STUDIES ON THE SULPHATASES.

A Thesis

presented by

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for the degree

of

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of the

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CONTENTS.

INTRODUCTION.

Although the occurrence of sulphate esters in natural products had been recognised since the work of Baumann (1976) the existence of the corresponding hydrolytic enzymes, the sulphatases, was not appreciated until much more recently. The first suggestion of the occurrence of a sulphatase was provided by the work of Derrien (1911) who showed that the mollusc Murex trunculus could form indigo from potassium indoxyl sulphate, but it was not until 1923 that the existence of a sulphatase was definitely proved by the work of Neuberg & Kurono. These authors showed the hydrolysis of potassium indoxyl sulphate by a sulphatase present in Taka-diastase, a commercial preparation obtained from the mould Aspergillus oryzae. This slow start to the study of the sulphatases was followed by a period of rapid development due almost entirely to Neuberg and his school in Berlin and to Soda and his pupils in Tokyo. These workers showed the existence of sulphatases of several different types, each one being characterised by its substrate specificity. The first of these types to be studied in detail was that of the arylsulphatases, discovered by Neuberg & Kurono in Taka-diastase

and subsequently demonstrated in many animal tissues. These enzymes were soon shown to be specific catalysts for the hydrolysis of a wide range of aryl sulphates. The next member of the group to be discovered was the rather obscure enzyme myrosulphatase, hydrolysing sinigrin and the related mustard oil glycosides: this enzyme was found in the seeds of white mustard (Sinapis alba) by Neuberg & Wagner (1926) and in horse and guinea-pig livers by the same authors in the following year. In 1931 Neuberg & Hofmann discovered the existence of a chondrosulphatase, hydrolysing chondroitin sulphuric acid, in certain putrefactive bacteria and in the same year Soda & Hattori described a glucosulphatase, hydrolysing glucose-6-sulphate, in tropical marine molluscs. Thus by 1931 four of the presently known types of sulphatase had been discovered. After this initial period of intensive study, however, interest in these enzymes waned and until recently there were no significant advances in this field.

Since this early work only one further type of sulphatase has definitely been characterised. This is steroid sulphatase, hydrolysing potassium

dehydroepiandrosterone sulphate, which was shown to occur in the digestive juices of the snail Helix pomatia and in mammalian livers by Henry, Thevenet & Jarrige (1952) and by Gibian & Bratfisch (1956) respectively. There is also some indirect evidence which suggests the existence of an alkylsulphatase, hydrolysing alkyl sulphates. Vlitos (1953) has shown that Bacillus cereus mycoides can convert 2,4-dichlorophenoxyethyl sulphate into the corresponding phenoxyacetic acid. The metabolic pathway has not been investigated but it would seem that prior to oxidation removal of the sulphate group must occur, presumably by an enzymic mechanism as the sulphate ester is quite stable in vitro. There are also references in the Japanese literature to a choline sulphatase, hydrolysing choline sulphate ester. This claim is based on the observation (Egami & Itabashi, 1951) that Aspergillus oryzae can utilise choline sulphate as a source of inorganic sulphate. Such a choline sulphatase might well be a general alkylsulphatase similar to the one postulated to occur in B. mycoides. Kahane & Sirchis (1953) claimed that the oral administration of choline

sulphate to guinea-pigs caused an increased urinary excretion of inorganic sulphate which would suggest the occurrence of a choline sulphatase in this species. Unfortunately, attempts to repeat these observations in this laboratory have not been successful.

At present, therefore, five types of sulphatase are known to exist and the occurrence of at least one other type seems to be highly probable. These enzymes are of very widespread distribution, as is summarised in the following table. References are only given to work which has appeared since the recent review of the sulphatases by Dodgson (1957).

Arylsulphatase	Animals, fungi, bacteria.
	Sinapis nigra (Baum & Dodgson, 1957).
Myrosulphatase	Mammals, Sinapis spp.
Chondrosulphatase	Mammals, molluscs, bacteria.
Glucosulphatase	Mammals, molluscs.
Steroid sulphatase	Mammals (Gibian & Bratfisch, 1956).
	Molluscs.
Alkylsulphatase	Fungi, bacteria.

Not all of these groups have been investigated

in detail and the only one which has been exhaustively studied is that of the aryl-sulphatases. These are by far the most widespread of the sulphatases and they have been found in all animals, vertebrate or invertebrate, so far studied, as well as in many other types of organism.

