Studies on Sulphatases

22. THE ANOMALOUS KINETICS OF ARYLSULPHATASE *A* OF HUMAN TISSUES: INTERPRETATION OF THE ANOMALIES*

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The anomalous kinetics of human arylsulphatase *A* have been described in the preceding paper (Baum, Dodgson & Spencer, 1958). This paper is concerned with the effect of various other factors on the activity of the enzyme and suggests a possible explanation of the observed anomalies.

EXPERIMENTAL AND RESULTS

Materials and methods of assay. Substrate and enzyme preparations and the method of enzyme assay were as described in Baum *et al.* (1958). The enzyme preparation generally used was that obtained from acetone-dried human liver (preparation 1).

Influence of the reaction products on the time course of the reaction

The anomalous kinetics of human arylsulphatase *A* are apparently dependent on a slow inactivation of the enzyme in the presence of substrate and the subsequent partial restoration of enzyme activity as the reaction is allowed to proceed. It was possible that the latter phenomenon might be related to the accumulation of one or both of the products of reaction, i.e. 4-nitrocatechol and SO_4^{2-} ions. It was therefore of interest to study the influence of these products on the course of the reaction.

Effect of inorganic sulphate. Fig. 1 shows the effect of SO_4^{2-} ions on the time course of the enzymic desulphation of 5 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) by arylsulphatase *A* in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. It will be observed that at concentrations up to 2·5 mM, SO_4^{2-} ion has little effect on the initial part of the time–activity curve (stage I, see Baum *et al.* 1958). On the other hand, SO_4^{2-} ion exerts a considerable activating effect on the later stages of the enzymic reaction. At higher concentrations of SO_4^{2-} ion, enzyme activity during stage I is inhibited appreciably but again there is marked activation during the later stages.

Fig. 2 shows the effect of 0·5 mM-Na_2SO_4 on the pH–activity curves of the enzyme when measured over varying incubation periods. The curves obtained under identical experimental conditions, but in the absence of SO_4^{2-} ions, are also shown. Although enzyme activity at pH 4·4 is only slightly affected by SO_4^{2-} ions, the latter has a considerable activating effect on the enzyme at pH 5, particularly after the first 30 min. of the reaction (i.e. during stages II and III). As a result of this activation, the pH curve, irrespective of the period of incubation, always shows two peaks of activity. By suitably modifying the experimental conditions, similar results can be obtained with

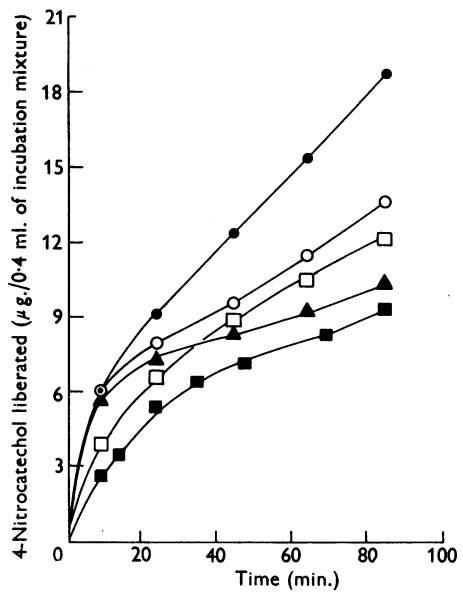


Fig. 1. Effect of SO_4^{2-} ions on the time–activity curves for human arylsulphatase *A* (ten times dilution of preparation 1) acting at 37·5° on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. Concentrations of Na_2SO_4 in the incubation mixture were 0·25 mM (○), 1·25 and 2·5 mM (●), 12·5 mM (□), 25 mM (■). A control curve (▲), where no Na_2SO_4 was added, is also shown.

* Part 21: Baum, Dodgson & Spencer (1958).

potassium *p*-nitrophenyl sulphate (NPS) as the assay substrate; then the peak at pH 6·1 becomes predominant (cf. Fig. 6 of Baum *et al.* 1958).

The effect of adding SO_4^{2-} ions to the incubation mixture during stage II of the reaction is shown in Fig. 3*A*. The enzyme was allowed to act against 5 mM-NCS in 0·25 M sodium acetate–acetic acid buffer, pH 5, the concentration of enzyme used being such that approx. 7 μg. of nitrocatechol was liberated in 80 min. by 0·4 ml. of the incubation mixture. At this time, stage II of the reaction was well established and less than 5 μg. of SO_4^{2-} ion/0·4 ml. of incubation mixture had been liberated. SO_4^{2-} ion was now added to the reaction vessel (0·01 ml. of 25 mM-Na_2SO_4 soln./ml. of incubation mixture present), the concentration of SO_4^{2-} ions in the mixture thereby being increased approximately threefold. A similar volume of water was added to the control experiment. It will be seen from Fig. 3*A* that SO_4^{2-} ions caused a rapid increase in enzyme activity.

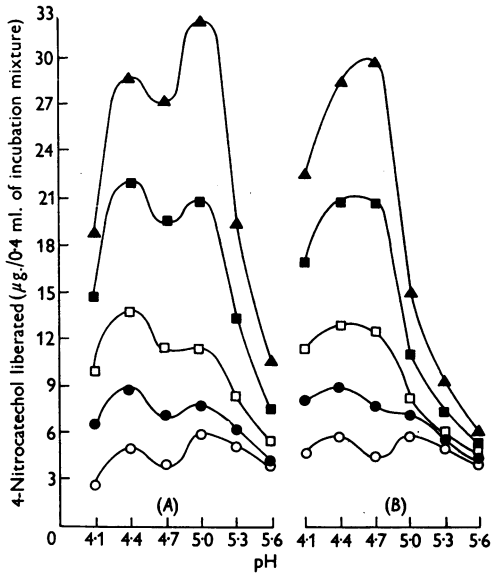


Fig. 2. Effect of SO_4^{2-} ions on the pH–activity curves for human arylsulphatase *A* plotted for various time intervals. The enzyme (ten times dilution of preparation 1) was acting on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer at 37·5°. (*A*) pH–Activity curves in the presence of 0·5 mM-Na_2SO_4; (*B*) normal pH–activity curves. ○, 10 min.; ●, 30 min.; □, 75 min.; ■, 155 min.; ▲, 240 min.

