Thanks are due to Dr G. A. Levvy for his stimulating and critical interest in this work; to Dr G. Halliwell for suggesting the butanol method of enzyme extraction, assistance at the commencement of the investigation and for providing the samples of rumen liquor used; to Dr B. H. Howard for advice on chromatography; to Mr I. Strachan and Miss E. F. Wallace for technical assistance. Acknowledgement is also due to several colleagues for comments on the manuscript.

REFERENCES

Aitken, R. A., Eddy, B. P., Ingram, M. & Weurman, C. (1956). *Biochem. J.* **64**, 63.

Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.

Chargaff, E., Levine, C. & Green, C. (1948). *J. biol. Chem.* **175**, 67.

Conchie, J. (1954). *Biochem. J.* **58**, 552.

Conchie, J. & Levvy, G. A. (1957). *Biochem. J.* **65**, 389.

Conchie, J., Levvy, G. A. & Marsh, C. (1956). *Biochem. J.* **62**, 24P.

Crook, E. M. & Stone, B. A. (1957). *Biochem. J.* **65**, 1.

De Whalley, H. C. S., Albon, N. & Gross, D. (1951). *Analyst*, **76**, 287.

Festenstein, G. N. (1957). *Biochem. J.* **65**, 23P.

Grassmann, W., Zechmeister, L., Tóth, G. & Stadler, R. (1933). *Liebigs Ann.* **503**, 167.

Halliwell, G. (1957a). *J. gen. Microbiol.* **17**, 153.

Halliwell, G. (1957b). *J. gen. Microbiol.* **17**, 166.

Hash, J. H. & King, K. W. (1954). *Science*, **120**, 1033.

Holden, M., Pirie, N. W. & Tracey, M. V. (1950). *Biochem. J.* **47**, 399.

Holden, M. & Tracey, M. V. (1950). *Biochem. J.* **47**, 407.

Jermyn, M. A. (1952a). *Aust. J. sci. Res.* B, **5**, 409.

Jermyn, M. A. (1952b). *Aust. J. sci. Res.* B, **5**, 433.

Jermyn, M. A. (1955a). *Aust. J. biol. Sci.* **8**, 541.

Jermyn, M. A. (1955b). *Aust. J. biol. Sci.* **8**, 577.

Karunairatnam, M. C. & Levvy, G. A. (1951). *Biochem. J.* **49**, 210.

Kitts, W. D. & Underkofler, L. A. (1954). *J. Agric. Fd Chem.* **2**, 639.

Kooiman, P., Roelofsen, P. A. & Sweeris, S. (1953). *Enzymologia*, **16**, 237.

Levinson, H. S., Mandels, G. R. & Reese, E. T. (1951). *Arch. Biochem. Biophys.* **31**, 351.

Levvy, G. A. (1946). *Biochem. J.* **40**, 396.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.

Morton, R. K. (1955). In *Methods of Enzymology*, vol. 1, p. 40. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Nelson, N. (1944). *J. biol. Chem.* **153**, 375.

Reese, E. T. & Mandels, G. R. (1957). Pioneering Research Division Quartermaster Research and Development Centre Natick. *Massachusetts Research Report, Microbiology Series*, no. 17.

Siu, R. G. H. (1951). *Microbial Decomposition of Cellulose.* New York: Reinhold Publishing Corporation.

Somogyi, M. (1952). *J. biol. Chem.* **195**, 20.

Stone, B. A. (1957). *Biochem. J.* **66**, 1P.

Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.

Whitaker, D. R. (1953). *Arch. Biochem. Biophys.* **43**, 253.

Whitaker, D. R. (1956). *Canad. J. Biochem. Physiol.* **34**, 102.

Willstätter, R. & Schudel, G. (1918). *Ber. dtsch. chem. Ges.* **51**, 780.