When the present work was commenced in 1950 very little information was available on the sulphatases of mammalian tissues and it had been assumed by several workers that the mammalian enzymes were similar in properties to the arylsulphatase of Taka-diastase which had been studied to a much greater extent. This assumption was quite unjustifiable, the more so as the early work of Neuberg had shown quite conclusively that the sulphatases of Taka-diastase and of mammalian liver were quite distinct. The general purpose of the work which was envisaged was an investigation of the properties of mammalian arylsulphatase with a view to elucidating the function of the enzyme in the body. As it transpired, the problem was very much more complex than had been expected and only the first of the objectives has been attained with

any degree of success.

In the early stages of the work the substrate used was either phenyl sulphate or m-tolyl sulphate and the enzymic activity was followed by the colorimetric determination of the liberated phenol by means of Folin & Ciocalteu's reagent. This stage of the investigation was not successful. The few significant results obtained in a study of the arylsulphatase of mouse liver indicated that the enzyme was present only in small amounts, that it was insoluble and that it was localised in the microsomes. Many of the difficulties were due to the fact that the tissue preparations had to be used in high concentrations because of the low activity of the enzyme: this meant that the blank values, due to endogenous phenols, were very high. In attempts to overcome this difficulty p-aminophenyl sulphate and phenolphthalein sulphate were synthesised but unfortunately neither compound was hydrolysed at a significant rate by mouse liver arylsulphatase, although both were attacked by the sulphatase of Taka-diastase. In 1951 Smith described the preparation of potassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) and its use as a substrate in the assay of the

sulphatase of Taka-diastase. This method was adapted for the study of mammalian arylsulphatase and was applied with immediate success. The initial experiments with mouse liver indicated the presence of a complex soluble arylsulphatase localised mainly in the mitochondria. The differences between the results obtained using phenyl sulphate on the one hand and nitrocatechol sulphate on the other were very striking and it was apparent that to investigate these differences thoroughly it would be advantageous to separate the enzymes involved. Ox liver was therefore chosen as the starting material for the preparation of the arylsulphatases on a large scale. The results of this work are described in the following section.

Although the main part of the work was concerned with the arylsulphatases of ox liver two other aspects of the more general metabolism of sulphate esters were studied and these results are also reported below.

The first of these subsidiary topics was that of molluscan steroid sulphatase. This problem was studied primarily from the standpoint of the specificity of the enzyme in view of its

possible importance as a means of hydrolysing urinary steroid sulphates prior to their assay. During the course of the investigation a sensitive method for the determination of steroid sulphates was developed and this method was applied to the second problem, that of the synthesis of steroid sulphates by rat liver. At that time there had been no investigation of the formation of steroid sulphates in vitro although the synthesis of aryl sulphates by liver preparations had been intensively studied. This work was of especial interest in view of the suggestion of Anderton, Smith & Williams (1948) that mammalian tissues could form the sulphate esters only of phenols and therefore could not directly synthesise compounds of the type of androsterone sulphate, the parent steroids of which are alcoholic in character.

EXPERIMENTAL SECTION.

The experimental section of this thesis consists of the following series of published papers together with one (Number 7) which is at present in the press.

1. The Sulphatase of Ox Liver. 1. The complex nature of the enzyme.
   Roy, A.B. (1953). Biochem. J. 53, 12.
2. The Sulphatase of Ox Liver. 2. The purification and properties of sulphatase A.
   Roy, A.B. (1953). Biochem. J. 55, 653.
3. The Sulphatase of Ox Liver. 3. Further observations on sulphatase B and an investigation of the origin of fractions A and B.
   Roy, A.B. (1954). Biochem. J. 57, 465.
4. The intracellular Distribution of Sulphatase.
   Roy, A.B. (1954). Biochim. Biophys. Acta, 14, 149.
5. The Sulphatase of Ox Liver. 4. A note on the inhibition of sulphatases A and B.
   Roy, A.B. (1955). Biochem. J. 59, 8.
6. The Sulphatase of Ox Liver. 5. Sulphatase C.
   Roy, A.B. (1956). Biochem. J. 64, 651.
7. The Sulphatase of Ox Liver. 6. Steroid sulphatase.
   Roy, A.B. (1957). Biochem. J. (In the press).

8. The Kinetics of Sulphatase A.

Roy, A.B. (1956). Biochem. J. 62, 35P.

9. The Occurrence of Nitropyrogallol Disulphate in Preparations of Nitrocatechol Sulphate.

Roy, A.B. & Kerr, L.M.H. (1956) Nature, 178, 376.