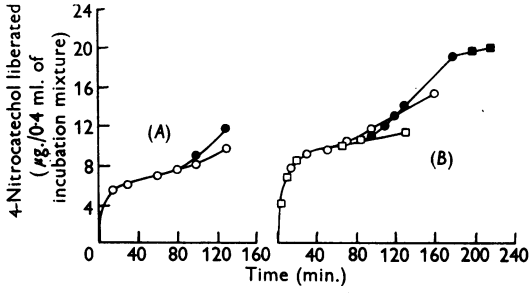


Fig. 3. Effect on the time–activity curves for human arylsulphatase *A* of Ba^{2+} and SO_4^{2-} ions when added at various stages during the reaction. (*A*) Addition of SO_4^{2-} ions during stage II of the reaction: ○, control; ●, SO_4^{2-} ions added. (*B*) Addition of Ba^{2+} ions at zero time followed by successive additions of excess of SO_4^{2-} ions and excess of Ba^{2+} ions: ○, control; □, Ba^{2+} ions added at zero time; ●, excess of SO_4^{2-} ions added; ■, excess of Ba^{2+} ions added.

These experiments suggested that the liberation of SO_4^{2-} ions during the course of the enzymic reaction could be at least partly responsible for the apparent reactivation of arylsulphatase *A* which occurs during stage III. Further support for this view was obtained by examining the effect of Ba^{2+} ions on the course of the reaction. In the presence of 3 mM-barium acetate, little or no reactivation of the enzyme occurred compared with a control experiment (Fig. 3*B*). Reactivation of the barium-treated enzyme could be achieved by adding to this incubation mixture 0·01 ml. of an aqueous solution of Na_2SO_4, the concentration of which was adjusted so as to provide a 0·6 mM excess of SO_4^{2-} ions (Fig. 3*B*). Subsequently the enzyme could again be inactivated by addition of a slight excess of barium acetate. It is clear from Fig. 3*B* that Ba^{2+} ion has little or no effect on stage I of the enzyme reaction. It will be recalled that at concentrations up to 2·5 mM, SO_4^{2-} ion was also without effect on stage I of the reaction (cf. Fig. 1). Other experiments showed that when SO_4^{2-} ion was present in the incubation mixture from the beginning of the reaction in sufficient concentrations to cause inhibition during stage I, addition of a slight excess of Ba^{2+} ions during the early stages of the reaction merely abolished the inhibition. If the Ba^{2+} ions were added during the later stages immediate inactivation resulted. It follows therefore that whatever the nature of the inactivation process, SO_4^{2-} ion does not prevent its occurrence.

Fig. 2 suggests that the effect of SO_4^{2-} ions on the course of the reaction was less marked at pH 4·4 than at 5·0. It was therefore of interest to examine the effect of Ba^{2+} ions on the time course of the reaction at pH 4·4. The results of this experiment are shown in Fig. 4, from which it can be seen that stage II was achieved more slowly at the lower pH. When the enzyme reaction was allowed to attain stage II at pH 5 in the presence of Ba^{2+} ions, readjustment of the pH of the reaction mixture from 5·0 to 4·4 at this point did not result in any immediate increase in enzyme activity. It was therefore concluded that the rate of inactivation

was greater at the higher pH but that the overall course of the reaction was the same.

Effect of sulphate in the absence of 4-nitrocatechol. It will be shown later that 4-nitrocatechol, the second reaction product, also affects the time course of the reaction. For this reason an attempt was made to confirm the effect of SO_4^{2-} ion on the inactivated enzyme in the absence of 4-nitrocatechol. Arylsulphatase *A* (0·6 ml. of preparation 1, see Baum *et al.* 1958) was incubated at 37·5° in a stoppered 15 ml. centrifuge tube for 3 min. with 0·6 ml. of 50 mM-barium acetate soln. and 2·8 ml. of water. At zero time, 2 ml. of a previously warmed 15 mM solution of NCS in 0·75 M sodium acetate–acetic acid buffer, pH 5, was added to the reaction vessel. The time course of the reaction was followed and, after 30 min., when stage II of the reaction was well established, the incubation mixture was rapidly chilled to 0°. The liberated 4-nitrocatechol was then carefully extracted (by gentle inversion of the tube) with four successive 6 ml. portions of ice-cold ether. Addition of 1 ml. of N-NaOH to a sample (0·2 ml.) of the extracted mixture showed that the liberated 4-nitrocatechol had been removed. To 4 ml. of the mixture was added 0·1 ml. of 0·2 M-Na_2SO_4, to give a slight excess of SO_4^{2-} ions, corresponding to that liberated during the enzymic reaction, and the precipitated $BaSO_4$ was removed by centrifuging. A portion (1·8 ml.) of the supernatant solution was treated with 0·01 ml. of 0·2 M-Na_2SO_4 (to give an excess of approx. mM-SO_4^{2-} ions), and to a similar portion was added 0·01 ml. of water. Both solutions were then replaced in the water bath at 37·5° and the time courses of the reactions were followed in the usual manner. The results (Fig. 5) show that SO_4^{2-} ion still caused a rapid reactivation of the enzyme when the amounts of 4-nitrocatechol present were negligible.

Effect of 4-nitrocatechol. Fig. 6 shows the effect of 4-nitrocatechol on the time course of the enzyme reaction. In concentrations up to 0·5 mM, 4-nitrocatechol has little effect on stage 1 of the reaction but causes appreciable activation during the later stages. This activation can occur in the absence of

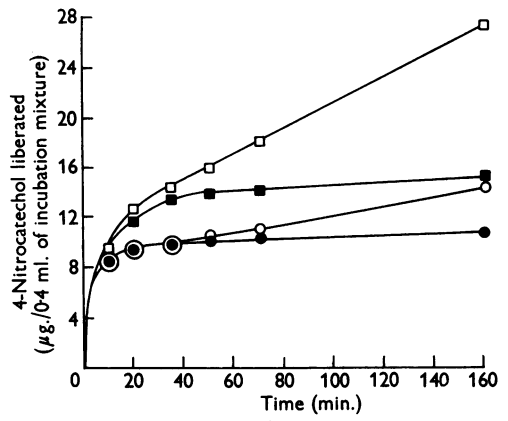


Fig. 4. Time–activity curves for human arylsulphatase *A* acting on 5 mM-NCS at 37·5° in 0·25 M sodium acetate–acetic acid buffer, pH 5·0 and 4·4, in the presence and absence of 3 mM-barium acetate. ○, pH 5 in the absence of Ba^{2+} ions; ●, pH 5 in the presence of Ba^{2+} ions; □, pH 4·4 in the absence of Ba^{2+} ions; ■, pH 4·4 in the presence of Ba^{2+} ions.

