2. Each oil contained similar quantities of saturated acids (33–35 %), mainly palmitic (24–27 %), but differed in the proportions of the various unsaturated acids. These differences are particularly evident in the content of hexadecenoic acid, octadecadienoic acid, and the unsaturated C_{20-22} acids.

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The Sulphatase of Ox Liver

3. FURTHER OBSERVATIONS ON SULPHATASE B AND AN INVESTIGATION OF THE ORIGIN OF FRACTIONS A AND B

By A. B. ROY

Department of Biochemistry, University of Edinburgh

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The previous communications of this series have described the occurrence of two fractions exhibiting sulphatase activity in an aqueous extract of an acetone powder of ox liver, and the purification of one of these fractions, sulphatase A (Roy, 1953*a*, *b*). The present paper describes the further purification of the second fraction, sulphatase B, as a preliminary to the study of the relationship between the two fractions, more especially the possibility of artifact formation during their preparation. This is of particular interest as Dodgson, Spencer & Thomas (1953) have not detected two fractions in their preliminary study of rat liver sulphatase, although unpublished work from this laboratory has shown that two fractions, comparable to fractions A and B of ox liver, can be isolated from an acetone powder of rat liver by the methods already described (Roy, 1953*a*).

EXPERIMENTAL

Preparation of the enzyme

The general methods of preparing the acetone-powder extract and of fractional precipitation with acetone or $(NH_4)_2SO_4$ were as described previously (Roy, 1953*a*, *b*). Protein estimations were kindly performed by Dr L. M. H. Kerr using a slight modification of the Folin phenol-method

of Lowry, Rosenbrough, Farr & Randall (1951). In what follows, one sulphatase B unit (s.u.) is defined as the amount of enzyme which will liberate 1 μ g. nitrocatechol under the standard conditions described below.

Stage B. This was obtained by the precipitation of 800 ml. aqueous extract with 43% (v/v) acetone in phosphate buffer, pH 7, at -9° as described by Roy (1953*a*). The precipitate so obtained was dissolved in water and dialysed overnight against running tap water at room temperature, giving 400 ml. of sulphatase B (700 000 s.u., 25 s.u./mg. protein).

Stage B-1. To 400 ml. sulphatase B were added 26 ml. 0.5M sodium acetate, pH 6.5, and 4.5 ml. 0.3M- $CaCl_2$. The enzyme was then precipitated by the addition of 220 ml. acetone (34%, v/v, final concentration), the temperature being lowered to -9° during the process. After equilibration at that temperature the precipitate was centrifuged off, dissolved in 100 ml. water and dialysed as above. A copious inactive precipitate was separated, giving 250 ml. sulphatase B (stage B-1; 500 000 s.u., 36 s.u./mg. protein).

Stage B-2. 250 ml. B-1 were made 0.3 saturated with respect to $(NH_4)_2SO_4$ by the slow addition of 55 g. solid $(NH_4)_2SO_4$. After standing a few hours the precipitate was centrifuged off and discarded. The supernatant was made 0.5 saturated with respect to $(NH_4)_2SO_4$ by the addition of the calculated amount of solid and after equilibration the active precipitate was centrifuged off, dissolved in water and dialysed overnight. This $(NH_4)_2SO_4$ fractionation was then repeated on the dialysate, giving a clear solution of sulphatase B-2 (200 000 s.u., 190 s.u./mg. protein).

Estimation of sulphatase B activity

The substrate used was dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) and the enzymic activity was followed by the colorimetric estimation of the liberated 4-nitrocatechol (Robinson, Smith, Spencer & Williams, 1952; Roy, 1953a). A buffered substrate solution was prepared by dissolving 250 mg. nitrocatechol sulphate (dihydrate) in approximately 5 ml. hot water; after adding 0.58 ml. *N*-HCl and 3 ml. 0.5M acetate buffer, pH 5.7, the volume was made up to 9 ml. with water, giving a 0.08M solution of nitrocatechol sulphate, pH 5.7. This solution was stored at 37°, as below this temperature crystallization occurred. For assay, 0.2 ml. enzyme solution was mixed with 0.6 ml. buffered substrate, both solutions being at 37°, and the reaction mixture incubated 1 hr. at that temperature. The final substrate concentration was therefore 0.06M nitrocatechol sulphate in 0.15M acetate buffer, pH 5.7. After incubation the solution was deproteinized with 3 ml. 2% (w/v) phosphotungstic acid in 0.1N-HCl and the liberated nitrocatechol estimated colorimetrically by means of the red colour developed in an alkaline quinol solution. Assays were run in duplicate along with appropriate blanks. Under the above conditions the reaction velocity was directly proportional to the enzyme concentration and the degree of hydrolysis of the substrate to the time of incubation for periods of up to 2 hr. If the protein-free supernatant from the phosphotungstic acid precipitation was left for some time before making alkaline, a copious white precipitate slowly separated. No attempt was made to remove this precipitate as it immediately dissolved in the alkaline quinol. The precipitation was apparently due to a high concentration of electrolytes.