10. The Kinetics of Sulphatase A.

Roy, A.B. (1957). Exper. 13, 32.

11. The Steroid Sulphatase of Patella vulgata.

Roy, A.B. (1954). Biochim. Biophys. Acta, 15, 300.

12. The Steroid Sulphatase of Patella vulgata.

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Roy, A.B. (1956). Biochem. J. 63, 294.

14. Le Métabolisme des Sulfates de Stéroïdes.

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The Sulphatase of Ox Liver

1. THE COMPLEX NATURE OF THE ENZYME

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(*Received* 2 *May* 1952)

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.

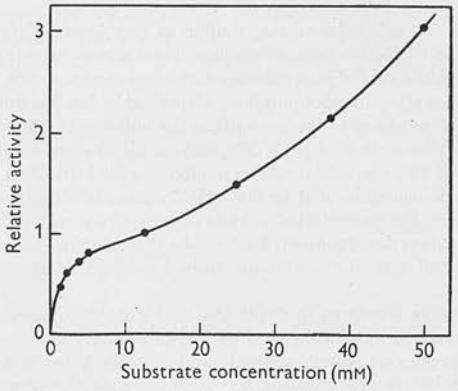

Fig. 1. Effect of varying substrate concentrations on reaction velocity. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. unfractionated sulphatase preparation. Incubated 1 hr. at 37° in 0·05 M-citrate buffer, pH 6·0.

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.

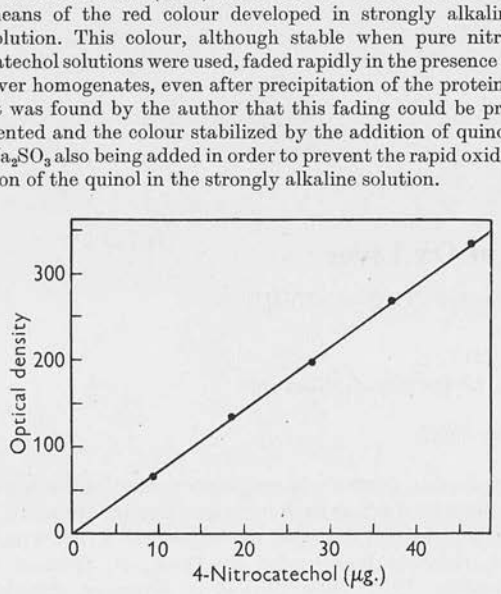

Fig. 2. Calibration curve of 4-nitrocatechol estimation.

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.

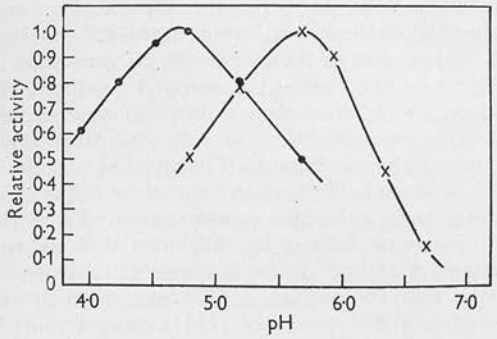

Fig. 3. Effect of pH on reaction velocity. Incubated for 1 hr. at 37° in 0·15M-acetate buffers of varying pH. Substrate concentrations 0·003 and 0·033M-nitrocatechol sulphate for fractions *A* and *B* respectively. Final volume 0·8 ml. containing 0·2 ml. enzyme solution. 'Relative activity 1·0' corresponds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

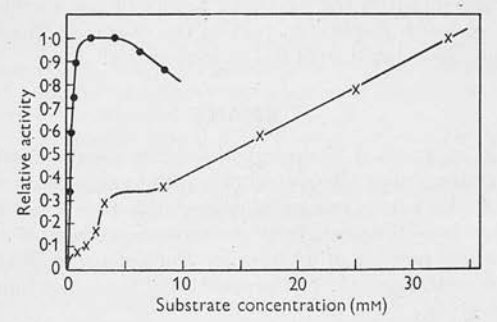

Fig. 4. Effect of varying substrate concentration on reaction velocity. Incubated for 1 hr. at 37° with varying concentrations of nitrocatechol sulphate in 0·15M-acetate buffers of pH 4·7 and 5·7 for fractions *A* and *B* respectively. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme 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.