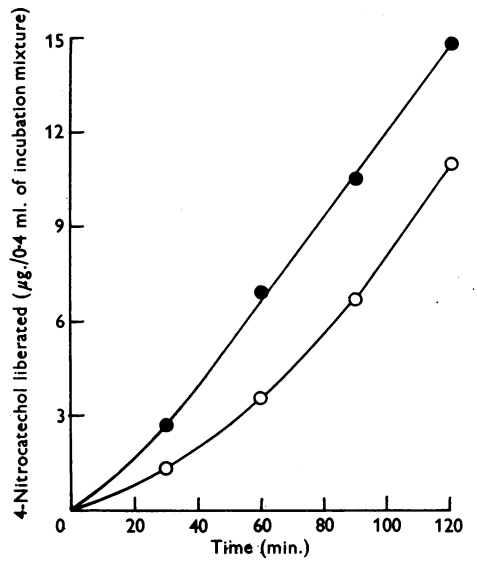


Fig. 5. Effect of SO_4^{2-} ions on the time–activity curve for 'inactivated' human arylsulphatase *A* acting on NCS. The experiment is described in detail in the text. ○, Slight excess of SO_4^{2-} ions; ●, mM-SO_4^{2-} ions.

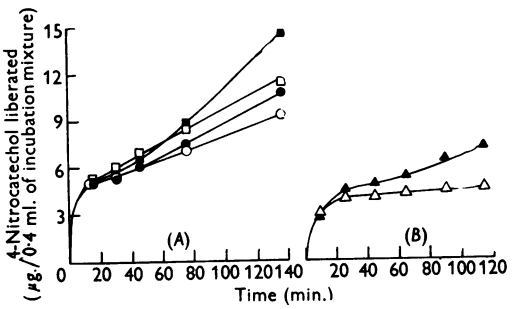


Fig. 6. Effect of 4-nitrocatechol on the time–activity curves for human arylsulphatase *A* acting on 5 mM-NCS at 37·5° in the presence of: (*A*) 0·25 M sodium acetate–acetic acid buffer, pH 5, and (*B*) 0·25 M sodium acetate, barium acetate–acetic acid buffer (3 mM-Ba^{2+} ions), pH 5. Concentrations of 4-nitrocatechol in the incubation mixtures were: (*A*) nil (○), 0·2 mM (●), 0·4 mM (□), 0·5 mM (■), and (*B*) nil (△), 0·4 mM (▲).

SO_4^{2-} ions since the effect is also observed when Ba^{2+} ion is present in the reaction mixture (Fig. 6*B*).

Effect of other compounds on the time course of the reaction

Fig. 7 shows the effect of Na_3PO_4 and $Na_4P_2O_7$ on the course of the enzyme reaction. When present in relatively high concentrations both compounds were potent inhibitors of enzyme activity. At lower concentrations they tended to inhibit during stage I of the reaction only and to activate during the later stages. Similar though less-marked results were obtained with NaF. It was possible to select concentrations of these compounds at which stage I of the reaction was virtually unaffected by their presence but activation still occurred during the later stages. Moreover, the compounds were capable of reactivating the enzyme when added to a normal enzyme–substrate mixture during stage II. It is clear that the effects of these compounds are very similar to the effect produced by SO_4^{2-} ion although quantitative differences are apparent. Thus traces of $Na_4P_2O_7$ affect the enzyme reaction markedly and, indeed, when present in concentrations of 0·1 mM, the compound apparently completely abolished the anomalous kinetics.

Fig. 7 also shows the effects of varying concentrations of urea on the time course of the reaction. Increasing concentrations of urea increased the rate of inactivation of the enzyme but did not eliminate stage III. Preincubation with urea for 20 min. before addition of substrate did not affect these results.

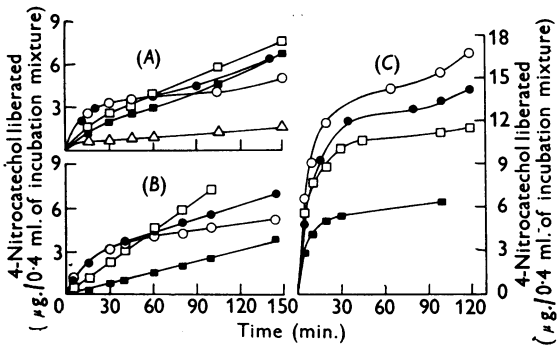


Fig. 7. Effect of PO_4^{3-} ions, $P_2O_7^{4-}$ ions and urea on the time–activity curve for human arylsulphatase *A* acting on 5 mM-NCS at 37·5° in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. (*A*) Preparation 1 diluted 20 times and assayed in the presence of the following concentrations of Na_3PO_4: nil (○), 0·1 mM (●), 0·5 mM (□), 1·25 mM (■) and 12·5 mM (△). (*B*) Preparation 1 diluted 20 times and assayed in the presence of the following concentrations of $Na_4P_2O_7$: nil (○), 0·01 mM (●), 0·1 mM (□) and mM (■). (*C*) Preparation 1 diluted five times and assayed in the presence of the following concentrations of urea: nil (○), 6% (●), 10% (□) and 20% (■).

Attempted separation of the inactive form of the enzyme

The collective results indicated that human arylsulphatase *A* was slowly converted into an inactive form by incubation with substrate. The inactive form of the enzyme could subsequently be partially reactivated by small amounts of SO_4^{2-} ions and 4-nitrocatechol, the products of the enzymic reaction. Partial reactivation could also be achieved by the addition of small amounts of certain other compounds which, when present in higher concentrations, were potent inhibitors of enzyme activity.