Electrophoresis on paper

Paper electrophoresis was carried out in an apparatus developed from that of Latner (1952). Approximately 0.07 ml. of the solution to be analysed (containing approximately 4 mg. protein) was lined on a strip (50 × 6 cm.) of Whatman no. 100 paper and run for 16 hr. at 200 v in a veronal-acetate-HCl buffer of suitable pH, usually pH 7.1. The various components were localized by cutting the paper into 1 cm. strips and assaying portions of these strips (usually 1 × 3 cm.) as described below.

Total protein. The strip was immersed in 1.0 ml. diluted Folin and Ciocalteu reagent (British Drug Houses reagent diluted 1 in 10 with water) and allowed to stand for 15 min.; 1 ml. *N*-Na₂CO₃ was then added and the tubes were incubated 15 min. at 37° to develop the colour, the intensity of which was indicative of the amount of protein present.

Sulphatases A and B. These were localized by incubating the strips for a suitable time (normally 1 hr.) at 37° in 0.8 ml. acetate-buffered nitrocatechol sulphate, 0.003M, pH 5.0, for sulphatase A, and 0.06M, pH 5.7, for sulphatase B. After incubation, the reaction was stopped with phosphotungstic acid and the colour developed with alkaline quinol as usual.

Acid phosphatase. The strips were incubated for 1 hr. at 37° in 0.8 ml. 0.0025M disodium *p*-nitrophenyl phosphate in 0.15M acetate buffer, pH 5.0. Phosphatase activity was detected by the addition of 8 ml. 0.6N-Na₂CO₃ to develop a yellow colour in these tubes containing liberated *p*-nitrophenol.

Esterase. This was detected by the method of Huggins & Lapiere (1947) by incubating the strips in 2 ml. 0.06M phosphate buffer, pH 7, 2 ml. 0.066M *p*-nitrophenyl acetate, and 6 ml. water at 20°. In the presence of an esterase a yellow colour developed.

All these methods could be made semi-quantitative by reading the intensity of colours in the Spekker Absorptiometer after removing the filter paper by centrifuging.

RESULTS

Properties of sulphatase B

Effect of variations in substrate concentration. The effect of variations in substrate concentration on the reaction velocity is shown in Fig. 1. It is obvious that the maximum velocity has not been reached even at a substrate concentration of 0.06M nitrocatechol sulphate. The results give a good fit of the Lineweaver & Burke (1934) equations, and a number of experiments gave values of K_m between 0.06M and 0.08M nitrocatechol sulphate. It is clear that the optimum substrate concentration must be exceptionally high, in the region of 0.2M nitrocatechol sulphate, a region in which it is impossible to work owing to the limited solubility of the substrate.

Effect of pH. Buffered substrate solutions were made up as described above using acetate buffers of varying pH and the appropriate amount of *N*-HCl, as determined from the titration curve of nitro-

Fig. 1. Effect of varying substrate concentration on the reaction velocity. Incubated 1 hr. at 37°, with varying concentrations of nitrocatechol sulphate in 0.15M acetate buffer, pH 5.7. Final volume of reaction mixture 0.8 ml. containing 0.2 ml. enzyme solution; ●—●, plot of reaction velocity against substrate concentration; ×—×, the above data plotted according to the equation derived by Lineweaver & Burke (1934)

$$\frac{s}{v} = \frac{1}{V} s + \frac{K_m}{V},$$

where s is the substrate concentration, v and V the observed and maximum velocities respectively, and K_m the Michaelis constant.

catechol sulphate. As Fig. 2 shows, the optimum pH was 5.7 under such conditions. The use of acetate buffers of such high pH values is justified by the very strong buffering action of nitrocatechol sulphate in that region, the phenolic group having a pK of approximately 6.4.

When citrate buffers were used in place of acetate, a considerable inhibition occurred, but the position of the optimum was unchanged at pH 5.7.