Studies on Sulphatases

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

BY H. BAUM, K. S. DODGSON AND B. SPENCER

Department of Biochemistry, University of Wales, Newport Road, Cardiff

The anomalous kinetics shown by partially purified preparations of the arylsulphatase *A* of ox liver when acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) were first noted by Roy (1953*a*, *b*). When measured over an incubation period of 1 hr. the velocity of the enzymic reaction was linearly related to enzyme concentration only when the latter was raised to the power of 3/2. This effect was attributed to the ability of the enzyme to exist in several polymeric forms. Similar anomalous kinetics were observed by Dodgson, Spencer & Wynn (1956) for the

corresponding enzyme of human liver, and it was subsequently possible to show (Dodgson & Spencer, 1956*a*) that the fundamental anomaly was that the enzymic reaction was not of zero order when low concentrations of enzyme were used. These findings were later confirmed for the ox enzyme (Roy, 1956). Meanwhile, it was noted (Roy, 1956; Roy & Kerr, 1956) that the NCS used in these studies contained appreciable amounts of a sulphated impurity which was identified as nitropyrogallol disulphate. When NCS which had been 'purified' via the methylene-blue salt or by paper electrophoresis was used as assay substrate, the ox-liver enzyme no longer exhibited anomalous kinetics.

* Part 20: Spencer (1958).

Subsequently, Dodgson & Spencer (1956b) showed that the introduction of contaminating materials during these purification procedures was the fundamental reason for the disappearance of the anomalous kinetics. These workers eliminated nitropyrogallol disulphate from preparations of NCS by repeated recrystallization of the latter in the form of the monopotassium salt. Human arylsulphatase *A* still exhibited anomalous kinetics towards the purified substrate.

The properties of the human enzyme have been extensively investigated in these laboratories but have not previously been reported in detail because, until recently, it was difficult to provide an adequate explanation of the findings. However, Roy (1957) has recently summarized his findings for ox-liver arylsulphatase *A*, and it now seems important to present this account of the properties of the human enzyme in view of the many additional observations made.

MATERIALS AND METHODS

Substrates. Monopotassium *p*-nitrophenyl sulphate (NPS) and monopotassium *p*-acetylphenyl sulphate (APS) were prepared by the method of Burkhardt & Lapworth (1926). Dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) was prepared according to the directions of Roy (1953a) and purified by the method of Dodgson & Spencer (1956b). All substrates were repeatedly recrystallized until free from inorganic sulphate.

Preparations of arylsulphatase A

Concentrates of human arylsulphatase *A* were made in several ways, but, irrespective of the method of preparation, similar results were always obtained.

Preparation 1. Human liver, obtained within 48 hr. after death, was acetone-dried (see Dodgson, Rose, Spencer & Thomas, 1957), and the resultant powder sieved (40 mesh) to remove connective tissue. The powder (60 g.) was suspended in 300 ml. of 0·1 M sodium acetate–acetic acid mixture, pH 7, with the aid of a Townson and Mercer macerator and the suspension was incubated at 37·5° for 15 min. before centrifuging for 15 min. at 2000 g. The debris was washed with a further 200 ml. of the acetate mixture and, after centrifuging as before, the two supernatants were combined and further clarified by centrifuging at 5000 g for 20 min. The clear supernatant was cooled to 0° and acetone, previously cooled to −5°, was added with stirring over a period of 30 min. until the final concentration of acetone was 45 % (v/v). After standing for 30 min. at −5° the precipitate was removed by centrifuging at 2000 g and −5°. To the clear supernatant was added 4 ml. of 0·2 M-CaCl₂ soln. and acetone was added as before until the concentration was 50 % (v/v). After standing at −5° for 30 min. the precipitate was separated by centrifuging and discarded. The concentration of acetone in the clear supernatant was then increased to 60 % (v/v) and, after standing as before, the precipitated material was separated by centrifuging, dissolved in 30 ml. of water and dialysed