Attempts were now made to separate and study the inactive form of the enzyme.

Separation by dialysis. The enzyme (1 ml. of preparation 1, see Baum *et al.* 1958) was incubated at 37·5° with 3 ml. of 10 mM-NCS in 0·5 M sodium acetate–acetic acid buffer, pH 5. After 1 hr. the incubation mixture ('inactive' preparation) was removed from the water bath and dialysed for 24 hr. against running water in order to remove unchanged NCS and the reaction products. A control experiment was made in which the buffered substrate was replaced by 0·5 M sodium acetate–acetic acid buffer, pH 5. The two diffusates were each diluted to 6 ml. with water and portions (2 ml.) were incubated at 37·5° with 2 ml. of 10 mM-NCS in 0·5 M sodium acetate–acetic acid buffer, pH 5, the time course of the reaction being followed in the usual way.

Results of a typical experiment are shown in Fig. 8, from which it can be seen that although the initial reaction rate of the 'inactive' preparation

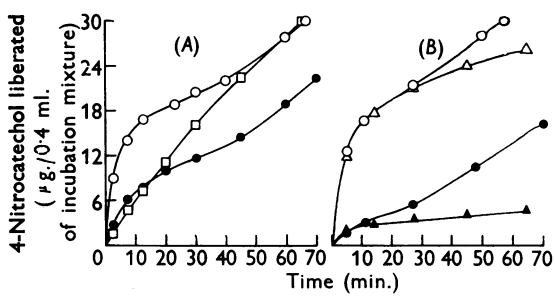


Fig. 8. Time–activity curves for preparations of the 'inactive' modification of human arylsulphatase *A*. Details of the experiments are described in the text. (*A*) Preparations subjected to the dialysis procedure; control preparation (○), 'inactive' preparation (●), 'inactive' preparation in the presence of mM-SO_4^{2-} ions (□). (*B*) Preparations subjected to the acetone-precipitation procedure: control preparation (○), 'inactive' preparation (●), control preparation in the presence of Ba^{2+} ions (△), 'inactive' preparation in the presence of Ba^{2+} ions (▲).

was less than half that of the control, the final reaction rates were very similar. This was taken to indicate that in the former preparation a considerable amount of the enzyme was already present in the inactive form and was eventually reactivated by the SO_4^{2-} ions and 4-nitrocatechol liberated as a result of the activity of the residual active enzyme. When Na_2SO_4 (final concentration, mM) was added to the reaction mixture, the final reaction rate of the 'inactive' preparation was established almost immediately. The similarity of the final reaction rates was not unexpected since the same initial concentration of enzyme was present in each case. Similar results were obtained when the initial inactivation of the enzyme was carried out in the presence of Ba^{2+} ions.

Separation by acetone precipitation. Since the dialysis procedure outlined above was not completely satisfactory for the separation of the inactive form of the enzyme an attempt was made to precipitate the inactive form with acetone. Preliminary experiments showed that the partially inactivated enzyme obtained by the procedure described in the preceding section could be precipitated by acetone (final concentration 75 %, v/v) and redissolved without markedly altering the enzyme kinetics. This was a further indication that the inactive form of the enzyme was a comparatively stable entity. Attempts were therefore made to precipitate the inactive form directly from the incubation mixture.

The enzyme (1·5 ml. of preparation I, see Baum *et al.* 1958) was incubated at 37·5° with 4·5 ml. of 10 mM-NCS in 0·5 M sodium acetate–acetic acid buffer, pH 5. After 45 min. the reaction mixture was rapidly cooled to 0° and treated with acetone at −5° until the concentration of acetone was 80 % (v/v). The precipitate was separated by centrifuging at −2° and subsequently resuspended in 8 ml. of water ('inactive' preparation).

A control experiment was carried out in which the enzyme was incubated with buffer alone. Portions (2 ml.) of the two preparations were each incubated at 37·5° with 2 ml. of 10 mM-NCS in 0·5 M sodium acetate–acetic acid buffer, pH 5, and the time courses of the reactions followed as usual (Fig. 8). Simultaneously the time courses were examined in the presence of Ba^{2+} ions in order to eliminate the effect of traces of SO_4^{2-} ion which might have coprecipitated with the enzyme during the acetone treatment (Fig. 8). The time curves obtained in the presence of Ba^{2+} ions indicate that approx. 80 % of the substrate-treated enzyme had been inactivated, compared with the control. In the absence of Ba^{2+} ions the final reaction rates of the 'inactive' and control preparations were again similar although the initial reaction rate of the former was many times less than that of the control.

Reconversion of the 'inactive' enzyme into its original form

It was clear that the reactivation which occurred during stage III of the enzymic reaction could not be due to the partial re-formation of the original form of the enzyme. This follows from the fact that removal of SO_4^{2-} ions by the addition of Ba^{2+} ions during stage III resulted in an immediate loss of enzyme activity which could be restored on addition of a slight excess of SO_4^{2-} ions (see Fig. 3*B*). On the other hand, small amounts of Ba^{2+} ions, SO_4^{2-} ions and 4-nitrocatechol were without effect on stage I of the reaction (i.e. on the original form of the enzyme). This implies that stage III of the enzyme reaction represents the activity of a new form of the enzyme which is active only in the presence of 4-nitrocatechol or SO_4^{2-} ions (or certain other ions). This form of the enzyme predominates in the so-called 'inactive' enzyme preparations obtained by the dialysis or acetone-precipitation procedures.

Results obtained during the course of several preparations of the 'inactive' enzyme suggested that the dialysis procedure might be less efficient than acetone precipitation because of the tendency for some of the original enzyme to be re-formed from 'inactive' enzyme during the dialysis period. Attempts were therefore made to convert the 'inactive' enzyme back into its original form. Preliminary experiments showed that apparent reconversion of 'inactive' enzyme could be achieved by incubation with sodium acetate solutions at pH 8. Incubation with water alone was ineffective. Considerable reconversion of the 'inactive' enzyme could consistently be achieved by the procedure described below, although the extent of the reconversion varied somewhat from experiment to experiment.