Inhibition studies. These were normally carried out by dissolving the inhibitor to the required concentration in 0.17 M acetate buffer, pH 5.7, adjusting the pH as necessary. For assay, 0.1 ml. of the buffered inhibitor was mixed with 0.5 ml. buffered substrate and 0.2 ml. enzyme added, so that the final substrate concentration was 0.05 M nitrocatechol sulphate.

Like sulphatase *A*, sulphatase *B* was unaffected by 0.05 M-NaCl or KCl, and by 0.01 M-KCN. It was inhibited to 25% by 2.5×10^{-2} M- Na_2SO_4 and to 40% by 3×10^{-4} M- Na_2SO_3 . Investigation of the inhibition by these substances suggested that it was non-competitive with values of K_i of approximately 7×10^{-2} M- Na_2SO_4 and 5×10^{-4} M- Na_2SO_3 , respectively. Too much stress must not be laid on the non-competitive nature of the inhibition as the range of substrate concentrations studied was of necessity small. A number of sulphuric acid esters were studied as possible inhibitors of sulphatase *B*. The results are not reported as they showed nothing of interest, the inhibition in all cases being rather less than the inhibition of sulphatase *A* by the same concentration of any given ester (Roy, 1953*b*). Sulphatase *B* was not inhibited by a final concentra-

tion of 1 mg./ml. of the methiodide of *m*-dimethylaminophenyl methyl carbamate, a potent inhibitor of liver esterase (Stedman & Stedman, 1931). Sulphatase *A* is likewise uninhibited by this urethane.

Activation energy. The apparent activation energy for the hydrolysis of nitrocatechol sulphate by sulphatase *B* was determined at pH 5.7 and a substrate concentration of 0.06 M nitrocatechol sulphate by determining the enzymic activity at various temperatures between 20 and 40°. From the results, shown in Fig. 3, the activation energy was calculated (Arrhenius, 1889) to be 13000 cal./mole. As pointed out by Gibson (1953), on theoretical grounds, the true activation energy can only be determined at substrate concentrations approaching the optimum; at lower concentrations the determined value is greater than the true value by a variable amount, so that the above value of 13000 cal./mole is a maximal figure. It should be noted, however, that Mills, Paul & Smith (1953) claimed that the determined activation energy for the hydrolysis of phenyl glucuronide by β -glucuronidase was less than the true value at substrate concentrations below the optimum.

Electrophoretic investigation of sulphatase *B*. Preparations of sulphatase *B-2* had been shown in preliminary work (unpublished observations) to exhibit considerable acid phosphatase and esterase activity. It has in the past been implied that sulphatase might be identical with a phosphatase or an esterase (Hommerberg, 1931) and it was therefore decided to attempt the separation of the three enzymes electrophoretically.

Fig. 2. Effect of pH on reaction velocity. Incubated 1 hr. at 37° in 0.15 M acetate or citrate buffers. Substrate concentration 0.06 M nitrocatechol sulphate. Final volume of reaction mixture 0.8 ml. containing 0.2 ml. enzyme solution; ●—●, acetate buffer; ×—×, citrate buffer.

Fig. 3. Plot of log reaction velocity ($\log v$) against the reciprocal of absolute temperature ($1/T$) for two concentrations of enzyme. Substrate concentration 0.06 M nitrocatechol sulphate, pH 5.7 in 0.15 M acetate buffers, final volume of reaction mixture 0.8 ml. containing 0.2 ml. enzyme. Incubated 1 hr.

Fig. 4. Typical results of electrophoretic investigations of sulphatase preparations. Run 16 hr. at 200 v; pH 7.1 in veronal buffer.

The results of typical experiments are indicated in Fig. 4, which conclusively shows the non-identity of the three enzymes. For comparison, the migration under similar conditions of sulphatase *A* is shown, and it is obvious that this enzyme is also distinct. It was also possible to separate by electrophoresis the two types of sulphatase activity from a mixture of sulphatases *A* and *B*.

Origin of sulphatases A and B

The low affinity of sulphatase *B* for its substrate suggested the possibility that it might be an artifact produced by the acetone treatments utilized in its preparation. In fact, the possibility could not be excluded that both sulphatases were artifacts. This problem was investigated by a study of the kinetics of various types of enzyme preparation and by electrophoresis.