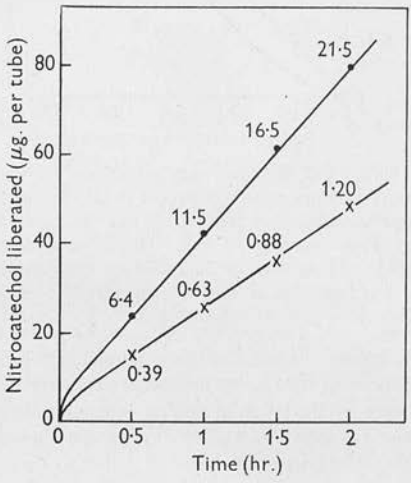

Fig. 5. Effect of time of incubation on degree of hydrolysis. In both cases the final volume of the reaction mixture was 0·8 ml. containing 0·2 ml. enzyme solution and the incubation carried out at 37°. For the assay of fraction *A* the substrate concentration was ·0·003M and the buffer 0·15M-acetate, 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.

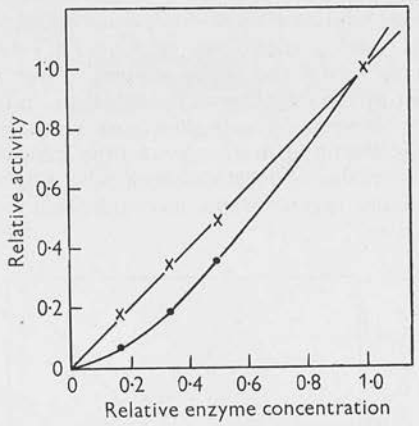

Fig. 6. Effect of enzyme concentrations on reaction velocity. Conditions as in legend of Fig. 5 except that the enzyme concentration was varied and the time of incubation kept constant at 1 hr. 'Relative velocity 1·0' represents a liberation of 75 and 51 μg. nitrocatechol per tube for fractions *A* and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

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The Sulphatase of Ox Liver

2. THE PURIFICATION AND PROPERTIES OF SULPHATASE A

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The first paper of this series (Roy, 1953) reported the presence of two distinct fractions exhibiting sulphatase activity in an aqueous extract of an acetone powder of ox liver, and gave preliminary data on the properties of the two crude enzymes, which were separated by fractional precipitation with acetone. The present paper describes the further purification and properties of fraction *A*, now referred to as sulphatase *A*.

EXPERIMENTAL

Preparation of sulphatase A

The starting material was an acetone powder of ox liver prepared as described previously (Roy, 1953). Cold acetone fractionation was carried out in an apparatus similar to that described by Askonas (1951) using thin aluminium vessels to ensure rapid heat transfer. Precipitation with $(NH_4)_2SO_4$ was effected by adding to the solution, with constant stirring, the calculated amount of solid $(NH_4)_2SO_4$; after the salt had dissolved the mixture was kept 4–6 hr. at room temperature before the precipitated protein was removed by centrifuging. Enzyme solutions were dialysed in Visking cellophan tubing against running tap water at room temperature unless otherwise specified.

Unfractionated extract. 60 g. of the acetone powder were incubated for 1 hr. at 37° with 400 ml. water and the insoluble material removed by centrifuging. The debris was washed with a further 150 ml. water and the combined supernatants were clarified by centrifuging for 30 min. at 8000 *g*.

Stage A. To 400 ml. unfractionated extract was added 40 ml. 0·2 M-phosphate buffer, pH 7·0 (final pH 6·8) and the volume made up to 450 ml. After bringing to 0°, 340 ml. cold acetone were slowly added, the temperature being lowered to −9° during the process. After equilibration at −9° for 30 min., the precipitate of fraction *B* (Roy, 1953) was centrifuged off at the same temperature and discarded. To the supernatant (650 ml.) kept at −9° were added 15 ml. of the phosphate buffer, followed by a further 280 ml. of acetone. The mixture was equilibrated as before and the precipitate of crude sulphatase *A* centrifuged off at −9°, dissolved in 75 ml. water and dialysed overnight to give approximately 140 ml. of a clear red solution of sulphatase *A*.

Stage A-1. To 140 ml. *A* were added 10 ml. 0·5 M-sodium acetate, pH 6·5 (final pH 7·0) and 1·5 ml. 0·3 M-$CaCl_2$. The solution was then precipitated with 115 ml. acetone at −9° as described above, and the inactive precipitate discarded; the supernatant, kept at −9°, was treated with a further 115 ml. acetone, the precipitate centrifuged off as before, dissolved in 25 ml. water, and dialysed overnight to give 40 ml. of a solution of sulphatase *A*-1.