A preparation of the 'inactive' form of the enzyme was made by the acetone-precipitation procedure described previously except that the precipitate was resuspended in only 4 ml. of water. A suitable control preparation was also made. A portion (1 ml.) of each preparation was treated with 0·8 ml. of M-sodium acetate, pH 8, and incubated for 2 hr. at 37·5° before allowing to stand for 18 hr. at room temperature. Subsequently, the pH of each solution was adjusted to 5 by the addition of 0·2 ml. of M-acetic acid. The time–activity curves of these solutions were then examined in the usual way after adding 2 ml. of 10 mM-NCS in 0·5 M sodium acetate–acetic acid buffer, pH 5, to which had been added sufficient $BaCl_2$ to give a final concentration of 5 mM-Ba^{2+} ions. A further portion of each preparation was treated exactly as described above except that the 20 hr. incubation period in the presence of acetate at pH 8 was omitted. Ba^{2+} ion was included in all the final reaction mixtures in order to eliminate the activating effect of SO_4^{2-} ions on the 'inactive' form of the enzyme. Any activity observed therefore is largely a measure of the amount of enzyme present in the original form.

The results of these experiments are shown in Fig. 9. It will be seen that the 20 hr. incubation at pH 8 caused a slight inactivation of the control preparation (Fig. 9, curves 1 and 2), whereas the initial reaction rate of the 'inactive' enzyme preparation was increased considerably by this treatment (Fig. 9, curves 3 and 4). This indicates that 'inactive' enzyme has been reconverted into its original form.

Effect of sodium pyrophosphate on the formation of the 'inactive' enzyme

It now seemed important to consider the effect of $P_2O_7^{4-}$ ions on the formation of the 'inactive' modification of the enzyme. Fig. 7 shows that when present in concentrations greater than 0·1 mM, $P_2O_7^{4-}$ ions abolished the anomalous time–activity curve for arylsulphatase *A*. This could mean that $P_2O_7^{4-}$ ion was inhibiting the enzyme but preventing its modification to the 'inactive' form. A study was therefore made of the effect of incorporating 0·1 mM-$Na_4P_2O_7$ into the initial incubation mixture when 'inactive' enzyme was prepared by the dialysis procedure described in an earlier section. The properties of the final preparation were identical with those of the 'inactive' preparation obtained by incubating enzyme and substrate alone. Incubation of arylsulphatase *A* with acetate buffer and $P_2O_7^{4-}$ ions in the absence of substrate gave, after dialysis, an unmodified preparation with kinetics identical with those of a control from which $P_2O_7^{4-}$ ion was omitted. Whenever dialysis was carried out in the presence of $P_2O_7^{4-}$ ions it was necessary to clarify the final preparations by centrifuging. This removed a precipitate containing sufficient adsorbed $P_2O_7^{4-}$ ions markedly to affect the time–activity curves and, indeed, sometimes to eliminate the anomalies completely.

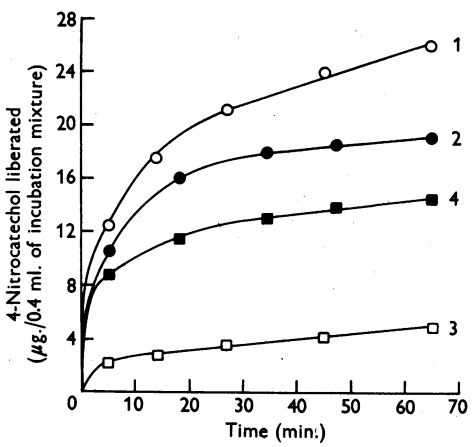


Fig. 9. Time–activity curves for human arylsulphatase *A* illustrating the reconversion of the 'inactive' modification of the enzyme into its original form. The experiment is described in detail in the text. ○, Control preparation; ●, control preparation after treatment with sodium acetate at pH 8; □, 'inactive' preparation; ■, 'inactive' preparation after treatment with sodium acetate at pH 8.

Obviously pyrophosphate does not prevent the formation of the 'inactive' modification of the enzyme. It will be recalled that similar conclusions were reached for SO_4^{2-} ions.

DISCUSSION

Any hypothesis about the anomalous kinetics of human arylsulphatase *A* must explain firstly the effects of temperature, pH and substrate concentration on the time course of the reaction (see Baum *et al.* 1958); and secondly, the effects of 4-nitrocatechol, SO_4^{2-} ions and certain other ions. Moreover, the hypothesis must account for the fact that it is possible to isolate an apparently modified form of the enzyme after treatment with substrate and subsequently to reconvert the modified enzyme into its original form.

A possible explanation of the anomalous time course of the enzyme reaction is that the enzyme preparation contains two distinct arylsulphatases, the more active one being rapidly but reversibly inactivated in the presence of substrate at 37·5°. Much of the kinetic data could then be explained by assuming an elaborate set of properties for these enzymes in terms of their response to temperature, pH and various ions. For example, in order to explain the fact that SO_4^{2-} ion in small amounts activates during the later stages of the reaction but is without effect during stage I, it would be necessary to assume that SO_4^{2-} ion activates the stable enzyme and inhibits the labile one in a compensating manner. The linear time–activity curve obtained when enzyme and substrate are incubated together in the presence of $Na_2P_2O_7$ (Fig. 7) would also be difficult to explain if the theory of two distinct arylsulphatases is accepted. The linearity and slope of this curve could be accounted for only by assuming either that $P_2O_7^{4-}$ ion prevents the inactivation of, but partially inhibits, the labile enzyme or that it prevents the latter from reacting with substrate in the first place. It has already been shown that modification of arylsulphatase *A* still occurs in the presence of $P_2O_7^{4-}$ ions and hence the second possibility would have to be invoked. It is difficult to visualize an inactivation of enzyme, which is known to take place only in the presence of substrate, being achieved under conditions where the enzyme is prevented from reacting with substrate. Paper electrophoresis (under varying experimental conditions) of a number of different preparations of human arylsulphatase *A* has failed to reveal the

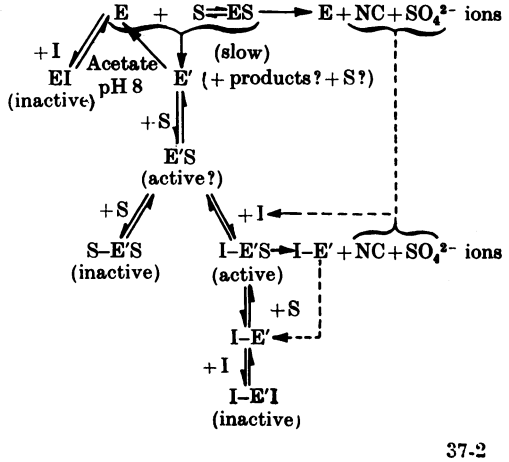
presence of a second enzyme, although this is not proof of homogeneity.