Study of the acetone-powder extract. A sample of an unfractionated extract of an acetone powder was fractionated with acetone as previously described (Roy, 1953a) and the two fractions so obtained were combined after dialysis overnight. A similar sample of unfractionated extract was dialysed without preliminary treatment, and the volumes of both dialysed samples were adjusted so that the enzyme concentrations should have been the same in both. The effect of variations in pH on the enzymic activity of both samples was determined in 0.15M acetate buffers and at substrate concentrations of 0.003M and 0.06M nitrocatechol sulphate. Fig. 5 shows the results of such an experiment and it can be seen that no significant change in the nature of the pH effects was brought about by the acetone treatment. It was therefore concluded that both sulphatases *A* and *B* existed in an aqueous extract of an ox-liver acetone powder.

Study of liver dispersions. A 5% dispersion of fresh ox liver in water, final pH 6.7, was prepared by treatment for 30 sec. in an Atomix Blender. A sample of the dispersion was brought to pH 4.7 with dilute acetic acid, stood for 10 min. at 37°, and the pH readjusted to 6.7 with dilute Na₂CO₃, giving the 'acetate-treated' enzyme. A similar sample was

Fig. 5. Effect of pH on reaction velocity of an unfractionated extract of an acetone powder of ox liver (●—● and ○—○, at substrate concentrations of 0.06M and 0.003M nitrocatechol sulphate, respectively) and of the combined fractions *A* and *B* isolated therefrom (×—× and △—△, at substrate concentrations of 0.06M and 0.003M nitrocatechol sulphate, respectively). Assay in 0.15M acetate buffers, general conditions as in Fig. 2.

centrifuged for 10 min. at 600 *g* after incubation as above giving two fractions, the supernatant, ('acetate-soluble') which was made up to the original volume of the sample, and the residue ('acetate-insoluble'), which was suspended in water and also made up to the original volume. The pH of both these fractions was also readjusted to 6.7. The pH/activity curves of these four fractions were then determined in 0.15M acetate buffers and a substrate concentration of 0.03M nitrocatechol sulphate. Fig. 6 shows the results of typical experiments. It is obvious that there is no significant difference in the shape of the pH curves of the untreated and acetate-treated enzymes, and that the curves of the acetate-soluble and acetate-insoluble fractions are both very different in nature from those of the first two preparations. Moreover, the summation of the pH curves of the acetate-soluble and acetate-insoluble fractions gave a curve almost identical with that of the acetate-treated enzyme. It should be noted that the recovery of nitrocatechol from liver dispersions prepared as described above is quantitative at all pH values studied.

Fig. 7 shows the effect of variations in substrate concentration on all four fractions. The pH was 5.5 in 0.15M acetate buffer. Again it is obvious that acetate treatment alone causes no change in the properties of the enzyme, and that the acetate-

soluble and acetate-insoluble fractions have different properties from the whole-liver dispersion.

The above experiments suggested that both sulphatases *A* and *B* were present in the liver dispersion, and that the acetate-soluble fraction was predominantly sulphatase *B*, while the acetate-insoluble fraction was probably a mixture of approximately equivalent amounts of sulphatases *A* and *B*.

Electrophoretic investigation of whole-liver extract. An approximately 20% dispersion of fresh ox liver in water was prepared as above and centrifuged at

30 000 *g* for 1 hr. at 0°. The clear red supernatant, which contained approximately 75% of the sulphatase activity, was separated and 0.1 ml. portions were taken for electrophoresis as described above. Sulphatase activities corresponding to sulphatase *A* and sulphatase *B* could clearly be detected in such an extract, as indicated in Fig. 4, although the latter activity appeared to move more slowly than purified sulphatase *B* under comparable conditions. As the ox liver was of necessity only available about 1 hr. after the death of the animal, post-mortem changes could not be ruled out and it seemed advisable to repeat the experiment using rat liver, which could be worked up immediately on killing the animal. Again both sulphatases were detected, although there appeared to be relatively less of sulphatase *A* than in ox liver. A similar pattern was also obtained after homogenizing the rat liver in a glass homogenizer (Potter & Elvehjem, 1936) as described by Dodgson *et al.* (1953).

DISCUSSION

Little need be said of the properties of sulphatase *B*, the only point of interest being its low affinity for nitrocatechol sulphate, K_m being approximately 0.07 *M*. This low affinity of an enzyme for its substrate is uncommon, the only obvious counterpart being the low affinity of certain esterases for the simpler esters. One conclusion might be that the sulphatase activity is a secondary action exhibited by an as yet unidentified hydrolase, although the activation energy of 13 000 cal./mole for the hydrolysis of nitrocatechol sulphate by sulphatase *B* is not very different from the corresponding figure for sulphatase *A*, and is very much less than that for the acid hydrolysis of aryl sulphuric acids, which is in the region of 23 000–27 000 cal./mole (Burkhardt, Ford & Singleton, 1936). This could be taken to indicate that a true sulphatase is involved. Table 1 summarizes some properties of sulphatases *A* and *B*.