Table 1. *The course of a typical preparation of sulphtase* A

Stage	Total activity (s.u.)	Activity (s.u./mg. N)
Unfractionated extract	126 000	38
A	150 000	330
A-1	120 000	3 000
A-2	90 000	—
A-3	40 000	6 700
A-4	29 000	40 000

Stage A-2. Sufficient solid $(NH_4)_2SO_4$ was added to *A*-1 to make the solution 30% saturated $(NH_4)_2SO_4$. After standing 5 hr., the active precipitate was centrifuged off, dissolved in 10 ml. water and dialysed overnight.

Stage A-3. *A*-2 was 20% saturated with $(NH_4)_2SO_4$, and the inactive precipitate centrifuged off. The supernatant was then 40% saturated with $(NH_4)_2SO_4$, the precipitate separated, dissolved in 5 ml. water and dialysed overnight, giving 8 ml. of a clear, faintly straw-coloured liquid.

Stage A-4. The clear solution of *A*-3 was dialysed at 0° against twelve or more changes of distilled water for 48 hr. and the white, flocculent precipitate of the enzyme was centrifuged off. This was extracted for 24 hr. at 0° with 4 ml. 0·1 M-NaCl, during which time the bulk of the enzyme passed into solution. The insoluble residue was centrifuged off, washed with 0·1 M-NaCl, and the combined supernatants stored at −10°.

The concentrated enzyme solutions so obtained were perfectly stable, even at 0°, but when diluted to a concentration suitable for assay as described below, the enzyme rapidly lost its activity. When so diluted, about 50% inactivation occurred on standing 24 hr. at 0° in the case of sulphatase *A*-4. Less highly purified preparations were considerably more stable.

Table 1 shows the course of a typical preparation of sulphatase *A*. One sulphatase *A* unit (s.u.) is defined as the amount of enzyme which, under the standard conditions described below, liberates (1 μg.)$^{\frac{2}{3}}$ nitrocatechol. The reason for raising the amount of nitrocatechol liberated to the power of $\frac{2}{3}$ is described below.

Preparation of sulphuric acid esters

The majority of the sulphuric acid esters were prepared by the method of Burkhardt & Lapworth (1926) using the chlorosulphonic acid-dimethylaniline complex in $CHCl_3$ solution as the sulphating agent. Dipotassium 2-hydroxy-5-nitrophenol sulphate (nitrocatechol sulphate) was prepared

as described previously (Roy, 1953). Steroid sulphates were prepared by sulphation with pyridine:sulphur trioxide (private communication from Dr J. Y. F. Paterson) as were the monosaccharide sulphates (Duff, 1949).

Estimation of enzymic activity

Routine estimations were carried out using the method previously described (Roy, 1953) with nitrocatechol sulphate as substrate. The total volume of the reaction mixture was 0·8 ml. containing 0·2 ml. enzyme solution, 0·2 ml. 0·5 M-acetate buffer, pH 4·9, and 0·4 ml. 0·006 M-nitrocatechol sulphate adjusted to pH 4·9 with 0·1 N-HCl, giving a final substrate concentration of 0·003 M-nitrocatechol sulphate in 0·13 M-acetate buffer. After incubating for 1 hr. at 37°, the solution was deproteinized with 3 ml. 2% phosphotungstic acid in 0·1 N-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 enzyme and substrate blanks. During typical assays approximately 50 μg. nitrocatechol were liberated, corresponding to a 10% hydrolysis of the substrate. Under the above conditions the degree of hydrolysis was linearly related to the time of incubation for periods of at least 2 hr. in the case of enzyme solutions purified to stage A-3. Some preparations purified to stage A-4 gave a decreasing rate of hydrolysis after 1 hr. incubation.

Use of substrates other than nitrocatechol sulphate. In all cases the general technique of the enzyme assay was similar to that described above, the principal difference being in the methods of estimating the liberated phenols. In the case of simple phenolic compounds (phenol, cresols, naphthols, 2-phenanthrol) the phenol was estimated colorimetrically using Folin & Ciocalteu reagent as described by Kerr, Graham & Levvy (1948) for the estimation of β-glucuronidase. To the reaction mixture at the end of incubation was added 3 ml. Folin & Ciocalteu reagent diluted 1 in 5 with water. Any precipitated protein was centrifuged off and 3 ml. of the supernatant pipetted into 5 ml. N-Na_2CO_3 for colour development. After standing for 15 min. at 37°, the blue colour was read in the Spekker absorptiometer using Ilford filter no. 608 (700 mμ.). With p-nitrophenyl sulphate, the liberated p-nitrophenol was estimated colorimetrically by means of the yellow colour developed in alkaline solution. The assay was similar to that described for nitrocatechol sulphate except that 3 ml. of the supernatant from the phosphotungstic acid precipitation was pipetted into 5 ml. N-Na_2CO_3 for colour development. The yellow colour was read in the Spekker absorptiometer using Ilford filter no. 601 (425 mμ.).