A second possibility is that the enzyme is acting in a manner analogous to that of chymotrypsin towards monopotassium *p*-nitrophenyl acetate and certain related esters. It has been shown (Macdonald & Balls, 1957; Dixon & Neurath, 1957) that, initially, the hydrolysis of these esters is accompanied by a simultaneous stoicheiometric acylation of the enzyme. This results in a rapid decrease in enzyme activity. Complete inactivation is never achieved, however, since the acylated enzyme undergoes a relatively slow spontaneous hydrolysis and this results in the establishment of a steady state of reaction which is considerably less than the initial rate. The time course of this enzyme reaction is thus, in some ways, similar to that observed for arylsulphatase *A*. When considered from a quantitative point of view, however, it is doubtful whether the mechanism of action of arylsulphatase *A* could be dependent on a sulphation of the enzyme analogous to the acylation of chymotrypsin, since it is almost certain that the initial reaction between arylsulphatase *A* and NCS is not restricted to a stoicheiometric process. Assuming a molecular weight of 10 000 for arylsulphatase *A*, then even if preparation 1 (see Baum *et al.* 1958) contained no other protein, at least 20 molecules of NCS would have been hydrolysed/molecule of enzyme during the initial reaction. Moreover, preparation 1 is by no means pure and for preparation 1*A* (see Baum *et al.* 1958) the corresponding figure is 200. Similar figures can be derived for purified preparations of ox-liver arylsulphatase *A* (see Roy, 1953). If arylsulphatase *A* was undergoing a sulphation analogous to the acylation of chymotrypsin this would therefore imply either that there were several 'active' sites per enzyme molecule or that rapid esterase activity without concomitant sulphation was proceeding at the same time. It is difficult to see how an inactive sulphated enzyme could be reactivated by SO_4^{2-} or other ions. Although a number of different nitrophenyl esters are capable of being hydrolysed by chymotrypsin, the sulphate ester is not (Macdonald & Balls, 1957). We have confirmed this observation and have made similar findings for NCS.

Other enzymes (e.g. the phenol oxidases, see Dawson & Tarpley, 1951) exhibit time–activity curves which are characterized by a short period of rapid activity followed by a reaction inactivation. However, a search of the literature has revealed no case of an enzyme having an anomalous time–activity curve which can be influenced by its reaction products in a manner analogous to that in which SO_4^{2-} ions and 4-nitrocatechol influence arylsulphatase *A*.

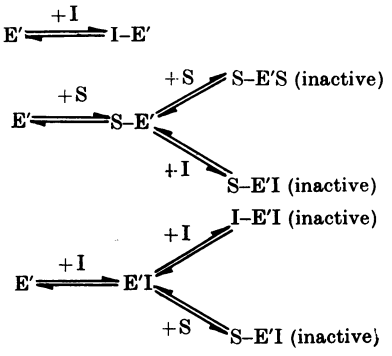
Roy (1957) has suggested that with ox arylsulphatase *A* the enzyme–substrate complex can break down not only into active enzyme plus products but also into an inactive enzyme plus products, both forms of enzyme being in equilibrium with the enzyme–substrate complex. There is an initial rapid formation of the inactive form of the enzyme, but, as the reaction products accumulate, active enzyme can be re-formed from the inactive form via the intermediary enzyme–substrate complex. However, he points out that this theory is at variance with the observation that SO_4^{2-} ion does not markedly influence the rate of initial inactivation. It is difficult to see how this theory could account for the reconversion into the original form of the 'inactive' enzyme preparation which was achieved by treatment with acetate alone during the course of the present work.

Although at this stage of the investigations any suggested explanation of the anomalous kinetics of human arylsulphatase *A* must be regarded as tentative, the following interpretation is consistent with the observed facts. During the interaction of enzyme and substrate a new substrate-binding site (there may be more than one such site per enzyme but only the simple case need be considered) is slowly exposed in the enzyme. The new site is capable of binding substrate and also the products of the enzyme reaction. Certain other compounds (PO_4^{2-}, $P_2O_7^{4-}$, F^- ions) which act as inhibitors towards the active centre of the enzyme can also bind to the new site. When substrate is bound to the new site of the modified enzyme the latter is virtually inactive. On the other hand, when the reaction products (or PO_4^{2-}, $P_2O_7^{4-}$ or F^- ions) are bound to the new site the modified enzyme is active, although the active centre may still be inhibited if these compounds are present in excess. Incubation of the modified enzyme with acetate buffer alone allows a slow rearrangement of the molecule to its original form. The proposed scheme may be represented diagrammatically as follows:



where E = the original form of the enzyme, S = substrate, E′ = modified enzyme. NC = 4-nitrocatechol, I = SO_4^{2-} ions, 4-nitrocatechol, PO_4^{3-}, $P_2O_7^{4-}$ or F^- ions. S (or I)–E′ represents binding to the new site in E′ and E′S (or I) binding to the active centre in E′.

For the purposes of clarity the following possible equilibria have been omitted from the scheme:



Although the kinetic complications inherent in such a scheme are formidable, it is nevertheless important to consider the scheme in greater detail and in relation to the experimental observations made. Thus in the scheme it is assumed that the slow formation of E′ from E in the presence of substrate is a process with an energy of activation which is greater than that of the normal esterase activity of the enzyme. The unusual behaviour of the enzyme towards variation in temperature (see Fig. 5 of Baum *et al.* 1958) can then be explained. At temperatures lower than 37·5°, although the esterase activity of E is reduced, the rate of formation of E′ (and hence the inactive S–E′S) is reduced to a still greater extent. Consequently, the activity of E is prolonged and, within limits, the overall sulphatase activity may therefore appear to increase with decrease in temperature.