overnight against tap water. The diffusate was clarified by centrifuging, the pH adjusted to 5 with a trace of acetic acid, and solid ammonium sulphate was slowly added (with stirring) until the solution was 35 % saturated with regard to ammonium sulphate. The pH was readjusted to 5 and the mixture allowed to stand for 5 hr. at room temperature. The precipitate was separated by centrifuging, dissolved in 20 ml. of water, dialysed for 18 hr. against tap water and subsequently for 24 hr. against several changes of distilled water (8 × 500 ml. at 2°). The cloudy solution was clarified by centrifuging and the colourless clear supernatant stored in the frozen state. The protein content of the preparation was usually in the region of 0·5 mg./ml. when measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Occasionally preparation 1 was further dialysed against several more changes of distilled water at 2°. Under these circumstances a precipitate sometimes appeared which, when extracted with 0·5 M sodium acetate–acetic acid buffer, pH 5, gave a solution which contained the bulk of the enzyme activity (preparation 1A). This preparation exhibited the same anomalous kinetics as preparation 1 and had about ten times the activity/wt. of protein. However, the preparation was somewhat unstable.

Preparation 2. This was identical with the first preparation as far as the precipitation of the 50–60 % (v/v) acetone fraction. This fraction was dissolved in water (20 ml.), dialysed overnight against tap water, clarified by centrifuging and concentrated to low bulk by freeze-drying. The concentrated solution was then subjected to horizontal-paper electrophoresis on several strips of Whatman no. 100 filter paper which had previously been treated with cetyl-pyridinium bromide as described by Dodgson & Spencer (1956c). Electrophoresis was carried out in the refrigerator for 18 hr. at 210 v in 0·1 M sodium acetate–acetic acid buffer, pH 5. Subsequently, arylsulphatase *A* was located on one of the strips (see Dodgson, Spencer & Thomas, 1955) and the enzyme was then eluted from the appropriate portion of the remaining strips with water. After concentrating to a suitable volume by freeze-drying, the enzyme solution was dialysed against tap water followed by distilled water as described for preparation 1.

Preparation 3. The arylsulphatase *A* present in freshly voided urine was separated from arylsulphatase *B* by the electrophoretic procedure of Dodgson & Spencer (1956c) and was subsequently eluted from several strips of filter paper with water. The eluates were combined and concentrated to low bulk by freeze-drying before dialysing as described for the other preparations.

Paper electrophoresis of the three preparations showed that in no case was arylsulphatase *B* (see Dodgson *et al.* 1956) present.

Other arylsulphatase preparations. The arylsulphatase of *Alcaligenes metalcaligenes* was concentrated according to the method of Dodgson, Spencer & Williams (1955), limpet (*Patella vulgata*) arylsulphatase was concentrated to give preparation *D* (see Dodgson & Spencer, 1953) and Taka-diastase (Parke, Davis and Co. Ltd.) was used as a source of fungal arylsulphatase. Human arylsulphatase *B* was prepared by the method of Dodgson & Wynn (1958), and the digestive juice of *Helix pomatia* was used as the source of snail arylsulphatase. All the preparations were dialysed for 18 hr. against tap water and subsequently for 24 hr. against several changes of distilled water (8 × 500 ml. at 2°).

Enzyme assay. The following method was employed in all cases irrespective of which substrate or enzyme preparation was used. The enzyme solution (3 ml., contained in a stoppered vessel) was pre-incubated for 3 min. at 37·5° before adding, as quickly as possible, an equal volume of a solution of the appropriate substrate (in 0·5 M sodium acetate–acetic acid buffer, adjusted to the appropriate pH) which had previously been warmed to 37·5°. Samples (0·4 ml.) were withdrawn at suitable time intervals and added immediately to 2 ml. of N-NaOH. After mixing, the liberated phenols were measured spectrophotometrically with the appropriate wavelength of maximum absorption (see Dodgson & Spencer, 1957a). Suitable control determinations were made.