More important is the question whether or not sulphatases *A* and *B* are present in liver as such. The kinetic studies described above lead to the conclusion that both enzymes are actual constituents of liver tissue, and this view is strongly supported by the results of the electrophoretic studies. This

Table 1. *Comparison of sulphatases A and B*

In both cases the substrate was nitrocatechol sulphate.

	Sulphatase <i>A</i>	Sulphatase <i>B</i>
pH optimum (in acetate)	4.9	5.7
Optimum substrate concn.	0.003 <i>M</i>	> 0.10 <i>M</i>
K_m	8×10^{-4} <i>M</i>	7×10^{-2} <i>M</i>
$K_i^m \text{SO}_4^{2-}$	7×10^{-4} <i>M</i>	7×10^{-2} <i>M</i>
$K_i \text{SO}_3^{2-}$	2×10^{-6} <i>M</i>	5×10^{-4} <i>M</i>

Fig. 6. Effect of pH on reaction velocity of sulphatase in a dispersion of ox liver before and after various treatments; for details of which see text. Assay in 0.15 *M* acetate buffers, substrate concentration 0.03 *M* nitrocatechol sulphate; general conditions as in Fig. 2; ●—●, whole dispersion of ox liver; ×—×, 'acetate-treated'; △—△, 'acetate-insoluble'; ○—○, 'acetate-soluble'; ---, summation of 'acetate-soluble' and 'acetate-insoluble' curves.

Fig. 7. Effect of varying substrate concentration on the reaction velocity of sulphatase in a dispersion of ox liver before and after various treatments. Assay in 0.15 *M* acetate buffer, pH 5.5, general conditions as in Fig. 1; ●—●, whole dispersion; ×—×, 'acetate-treated'; △—△, 'acetate-insoluble'; ○—○, 'acetate-soluble'.

contrasts with the findings of Dodgson *et al.* (1953) who did not detect two enzyme fractions in their study of rat liver sulphatase, although both sulphatases *A* and *B*, isolated from ox liver, will hydrolyse potassium *p*-acetylphenyl sulphate, the substrate used by these workers. It is possible, however, that the method used by Dodgson *et al.* (1953) would not show the presence of two enzymes. Simple kinetic studies on a whole-liver dispersion would not necessarily show that two enzymes were present unless these studies were designed for that purpose. Investigation of the effect of variations of pH and substrate concentration on the sulphatase activity of whole-liver preparations could well lead to the conclusion that only one enzyme was present as such curves are often remarkably smooth, although they are the resultants of two components, corresponding to sulphatases *A* and *B* respectively. Consideration of the pH curve published by Dodgson *et al.* (1953) shows that their results are not inconsistent with the view that two enzymes are present, as the curve shows a flattening in the region of pH 6.5–7.2, analogous to a flattening between pH 5.0 and 5.7 obtained on many occasions in the present study of whole liver dispersions.

The differing pH optima in the two sets of results are not difficult to explain if it be postulated that the formation of the enzyme–substrate complex is due to, or assisted by, electrostatic attraction between the —SO_4^- group of the substrate and a positively charged active centre on the enzyme molecule. At pH 7, the phenolic group of nitrocatechol sulphate is ionized and so might well compete with the —SO_4^- group for the active centre: below pH 5.5 the phenolic group is not appreciably ionized and so could not compete with the —SO_4^- grouping. On this basis it would be expected that the optimum pH for the hydrolysis of nitrocatechol sulphate would be lower than that for aryl sulphates not containing a second ionizable group. This is in agreement with the results reported above.

The most important conclusion is that results of studies of the distribution of sulphatase in various tissues must be accepted with considerable caution until these tissues have been studied with regard to their content of sulphatases *A* and *B*.

SUMMARY

1. A method is given for the partial purification of sulphatase *B* and the properties of the enzyme are described.

2. Sulphatases *A* and *B* have been shown by electrophoretic studies to be distinct from esterase and from acid phosphatase.

3. It has been shown that sulphatases *A* and *B* exist in an aqueous extract of fresh liver both from the ox and from the rat.

4. The difference between the above findings and those of other workers are discussed, and the need for caution in the interpretation of earlier results is stressed.

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