The anomalous pH–activity curves obtained for the enzyme (Fig. 4 of Baum *et al.* 1958) can also be explained in terms of the proposed reaction scheme. Not only is the sulphatase activity of E affected by pH but the rate of formation of E′ (and hence the inactive S–E′S) is also a pH-dependent process, being greater, for example, at pH 5 than at 4·4 (Fig. 4). The observed sulphatase activity at a given pH will therefore depend on the relative contribution of each process at that pH, and under these circumstances the finding that the pH–activity curves obtained during stage I of the reaction show two distinct optima is not altogether unexpected. The persistence of the optimum at pH 4·4 and the disappearance of that at 5·0 as the reaction proceeds reflects the fact that the rate of formation of E′ (and hence the inactive S–E′S) is greater at the latter pH. However, other factors complicate the situation when substantial amounts of E′ and reaction products have been formed, since the effects of pH on the reactions in which E′ and products are involved must also then be considered. When the effect of pH on enzyme activity is studied with high concentrations of enzyme, appreciable quantities of the reaction products accumulate even during the initial stage of the reaction. This is reflected in the different pH–activity curves which are obtained at these higher enzyme concentrations (Fig. 4 of Baum *et al.* 1958) and which represent the sum of a number of different pH effects. When one of the reaction products (i.e. SO_4^{2-} ion) is present in the incubation mixture in appreciable amounts from the beginning of the reaction, the pH–activity curves are modified to an even greater extent (Fig. 2).

According to the proposed scheme, when the conversion of E into E′ is complete, the overall rate of reaction is governed by the competition between substrate and reaction products for the newly formed substrate-binding site which is present in E′. The relative proportions of substrate and products will thus determine the amount of E′ which is present in the I–E′S (active) form. At high substrate concentrations a high proportion of the modified enzyme will be in the S–E′S (inactive) form and this is in agreement with the observation that the substrate concentration–activity curve obtained during stage III of the enzyme reaction is typical of that for inhibition by excess of substrate (see Fig. 3 of Baum *et al.* 1958). In contrast, at high concentrations of enzyme, the reaction products will accumulate rapidly and will be present, even during the initial stages of the reaction, in concentrations sufficiently great to compete strongly with substrate for the new site in E′ immediately the latter is formed. Under these conditions the observed sulphatase activity during stage III of the reaction is relatively greater than when small amounts of enzyme are present (Fig. 1 of Baum *et al.* 1958).

Stage II of the enzyme reaction is most marked at low enzyme concentrations (Fig. 1 of Baum *et al.* 1958), and this is in accord with the reaction scheme. At low enzyme concentrations when formation of E′ is complete only small amounts of the reaction products will be present in the reaction mixture. The amount of I–E′S which can be formed at this stage will therefore be small. Nevertheless, the small amount present will constantly be forming products and the reaction at this stage becomes autocatalytic, leading eventually to stage III when a relatively high proportion of the enzyme is in the I–E′S (active) form.

From the above considerations it is clear that the presence of small amounts of SO_4^{2-} ions at the

beginning of the enzyme reaction would tend to eliminate stage II and to accentuate stage III. This is in agreement with the experimental observations (Fig. 1). However, when present in high concentrations from the beginning of the reaction, SO_4^{2-} ion competes with substrate not only for the new site in E′ but also for the active centres of both E and E′. The result of this would be inhibition during stage I and, depending on SO_4^{2-} ion concentration, reduced activation or even inhibition during the later stages (cf. Fig. 1). The activation which results from the addition of small amounts of SO_4^{2-} ion to preparations of 'inactive' enzyme (i.e. E′, see Fig. 8) or to incubation mixtures during later stages of the enzyme reaction (Fig. 3) is consistent with the proposed scheme.

If Ba^{2+} ion is present in the incubation mixture from the beginning of the reaction, then the effects due to SO_4^{2-} ions are abolished and stage II is thereby prolonged (Fig. 3). Indeed, the effects of adding Ba^{2+} ions to any reaction mixture at any stage are explicable in terms of the elimination of sulphate.

It seems likely that 4-nitrocatechol affects the enzyme reaction in a way comparable with that observed for SO_4^{2-} ions (Fig. 6). However, in view of experimental difficulties associated with the sensitivity of the assay procedure, no detailed study has been made of the effects of added 4-nitrocatechol.

The effects of PO_4^{3-}, $P_2O_7^{4-}$ and F^- ions on the enzymic reaction are apparently similar to those observed for SO_4^{2-} ions (see Fig. 7), although quantitative differences are apparent. However, the linear time–activity curves obtained when $P_2O_7^{4-}$ ion is present in the incubation mixture in concentrations greater than 0·1 mM are of considerable interest, since it is clear from the experimental results that conversion of E into E′ still occurs under these conditions. The most likely explanation of these findings which is in keeping with the proposed scheme is that the affinity of $P_2O_7^{4-}$ ion for the new site in E′ is so great that, under these conditions, S–E′S is never formed. The linearity of the time–activity curve may also mean that I–E′ has the same activity as E. The observation that the reaction rate in the presence of $P_2O_7^{4-}$ ion is less than that during stage I of the reaction in the absence of $P_2O_7^{4-}$ ion would then be interpreted as being due to the equal inhibition of the active centres of E and I–E′ by free $P_2O_7^{4-}$ ion.

Although the experimental observations are in accord with the proposed reaction scheme further work will be necessary in order to establish it conclusively. Thus the nature of changes involved in the formation of E′ and the reconversion of E′ into E are at present obscure. However, the fact that urea appears to accelerate the production of the modified enzyme (Fig. 7) may indicate that the changes involve the rupture and re-formation of hydrogen bonds. The nature of the new site which is exposed when E is converted into E′ is of some interest since it appears to be capable of binding not only substrate and SO_4^{2-} ions but also 4-nitrocatechol.