Mention has already been made that similar results were obtained with all three enzyme preparations. However, for obvious reasons, preparation 1 was generally preferred and was used in all the experiments described below. When NCS was the substrate the kinetic anomalies were shown to best effect when enzyme activity was such that 3–9 μg. of 4-nitrocatechol was liberated in the initial 10 min. period/ 0·4 ml. of enzyme–substrate mixture, and the concentration of enzyme necessary to achieve this was determined by preliminary experiment. Generally speaking, the enzyme preparation had to be diluted about 10 times, although the activity varied appreciably from liver to liver.

RESULTS

Influence of various factors on the enzyme reaction

Time and enzyme concentration. Fig. 1 shows the time–activity curves for different concentrations of arylsulphatase *A* acting on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. At the lowest enzyme concentration there is an initial rapid enzyme reaction (stage I) which is followed by a period of very low but steady enzyme activity (stage II). At the higher enzyme concentrations, however, this second phase is not maintained and enzyme activity slowly increases again until it again reaches a steady rate (stage III), which, however, is less than that shown in stage I. At higher concentrations of enzyme than those shown in Fig. 1, the curve tends towards (but never achieves) a straight line. Similar curves are obtained after pre-incubation of the enzyme solution for 1 hr. at 37·5° before adding substrate, thus indicating that the phenomenon is at least partly dependent on the presence of substrate.

Fig. 2 shows the enzyme concentration–activity curves plotted from the data shown in Fig. 1. These curves vary appreciably with the time of incubation but in no case are they linear. Other experiments showed the relationship between enzyme concentrations and activity to be approximately linear only during the initial 2 or 3 min. of the reaction. The problems associated with the quantitative assay of arylsulphatase *A* in its purified form will be obvious from a consideration of Figs. 1 and 2.

Substrate concentration. Increasing substrate concentration results in increased enzyme activity during the initial stage (stage I) of the incubation
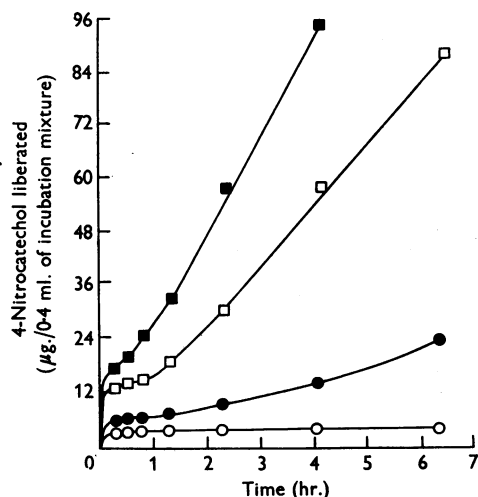

Fig. 1. Time–activity curves for different concentrations of human arylsulphatase *A* acting at 37·5° on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. The lowest relative enzyme concentration (RE) used was obtained by diluting preparation 1 fifteen times. ○, RE = 1; ●, RE = 2; □, RE = 4; ■, RE = 6.
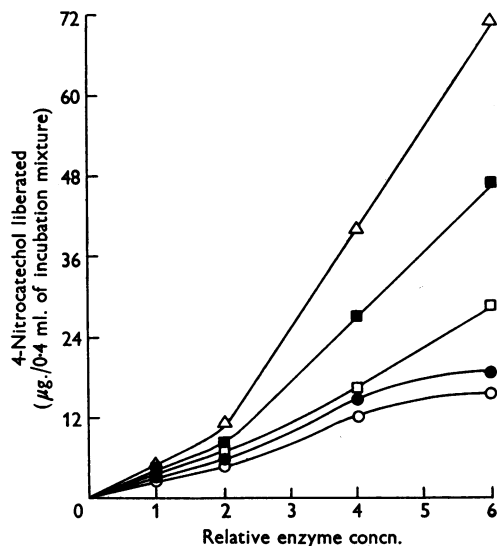

