REFERENCES

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Hartwell, G. A. (1926). *Biochem. J.* **20**, 751.
Hubbell, R. B., Mendel, L. B. & Wakeman, A. J. (1937). *J. Nutrit.* **14**, 273.
King, E. J. & Delory, G. E. (1940). *Enzymologia*, **8**, 278.
Lawrie, N. R. & Yudkin, J. (1949). *Biochem. J.* **45**, 438.
Long, C. (1951). *Biochem. J.* **50**, 407.
Lutwak-Mann, C. (1952). *Biochem. J.* **52**, 356.
Motzok, I. (1950). *Biochem. J.* **47**, 193.
Motzok, I. & Wynne, A. M. (1950). *Biochem. J.* **47**, 187.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Verzár, F. & McDougall, E. J. (1936). *Absorption from the Intestine.* London: Longmans Green and Co.
Westenbrink, H. G. K. (1934). *Arch. néer. Physiol.* **19**, 563.

The Sulphatase of Ox Liver

1. THE COMPLEX NATURE OF THE ENZYME

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The enzyme sulphatase present in many animal tissues (Fromageot, 1938) has up to the present been little studied in comparison with the corresponding enzyme of *Aspergillus oryzae*. In view of the importance of sulphuric acid esters in many metabolic processes in the animal body it appeared that the sulphatase of animal tissues might be worthy of a detailed study. The method of assay used in the present work was based on that of Robinson, Smith & Williams (1951) which utilizes potassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) as substrate, and follows the sulphatase activity by the colorimetric estimation of the liberated 4-nitrocatechol. This method is not applicable to whole homogenates of animal tissues, especially liver, as such homogenates are apparently capable of metabolizing nitrocatechol, a fact which has recently been noted by Dodgson, Spencer & Thomas (1952). The method is, however, suitable for the assay of sulphatase in the partly purified preparations used in the present investigations, as under such conditions the recovery of added nitrocatechol is quantitative.

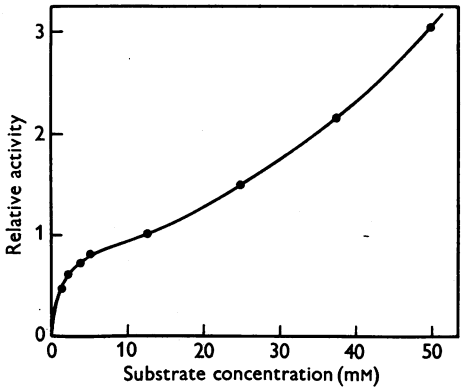

Fig. 1. Effect of varying substrate concentrations on reaction velocity. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. unfractionated sulphatase preparation. Incubated 1 hr. at 37° in 0·05 M-citrate buffer, pH 6·0.

Preliminary investigations using this substrate showed that crude sulphatase preparations gave a very anomalous substrate concentration-reaction velocity curve (Fig. 1) and it appeared that more than one enzyme might be present. This paper describes the preparation of two distinct sulphatase fractions from such crude solutions.

METHODS

Preparation of substrate

The method used was essentially that of Smith (1951), except that it was found more convenient to prepare the substrate as the dipotassium 2-hydroxy-5-nitrophenyl sulphate instead of the monopotassium salt.

To 70 g. KOH and 70 g. potassium persulphate dissolved in 1 l. water were added 30 g. *p*-nitrophenol. The reaction mixture was left 48 hr. at 37°, then acidified to pH 4 with H_2SO_4, and free phenols extracted with ether. The aqueous solution was then made strongly alkaline to litmus with KOH and concentrated *in vacuo* to about 300 ml. The solution, and any precipitate, was poured into 2 vol. acetone and the mixture filtered. After washing the residue with acetone-water (2:1, v/v), the combined filtrates were taken to dryness *in vacuo* and the residue recrystallized three times from water. Bright yellow crystals of dipotassium 2-hydroxy-5-nitrophenyl sulphate dihydrate were obtained with a yield

of about 3 g. (Found: C, 20·2; H, 1·87; N, 4·02; K, 23·0. Calc. for $C_6H_3O_7NSK_2.2H_2O$: C, 20·3; H, 2·03; N, 4·03; K, 22·5%.)