Of more immediate interest is the fact that, even after prolonged dialysis, preparations of arylsulphatase *A* to which $P_2O_7^{4-}$ ion has been added apparently retain sufficient $P_2O_7^{4-}$ ions (before centrifuging) to give linear time–activity curves. This may be of considerable significance since human-liver arylsulphatase *B* (Dodgson & Wynn, 1958), which exhibits apparently normal kinetics, is prepared from aqueous extracts of acetone-dried liver which also contain both arylsulphatase *A* and free and combined $P_2O_7^{4-}$ ions. The possibility that the two arylsulphatases might be interconvertible must therefore be considered. The properties of mixtures containing arylsulphatase *A* and $P_2O_7^{4-}$ ions are now being studied and attempts to interconvert arylsulphatases *A* and *B* are in progress.

SUMMARY

1. The anomalous time course of the reaction between human arylsulphatase *A* and dipotassium 2-hydroxy-5-nitrophenyl sulphate is markedly affected by the products of the reaction, i.e. 4-nitrocatechol and SO_4^{2-} ions. Both these products, when present in small amounts, are without effect on the initial stages of the enzyme reaction but exert an activating effect during the later stages of the reaction.

2. The effect of SO_4^{2-} ions can be abolished by addition of a slight excess of Ba^{2+} ions and subsequently restored by the further addition of a slight excess of SO_4^{2-} ions.

3. PO_4^{3-}, $P_2O_7^{4-}$ and F^- ions are similar to SO_4^{2-} ions with regard to their effect on the time course of the enzyme reaction. When present in higher concentrations, these ions inhibit enzyme activity.

4. A modified form of the enzyme can be separated from the incubation mixture at the end of the initial period of high enzyme activity. The modified form of enzyme can be reconverted into the original form by incubation with sodium acetate solution at pH 8.

5. A possible explanation of the anomalous kinetics of arylsulphatase *A* is offered in which it is suggested that the observed effects result from the slow exposure of a second substrate-binding site as the enzymic reaction proceeds. This second site can bind substrate to give a form of enzyme which is virtually inactive or it can bind SO_4^{2-}, PO_4^{3-} and $P_2O_7^{4-}$ ions and 4-nitrocatechol to give a form of enzyme which is still active. The final

rate of reaction is governed by the relative affinity of the new site for substrate or reaction products.

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Growth of *Acetobacter suboxydans* and the Oxidation of Aldoses, Related Carboxylic Acids, and Aldehydes

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Acetobacter suboxydans was first isolated by Kluyver & de Leeuw (1924) from beer. Referring to the oxidation of carbohydrates during aerobic growth, they described the organism as 'characterized by its slight intensity of oxidation which leads to an accumulation of intermediary products'. The discovery of the selective oxidation of polyols by growing cultures of *Acetobacter xylinium*, resulting in the accumulation of the corresponding ketoses, led to the enunciation of the well-known Bertrand rule that has been confirmed and extended by later workers studying *A. suboxydans*. Growth on aldoses as carbon source leads to the formation of the corresponding aldonic acids, and it is probable that gluconic acid is the initial product of the action of all *Acetobacter* species on glucose. More extensive oxidation of glucose to yield 5-oxogluconic acid during growth is a feature of a number of *Acetobacter* species, including *A. suboxydans*, and the concomitant formation of 2-oxogluconic acid by this organism has also been reported. These aspects have been reviewed recently by Rao (1957).

The oxidation of some carbohydrates by suspensions of *A. suboxydans* has been the subject of a number of investigations (Butlin, 1936, 1938*a*, *b*; Kluyver & Boezaardt, 1938; King & Cheldelin, 1952, 1953), and the present study, of which a preliminary communication has been made (Fewster, 1956), is designed to correlate and extend much of this work as well as to examine the effectiveness of various carbohydrates as energy sources for growth and how these, in turn, influence the oxidative activity of the harvested organisms. This first paper is concerned with growth of the organism and the oxidation of aldoses and a number of aldehydes and carboxylic acids.

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EXPERIMENTAL

Organism. The strain of *A. suboxydans* used was no. 621 of the American Type Culture Collection and was obtained from the National Collection of Type Cultures. It was maintained on malt wort (Walker & Tosic, 1946) containing chalk (2%, w/v; previously sterilized at 160° for 4 hr.) in suspension solidified with agar (2%, w/v) in sloped test tubes; the tubes were incubated at 30° for 48 hr.

Media

Media for growth tests. Semi-defined medium *B* (sterilized by autoclaving for 10 min. at 10 lb./in.^{2}) contained per litre: acid-hydrolysed casein (vitamin-free), prepared by the method of Snell & Rannefeld (1945), equivalent to 6 g. of original casein; DL-tryptophan, 100 mg.; L-cysteine hydrochloride, 100 mg.; KH_2PO_4, either 10 g. (medium B_1) or 27 g. (medium B_2); $MgSO_4,7H_2O$, 400 mg.; $FeSO_4$, $(NH_4)_2SO_4,6H_2O$, 30 mg.; $MnSO_4,4H_2O$, 20 mg.; calcium pantothenate, 2 mg.; nicotinic acid, 2 mg.; *p*-aminobenzoic acid, 27·5 μg.; Tween 80 (Honeywill and Stein Ltd., London S.W. 1), 1 ml.; the medium was adjusted to pH 6 with N-NaOH. Complex medium *C* (sterilized by autoclaving for 15 min. at 10 lb./in.^{2}) contained per litre: peptone (Evans Medical Supplies Ltd., Liverpool), 10 g.; yeast extract (Marmite Ltd., London), 10 g.; the pH of the medium was adjusted to 6 with N-NaOH.

Media for bulk growth of the organism. Two types of solid media were employed: semi-defined medium B_1 or complex medium *C* (90 ml.) was added to agar (2 g.) in a Roux bottle; the mixture was autoclaved for 15 min. at 10 lb./in.^{2} and to it was added 10 ml. of a sterile aqueous solution (50%, w/v) of the carbohydrate under investigation. To neutralize the acids produced during growth on an aldose, sterile chalk (2 g.) was added to each bottle and suspended