Fig. 2. Enzyme concentration–activity curves for human arylsulphatase *A* acting at 37·5° on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5, for various periods of time. The curves are plotted from the data shown graphically in Fig. 1. ○, 15 min.; ●, 45 min.; □, 60 min.; ■, 120 min.; △, 180 min.

period. In contrast, at substrate concentrations greater than 4 mM, increasing substrate concentration decreased the rate of enzymic hydrolysis during the later stages. Fig. 3 shows the substrate concentration–activity curve plotted for an incubation period of 10 min. (i.e. during stage I), from which it can be seen that the enzyme appears to be behaving according to the normal Michaelis–Menten equation. Fig. 3 also shows the effect of substrate concentration on the activity of the enzyme during stage III. Actually, the amounts of 4-nitrocatechol liberated between 90 and 130 min. were taken as a measure of the activity during this stage. The curve obtained is typical of that for inhibition by excess of substrate.

pH. The time course of the enzyme reaction is markedly affected by variation of pH, and hence pH–activity curves for arylsulphatase A vary considerably with the length of incubation period used. Fig. 4 shows the pH–activity curves plotted for various time intervals. During stage I the curves show two peaks of activity at pH 4·4 and 5·0 respectively but during stages II and III these peaks merge into a single peak at pH 4·4 which, in turn, shifts towards the region of pH 4·7 as the incubation proceeds. When the concentration of enzyme was increased so that approx. 15 μg. of 4-nitrocatechol was liberated in the initial 10 min. period/0·4 ml. of enzyme–substrate mixture, a

single pH optimum was obtained in the region of pH 4·8 (Fig. 4).

Temperature. The time–activity curves obtained at various temperatures for two different concentrations of enzyme acting on 5 mM-NCS at pH 5 are shown in Fig. 5. During the first few minutes of the reaction the enzyme behaves in the expected manner towards increase in temperature. However, as the reaction proceeds, the time–activity curves obtained at 20·5° and 30·5° cross those obtained at 37·5° and 50·5°, although the point at which this occurs varies somewhat with the enzyme concentration used in the experiment. Although not shown in Fig. 5, the greater rate of activity at 20·5° is prolonged for several hours, but during this

Fig. 4. pH–Activity curves for human arylsulphatase A plotted for various time intervals. The enzyme was acting on 5 mM-NCS in 0·25 M sodium acetate–acetic acid buffer at 37·5°. □, Preparation 1 diluted ten times; ■, preparation 1 diluted five times.
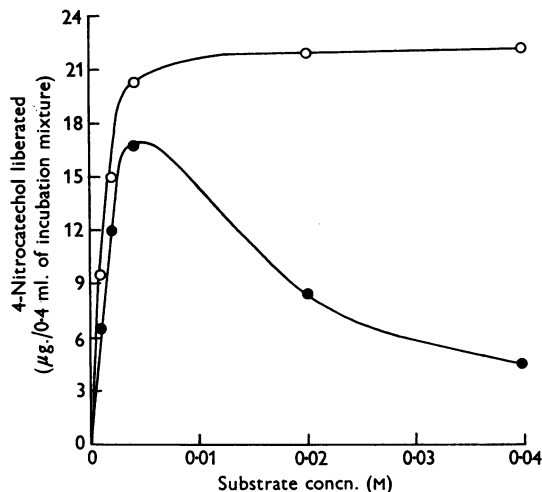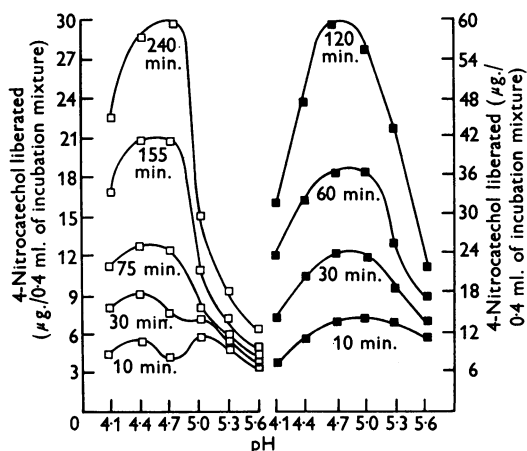
Fig. 3. Effect of substrate (NCS) concentration on the activity of human arylsulphatase A during stages I and III of the enzymic reaction. Enzyme activity was determined in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5, and the enzyme solution used was preparation I diluted five times. ○, Stage I (an incubation period of 10 min.); ●, stage III (plotted from the amounts of 4-nitrocatechol liberated between 90 and 130 min. of the incubation period; see text).
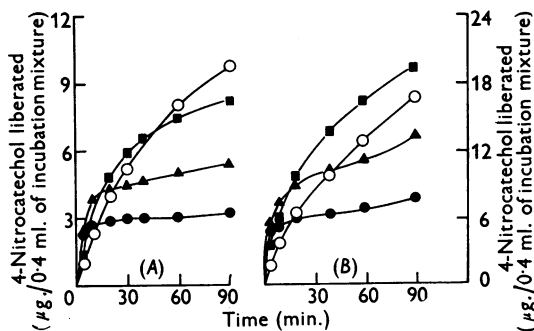
Fig. 5. Effect of temperature on the time–activity curves for human arylsulphatase A acting on 5 mM-NCS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 5. The enzyme solutions used were (A) preparation 1 diluted fourteen times and (B) preparation 1 diluted seven times. ○, 20·5°; ■, 30·5°; ▲, 37·5°; ●, 50·5°.