Estimation of 4-nitrocatechol

Robinson *et al.* (1951) estimated the 4-nitrocatechol by means of the red colour developed in strongly alkaline solution. This colour, although stable when pure nitrocatechol solutions were used, faded rapidly in the presence of liver homogenates, even after precipitation of the proteins. It was found by the author that this fading could be prevented and the colour stabilized by the addition of quinol, Na_2SO_3 also being added in order to prevent the rapid oxidation of the quinol in the strongly alkaline solution.

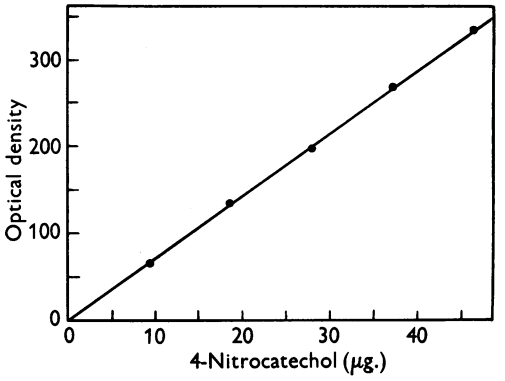

Fig. 2. Calibration curve of 4-nitrocatechol estimation.

The alkaline quinol reagent was made up immediately before use by adding 5 ml. of a 4% solution of quinol in 0·1 N-HCl (made up weekly) to 100 ml. of 2·5 N-NaOH containing 5% $Na_2SO_3.7H_2O$, giving a final quinol concentration of approximately 0·2%. The calibration curve (Fig. 2) was prepared by adding 0·2 ml. water to 0·6 ml. of standard 4-nitrocatechol solutions, followed by 3 ml. 2% phosphotungstic acid in 0·1 N-HCl. After mixing, 3 ml. of the solution were pipetted into 5 ml. freshly prepared alkaline quinol reagent. The red colours were read in a Spekker absorptiometer, using Ilford filter no. 604 (520 mμ.), against a reagent blank. The colour is stable for at least 3 hr., and the Lambert-Beer law is obeyed to an optical density of 0·7, corresponding to 100 μg. of nitrocatechol.

The intensity of the colour is independent of the concentration of the NaOH used within wide limits (1–5 N-NaOH) and of the presence or absence of Na_2SO_3. Quinol increases the intensity of the colour appreciably, but again the intensity is independent of the quinol concentration, within the limits of 0·1–0·3% in the alkaline reagent. When the phosphotungstic acid is pipetted into the alkaline quinol reagent a blue colour is produced, which fades rapidly and does not interfere with the estimations.

Preparation of the enzyme

Fresh ox liver was cut into 1 in. cubes and 100 g. portions were thrown into 500 ml. acetone at 0° in a chilled 'Atomix' blender: the mixture was homogenized for 1 min., filtered, and washed successively with 500 ml. portions of acetone and ether at 5°. After sucking dry at the pump the filter cake was broken up and dried *in vacuo* over P_2O_5. The material was sufficiently dry to powder and sieve within a few hours. When kept *in vacuo* at room temperature, the powder retained its enzymic activity for many weeks.

The enzyme solution was prepared by extracting 3 g. of the acetone powder with 20 ml. water for 1 hr. at 37°. The bulk of the debris was removed by centrifuging for 15 min. at 2000 rev./min. and the supernatant clarified by a further 30 min. centrifuging at 10 000 rev./min.