Study of inhibitory substances

The substance under consideration was dissolved in the 0·5 M-acetate buffer used in the routine assay described above to give any required concentration, after which the pH was checked (glass electrode) and if necessary adjusted to pH 4·9 with 0·1 N-HCl or NaOH. The buffered inhibitor solutions so obtained were used in place of the normal buffer in the routine assay, so that in all cases the enzyme was added to the previously mixed substrate and inhibitor.

RESULTS

In all the following experiments the enzyme used had been purified as far as stage A-3, unless otherwise specified in the text.

Effect of enzyme concentration on the reaction velocity. In the previous communication it was reported that in the case of crude sulphatase A (stage A) the rate of hydrolysis of nitrocatechol sulphate was not directly proportional to the enzyme concentration, whereas the relation was a linear one in the case of fraction B. It was at that time suggested that this anomaly might be due to the crude nature of the fraction, but it has now been found that highly purified preparations of sulphatase A also exhibit this effect, as is shown in Fig. 1. The effect is not due to the presence of an activator or coenzyme, as the addition of boiled enzyme solution to the assays makes no difference to the response to changes in enzyme concentration.

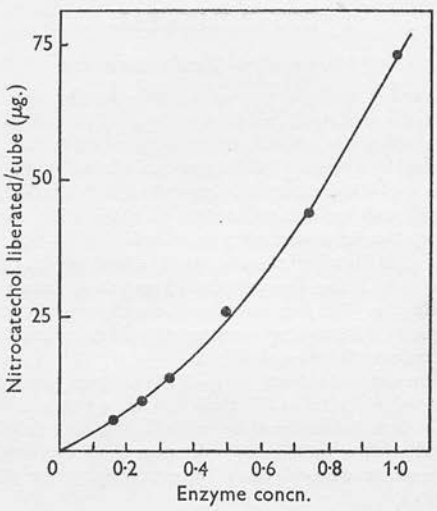

Fig. 1. Effect of enzyme concentration on reaction velocity. Final volume of reaction mixture: 0·8 ml., containing 0·2 ml. enzyme solution of varying concentration, 0·4 ml. 0·006 M-nitrocatechol sulphate and 0·2 ml. 0·5 M-acetate buffer, pH 4·9. Incubated 1 hr. at 37°.

Fig. 2 shows that although the reaction velocity is not proportional to the enzyme concentration it is, at least approximately, so related to the enzyme concentration raised to the power of $\frac{3}{2}$ over the range of enzyme concentration usually studied. This relationship appears to break down at higher enzyme concentrations, where the reaction velocity is even higher than would be expected from the relationship $v = kE^{\frac{3}{2}}$ (v is reaction velocity, E the enzyme concentration, and k a constant). Table 2 illustrates this point.

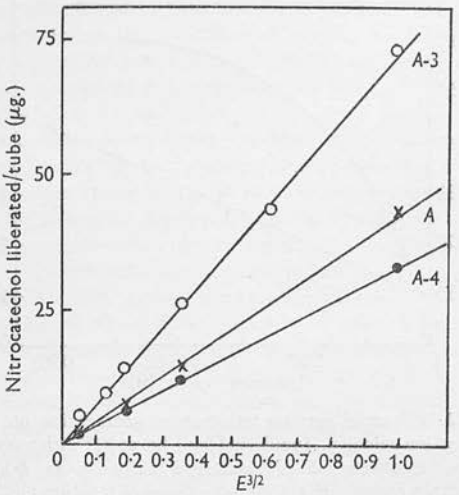

Fig. 2. Relationship between reaction velocity and the enzyme concentration raised to the power 3/2 ($E^{\frac{3}{2}}$). Conditions as in Fig. 1. The figures on the lines indicate the state of purification of the enzyme.