time the 37·5° time–activity curve shows an increasing upward trend and approaches the 20·5° curve and finally crosses it. This clearly indicates that thermal destruction of the enzyme is not an important factor in the production of these anomalous curves.

Behaviour of the enzyme towards other arylsulphatase substrates

Arylsulphatase *A* is a type II arylsulphatase (see Dodgson & Spencer, 1957b) which exhibits considerable affinity and activity towards NCS but only relatively low affinity and activity towards simpler arylsulphates such as NPS and APS. Nevertheless, by suitably adjusting the experimental conditions it can be shown that the enzyme also exhibits anomalous kinetics towards these other substrates. The pH–activity curves obtained for various incubation periods when the enzyme is acting against 50 mM-NPS are given in Fig. 6. Again two peaks of activity (at 5·3 and 6·1) are obtained over short incubation periods and these peaks give rise to a single peak in the region of pH 5·3 when the incubation period is prolonged. Similar results are obtained when APS is used as the substrate. Fig. 7 shows the time–activity curves obtained for different concentrations of enzyme acting on 50 mM-NPS at pH 6·1 and the enzyme concentration–activity curves for various incubation periods. The curves are very similar to those obtained with NCS, although it will be noted that the conditions of pH and enzyme and substrate concentration which have been employed to obtain these curves are very different from those necessary when NCS was the substrate. Similar results can be obtained with APS.