Fractionation of the enzyme

To 20 ml. of the aqueous extract prepared as above were added 2 ml. 0·1 M-phosphate buffer, pH 6·9; the mixture was cooled to 0°, and 16·5 ml. chilled acetone were slowly run in with stirring, the temperature being lowered to −9° during the process, giving a final acetone concentration of 43%, v/v. After standing 30 min. at −9°, the precipitate was removed by centrifuging at the same temperature and dissolved in 15 ml. water, giving a solution of fraction *B*. To the supernatant from this first precipitation (29 ml.), kept at −9°, was added 0·8 ml. of the phosphate buffer followed by 12·5 ml. chilled acetone, to give a final acetone concentration of 60%. The precipitate was removed by centrifuging as before and dissolved in 15 ml. water, giving fraction *A*.

Both fractions were dialysed overnight at room temperature against running water, and any insoluble material was removed by centrifuging. The solutions so obtained were diluted 5 times with distilled water to give enzyme concentrations suitable for assay by the method described below.

Estimation of enzymic activity

To 0·2 ml. 0·5 M-acetate buffer of the appropriate pH (4·7 and 5·7 in the case of fractions *A* and *B* respectively) was added 0·4 ml. of a solution of the substrate (0·006 and 0·066 M-nitrocatechol sulphate respectively for fractions *A* and *B*) adjusted to the same pH as the buffer with 0·1 N-HCl. The tubes were brought to 37°, and 0·2 ml. enzyme solution, also at 37°, was added. After incubating for 1 hr., 3 ml. 2% phosphotungstic acid in 0·1 N-HCl were added and, after mixing, the precipitated proteins removed by centrifuging. For colour development, 3 ml. of the clear supernatant were pipetted into 5 ml. alkaline quinol reagent as described above.

Assays were run in duplicate, and suitable blanks were obtained by incubating the buffered substrate in the absence of the enzyme, which was only added to the tubes immediately before precipitating the proteins with the phosphotungstic acid. The assays were read in the Spekker against the appropriate blanks.

In typical assays the amount of substrate hydrolysed during incubation was approximately 10 and 1% of that initially present in the case of fractions *A* and *B* respectively.

RESULTS

Effect of pH. The effect of pH was determined over the range 4–7 in 0·5 M-acetate buffers at a final substrate concentration of 0·003 M in the case of fraction *A* and 0·033 M in the case of fraction *B*. The substrate solutions were adjusted to pH values corresponding to those of the buffers used, as nitrocatechol sulphate is itself a strong buffer over the

pH range 5–7. This fact allowed the use of acetate buffers at the extreme alkaline end of their normal range. For the determination of the optimum pH, 0·2 ml. enzyme was added to 0·4 ml. substrate solution and 0·2 ml. 0·5M-acetate buffer, giving a final acetate concentration of 0·15M. Incubation and colour development were carried out as described above.

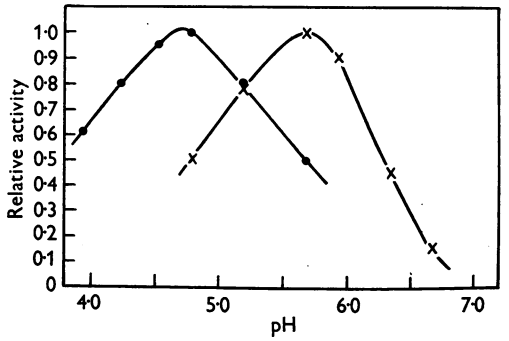

Fig. 3. Effect of pH on reaction velocity. Incubated for 1 hr. at 37° in 0·15M-acetate buffers of varying pH. Substrate concentrations 0·003 and 0·033M-nitrocatechol sulphate for fractions *A* and *B* respectively. Final volume 0·8 ml. containing 0·2 ml. enzyme solution. 'Relative activity 1·0' corresponds to a liberation of 39 and 56 μg. nitrocatechol per tube in the case of fractions *A* and *B* respectively. —●—●—, fraction *A*; —×—×—, fraction *B*.