Table 2. *Effect of enzyme concentration on reaction velocity*

E	$E^{\frac{3}{2}}$	$v_{obs.}$	$\frac{v}{E^{\frac{3}{2}}}$	$v_{calc.}$†
0·10	0·032	1·3	40·6	1·2
0·20	0·089	3·5	39·4	3·3
0·25	0·125	4·3	34·4	4·7
0·30	0·164	6·0	36·6	6·2
0·40	0·253	8·5	33·6	9·5
0·50	0·353	13·0	36·8	13·8
0·75	0·650	25·5	39·3	24·5
1·0	1·00	39·5	39·5	37·5
2·0	2·83	134·0	47·4*	106·0
3·0	5·20	243·0	46·8*	195·0
4·0	8·00	370·0	46·3*	300·0

* Omitted from calculation of mean value of $\frac{v}{E^{\frac{3}{2}}}$.

† $v_{calc.} = kE^{\frac{3}{2}}$, where k is the mean value of $\frac{v}{E^{\frac{3}{2}}}$.

It follows from the above relationship that in the case of enzyme assays at normal enzyme concentrations (liberating some 50 μg. nitrocatechol under the standard conditions) it is necessary to raise the amount of nitrocatechol liberated to the power of $\frac{2}{3}$ to obtain an approximate measure of the amount of enzyme present. One sulphatase unit is therefore defined as the amount of enzyme liberating $(1\ \mu g.)^{\frac{2}{3}}$ of nitrocatechol under the standard conditions described above.

Effect of changes in pH. The effect of varying pH on the activity of the enzyme is shown in Fig. 3, the assays being carried out in 0·13 M-acetate buffers at a final substrate concentration of 0·003 M-nitrocatechol sulphate. It is obvious from the figure that sulphatase *A* has a well-defined optimum at pH 4·9 in acetate buffers. With different preparations of the enzyme, the position of the optimum varied between pH 4·9 and 5·0. Fig. 3 also shows the effect of citrate buffers on the pH optimum of sulphatase *A*. In 0·13 M-citrate the pH optimum is displaced to approximately pH 5·9, and the reaction velocity at lower pH values is considerably depressed, compared with the velocity in acetate buffers of corresponding pH values. It would appear that the optimum at pH 5·9 in citrate is only an apparent one brought about by the very strong inhibition of the enzyme by citrate below pH 6.

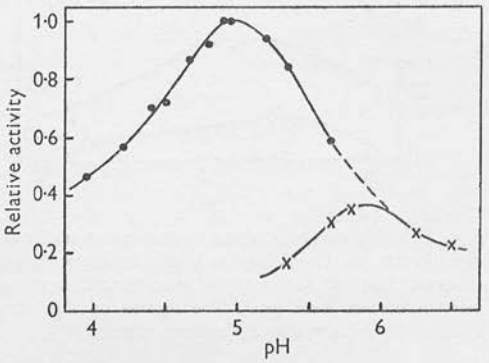

Fig. 3. Effect of pH on reaction velocity. Incubated for 1 hr. at 37° in 0·15 M-acetate or citrate buffers of varying pH. Substrate concentration 0·003 M-nitrocatechol sulphate. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution. ●—●, acetate buffer, ×—× citrate buffer.

Although several early preparations of sulphatase *A* gave pH-activity curves of the above type, more recent preparations have given curves of a very different nature, typical examples of which are shown in Fig. 4. This change is not due to any detectable alteration in the experimental procedure, which has been kept constant throughout the investigations, and it has not so far proved possible to repeat the preparations of specimens of sulphatase *A* with a simple pH curve. The complexity of the problem is indicated in Fig. 4, which shows that the shape of the pH curve varies markedly with changes in enzyme concentration. At high enzyme concentrations the enzyme activity is greatest at pH 5·2–5·3 with a subsidiary optimum at pH 4·8–4·9: as the enzyme concentration is lowered this latter optimum becomes more prominent and the optimum at pH 5·2 becomes subsidiary. Finally, in low enzyme concentrations the optimum at pH 5·2 disappears, that at pH 4·8 becomes less prominent, and the major optimum appears at pH 4·5–4·6. That is, as the enzyme concentration is lowered, the optimum shifts from pH 5·2 through pH 4·8 to 4·5.

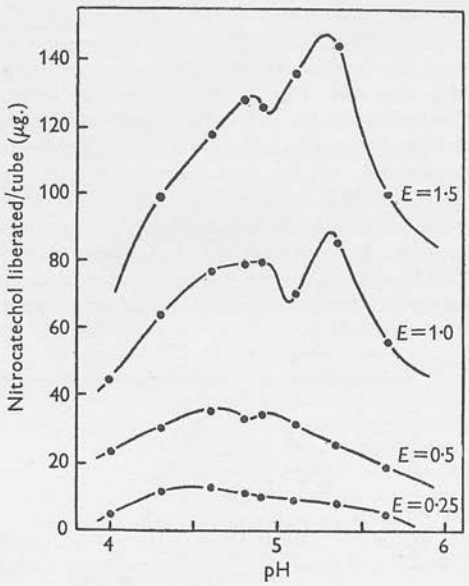

Fig. 4. Effect of varying enzyme concentration on the pH-activity curves. Conditions as in Fig. 3 except that the concentration of the enzyme was varied, and only acetate buffers were used. The figures on the curves indicate the relative enzyme concentrations.