Behaviour of other arylsulphatases towards NCS

Ox (Roy, 1953a) and rat (Dodgson & Spencer, unpublished work) tissues are known to possess an enzyme corresponding to human arylsulphatase *A*, and it seems likely that similar considerations will apply to other mammalian species. Arylsulphatases from other sources do not exhibit anomalous
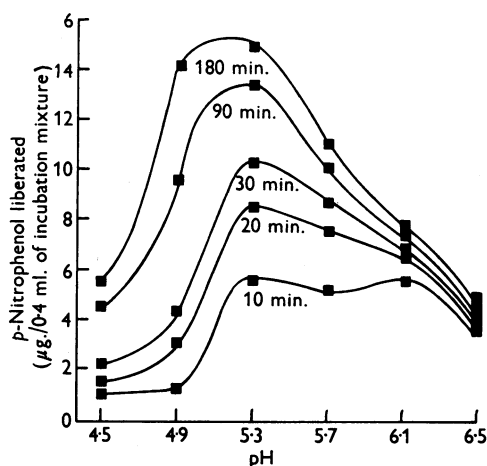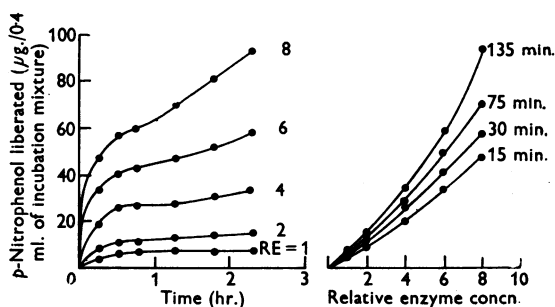
Fig. 7. Time–activity curves for different enzyme concentrations and enzyme concentration–activity curves for different time intervals for human arylsulphatase *A* acting on 50 mM-NPS in the presence of 0·25 M sodium acetate–acetic acid buffer, pH 6·1. Incubation was at 37·5° and the lowest relative concentration of enzyme (RE) used was concentrated (×2, by freeze-drying) preparation 1 diluted eight times.
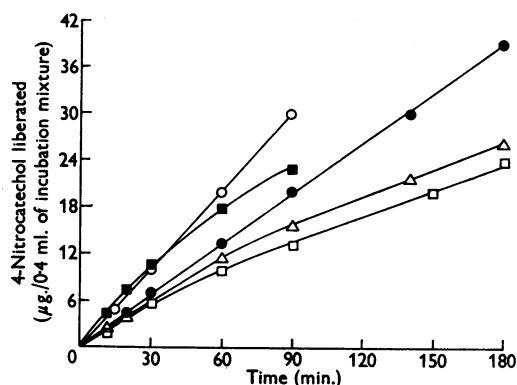
Fig. 8. Time–activity curves for arylsulphatases of different origin acting on NCS at 37·5° in the presence of 0·25 M sodium acetate–acetic acid buffer. The concentrations of the enzymes used were those necessary to liberate 3–9 μg. of 4-nitrocatechol/0·4 ml. of incubation mixture/20 min. as determined by preliminary experiments. The final concentrations of NCS and the pH at which the experiments were carried out are given below in parentheses. ○, Taka-diastase (2·5 mM, pH 6·0); ●, Alcaligenes metalcaligenes (15 mM, pH 8·0); □, Patella vulgata (10 mM, pH 5·6); ■, Helix pomatia (15 mM, pH 7·5); △, human arylsulphatase *B* (20 mM, pH 6·0).

Fig. 6. pH–Activity curves for human arylsulphatase *A* for various time intervals when acting at 37·5° on 50 mM-NPS in the presence of 0·25 M sodium acetate–acetic acid buffer. The enzyme solution used was preparation 1 diluted three times.

kinetics towards NCS. Fig. 8 shows the time–activity curves obtained for the arylsulphatases of Taka-diastase (*Aspergillus oryzae*), *Alcaligenes metalcaligenes*, *Patella vulgata* and *Helix pomatia*, with the appropriate conditions of pH and substrate concentration. Also included in the figure is human arylsulphatase *B*. In no case were anomalous time–activity curves obtained. Similar results were obtained when the experiments were carried out at pH values which varied on either side of the appropriate optimum by up to 2 pH units.