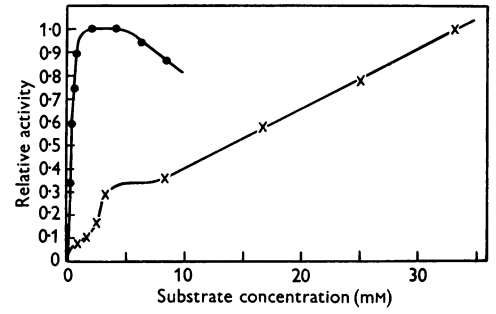

Fig. 4. Effect of varying substrate concentration on reaction velocity. Incubated for 1 hr. at 37° with varying concentrations of nitrocatechol sulphate in 0·15M-acetate buffers of pH 4·7 and 5·7 for fractions *A* and *B* respectively. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution. 'Relative activity 1·0' corresponds to a liberation of 31 and 65 μg. nitrocatechol per tube in the case of fractions *A* and *B* respectively. —●—●—, fraction *A*; —×—×—, fraction *B*.

The results are shown in Fig. 3, which indicates that the pH optima for fractions *A* and *B* are respectively 4·7 and 5·7 in 0·15M-acetate buffer, with nitrocatechol sulphate as substrate.

Effect of substrate concentration. Fig. 4 shows the effect of varying substrate concentration on the reaction velocity at a constant pH, i.e. 4·7 and 5·7 in the case of fractions *A* and *B* respectively.

With the fraction *A* there was an optimum substrate concentration of approximately 0·003M-nitrocatechol sulphate, above which concentration substrate inhibition occurred to some extent. With the *B* fraction there was no obvious substrate optimum within the limits studied. These limits were set by the solubility of the substrate and could only be overcome by radical changes in the method of assay. The hump in the curve for the *B* fraction at a concentration of 0·004M-nitrocatechol sulphate is presumably due to slight contamination by the *A* fraction.

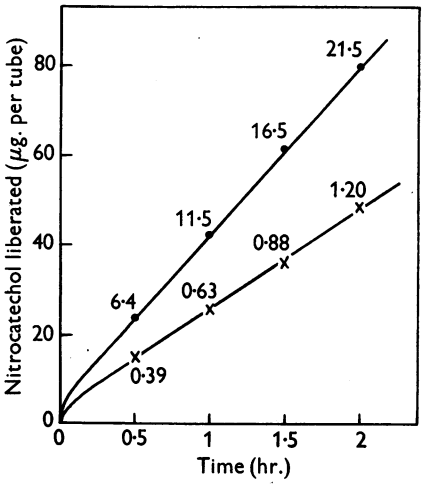

Fig. 5. Effect of time of incubation on degree of hydrolysis. In both cases the final volume of the reaction mixture was 0·8 ml. containing 0·2 ml. enzyme solution and the incubation carried out at 37°. For the assay of fraction *A* the substrate concentration was 0·003M and the buffer 0·15M-acetate, pH 4·7; for fraction *B* the corresponding figures were 0·033M and pH 5·7. The figure above each point represents the percentage hydrolysis of the substrate at the corresponding time. —●—●—, fraction *A*; —×—×—, fraction *B*.

Effect of time of incubation. Fig. 5 shows the effect of time of incubation on the degree of hydrolysis of the substrate, the enzymes working under optimal conditions of pH and substrate concentration. The degree of hydrolysis is essentially proportional to the time of incubation, apart from a slight decrease in the reaction velocity during the first 10 min. of incubation.

Effect of enzyme concentration. Fig. 6 shows the effect of varying enzyme concentrations on the reaction velocity under optimal conditions of pH and substrate concentration. With fraction *B*

there is a direct proportionality between the enzyme concentration and the reaction velocity; such a simple relationship does not appear to exist in the case of fraction *A*. This anomalous behaviour may well be due to the fraction being impure, although several preparations gave similar curves.

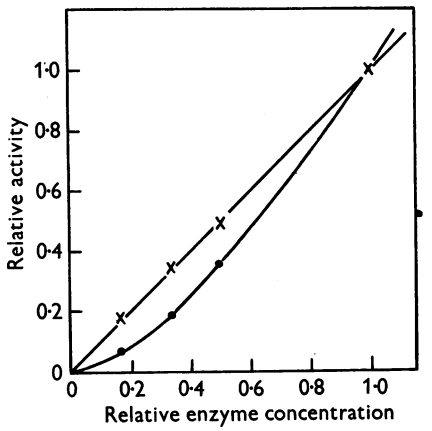

Fig. 6. Effect of enzyme concentrations on reaction velocity. Conditions as in legend of Fig. 5 except that the enzyme concentration was varied and the time of incubation kept constant at 1 hr. 'Relative velocity 1·0' represents a liberation of 75 and 51 μg. nitrocatechol per tube for fractions *A* and *B* respectively —●—●—, fraction *A*; — × — × —, fraction *B*.

Intracellular location of the enzyme. Preliminary data indicated that a considerable proportion of the sulphatase activity in a water homogenate of ox liver was insoluble, and a few experiments have been carried out with mouse liver in order to determine the localization of the sulphatase in the liver cell. These assays were carried out in 0·1 M-citrate buffer, pH 6, and in 0·05 M-nitrocatechol sulphate, thus determining essentially the activity of the *B* fraction. Cell fractionation was carried out in 0·25 M-sucrose, according to Schneider & Hogeboom (1950) and sulphatase estimations were carried out on the washed nuclear and mitochondrial fractions (sedimented at 700 and 5000 *g*. respectively) and on the supernatant from the above two fractions. Under the above conditions it was found that some 70% of the sulphatase activity was present in the mitochondria, the bulk of the remainder being present in the supernatant fraction. These figures must be regarded as semi-quantitative due, as pointed out above, to the destruction of nitrocatechol by tissue homogenates: they indicate, however, that a high proportion of the sulphatase activity is localized in the mitochondria.

DISCUSSION

The results reported above indicate that the sulphatase of ox liver is a more complex enzyme than has previously been thought, two distinct sulphatase fractions being present. The two fractions, so far only obtained in an impure state, differ in their pH optima and in their response to variations in substrate concentration; fraction *A* having a pH optimum of 4·7 in acetate buffer and an optimum substrate concentration of 0·003 M-nitrocatechol sulphate, whereas fraction *B* has a pH optimum of 5·7 in acetate buffer and an anomalous response to variations in substrate concentration. From the data presented it may be concluded that the sulphatase of animal tissues is very different in properties from the sulphatase of *A. oryzae* which has, according to Robinson *et al.* (1951), an optimum pH of 5·9 in acetate buffer, and an optimum substrate concentration 0·0025 M-nitrocatechol sulphate. This difference in properties has not been stressed by previous workers, many of whom (Huggins & Smith, 1947; Robinson *et al.* 1951) have applied methods based on kinetic data obtained with mould sulphatase to the assay of the corresponding enzyme in animal tissues. Much of the earlier work on the animal sulphatases is also vitiated by the lack of appreciation of the insoluble nature of the enzyme in water homogenates, part of the enzyme at least being associated with the mitochondria.

SUMMARY

1. A method is described for the assay of sulphatase, using nitrocatechol sulphate as substrate.

2. Two fractions exhibiting sulphatase activity have been obtained from an aqueous extract of an acetone powder of ox liver by fractional precipitation with acetone. The properties of these fractions are given.

3. A large part of the sulphatase activity is localized in the mitochondria, and is insoluble in water homogenates.

The author wishes to express his indebtedness to Prof. R. T. Williams who supplied details of his nitrocatechol method prior to its publication. Thanks are also due to Dr J. W. Minnis who carried out the micro-analysis.

REFERENCES

Dodgson, K. S., Spencer, B. & Thomas, J. (1952). *Biochem. J.* **51**, i.

Fromageot, C. (1938). *Ergebn. Enzymforsch.* **7**, 50.

Huggins, C. & Smith, D. R. (1947). *J. biol. Chem.* **170**, 391.

Robinson, D., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **49**, lxxiv.

Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 124.

Smith, J. N. (1951). *J. chem. Soc.* p. 2861.