DISCUSSION

Mention has already been made in the introduction to this paper that the anomalous kinetics of human (and ox) arylsulphatase *A* cannot be due to either polymerization of enzyme or the presence of impurities in the substrate as was originally suggested by Roy (1953*b*, 1956). The observation (Roy, 1953*b*) that the activity of the ox enzyme was linearly related to enzyme concentration raised to the power 3/2 resulted from the fortuitous selection of an incubation period of 1 hr. and is in no way a measure of enzyme polymerization, and the fact that the enzyme gives anomalous kinetics with purified NCS as well as other arylsulphates disposes of the second theory. Roy (1957) failed to obtain the anomalies characteristic of the hydrolysis of NCS when NPS was used as the substrate but this can almost certainly be attributed to the fact that by studying the reaction at pH 5 this author was inadvertently selecting a pH unfavourable to the anomalous kinetics, and indeed curves similar to those observed by Roy (1957) can be obtained with the human enzyme if the enzyme experiments are carried out at pH 5·0 rather than at 6·1. The finding that arylsulphatases from other sources do not exhibit anomalous kinetics towards NCS also suggests that substrate impurities cannot be responsible for the anomalies exhibited by arylsulphatase *A*.

The weight of the evidence clearly indicates that the anomalous kinetics are a feature of all mammalian arylsulphatases *A*. It is possible, of course, that the presence of two arylsulphatases in the enzyme preparations could give rise to anomalous kinetics of this sort but there are reasons for discarding this possibility and these will be mentioned in the next paper (Baum & Dodgson, 1958). Assuming then that the anomalies are peculiar to the enzyme it seems clear that, under the influence of substrate, a relatively slow inactivation of the enzyme occurs and this is in turn followed by an apparent partial reactivation of the enzyme. The succeeding communication provides further examples of the way in which these processes may be modified and suggests a possible explanation of the phenomenon.

SUMMARY

1. Preparations of human arylsulphatase *A* exhibit anomalous time–activity curves when incubated with dipotassium 2-hydroxy-5-nitrophenyl sulphate and other aryl sulphates.

2. These anomalies can be considerably modified by varying the pH or temperature of the enzyme reaction and by varying the concentration of enzyme and substrate.

3. The anomalous kinetics appear to be a feature peculiar to mammalian arylsulphatases *A* and are not shown by human arylsulphatase *B* or the arylsulphatases of limpets, *Alcaligenes metalcaligenes*, Taka-diastase and *Helix pomatia*.

REFERENCES

Baum, H. & Dodgson, K. S. (1958). *Biochem. J.* **69**, 573.
Burkhardt, G. N. & Lapworth, A. (1926). *J. chem. Soc.* p. 684.
Dodgson, K. S., Rose, F. A., Spencer, B. & Thomas, J. (1957). *Biochem. J.* **66**, 363.
Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 315.
Dodgson, K. S. & Spencer, B. (1956*a*). *Biochem. J.* **62**, 30 P.
Dodgson, K. S. & Spencer, B. (1956*b*). *Biochim. biophys. Acta*, **21**, 175.
Dodgson, K. S. & Spencer, B. (1956*c*). *Clin. Chim. Acta*, **1**, 478.
Dodgson, K. S. & Spencer, B. (1957*a*). *Meth. biochem. Anal.* **4**, 211.
Dodgson, K. S. & Spencer, B. (1957*b*). *Rep. Progr. Chem.* **53**, 318.
Dodgson, K. S., Spencer, B. & Thomas, J. (1955). *Biochem. J.* **59**, 29.
Dodgson, K. S., Spencer, B. & Williams, K. (1955). *Biochem. J.* **61**, 374.
Dodgson, K. S., Spencer, B. & Wynn, C. H. (1956). *Biochem. J.* **62**, 500.
Dodgson, K. S. & Wynn, C. H. (1958). *Biochem. J.* **68**, 387.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Roy, A. B. (1953*a*). *Biochem. J.* **53**, 12.
Roy, A. B. (1953*b*). *Biochem. J.* **55**, 653.
Roy, A. B. (1956). *Biochem. J.* **62**, 35 P.
Roy, A. B. (1957). *Experientia*, **13**, 32.
Roy, A. B. & Kerr, L. M. H. (1956). *Nature, Lond.*, **178**, 376.
Spencer, B. (1958). *Biochem. J.* **69**, 155.