This effect is quite constant, and ten entirely independent preparations at varying stages of purity have given similar results. Unfortunately, samples of the earlier preparations were not available for investigation of this problem.

The inhibition of sulphatase *A* by citrate suggested that the enzyme might require magnesium ions as an activator. As Smith (1950) showed that the electrophoretic behaviour of leucine aminopeptidase was altered in the presence of its activator, manganese, it seemed possible that the presence of magnesium might alter the shape of the pH-activity curve of sulphatase *A*, as any change in electrophoretic behaviour is likely to be accompanied by a corresponding shift in the position of the pH optimum. The effect of magnesium ions on the pH-activity curve of the enzyme was therefore investigated. No significant difference could be detected between the pH curve of the normal enzyme and enzyme which had been treated with 0·01 M-magnesium chloride for 30 min. before assay.

Effect of changes in substrate concentration. Fig. 5 shows the effect of varying substrate concentration on the reaction velocity. As previously reported for crude fraction *A*, the reaction velocity reached a maximum at a substrate concentration of 0·003 M-nitrocatechol sulphate. Fig. 5 also gives the results of a typical experiment plotted according to the method of Lineweaver & Burke (1934) and allows

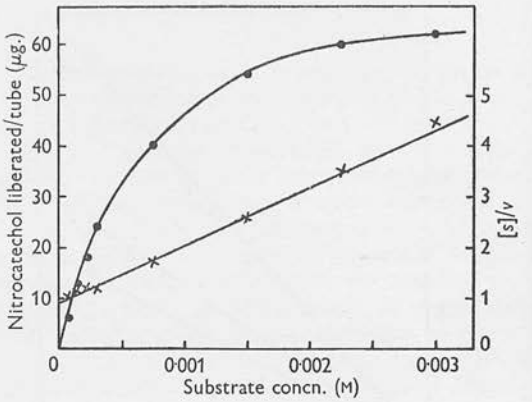

Fig. 5. Effect of varying substrate concentration on the reaction velocity. Incubated for 1 hr. at 37° with varying concentrations of nitrocatechol sulphate in 0·15 M-acetate buffer, pH 4·9. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution. ●—●, plot of reaction velocity against 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.

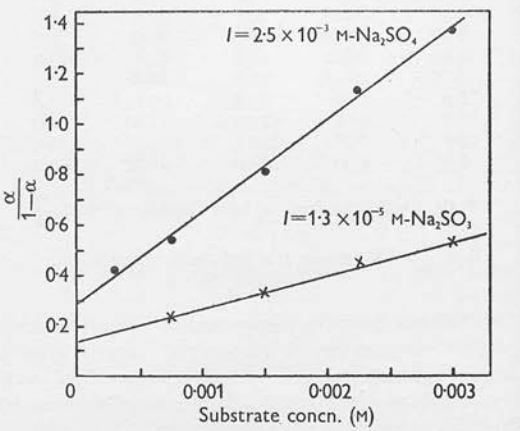

Fig. 6. Inhibition by Na_2SO_4 and Na_2SO_3. Conditions as in Fig. 5. Inhibitor concentration as indicated on the figure. Plotted according to the equation derived by Hunter & Downs (1945)

$$I\frac{\alpha}{1-\alpha}=K_i+\frac{K_i}{K_m}s,$$

where I and s are the inhibitor and substrate concentrations, α the fractional activity, K_m the Michaelis constant, and K_i the dissociation constant of the enzyme-inhibitor complex.

the calculation of the Michaelis constant, K_m. In the experiment shown the value of K_m is $7{\cdot}9 \times 10^{-4}$ M-nitrocatechol sulphate; a further two experiments gave values of $8{\cdot}2 \times 10^{-4}$ M- and $7{\cdot}6 \times 10^{-4}$ M-nitrocatechol sulphate.

As the above determinations were carried out with one of the early preparations, the experiments were repeated using a more recent enzyme preparation having multiple pH optima. The effect of substrate concentration was investigated with high and low concentrations of enzyme, at pH 5·2 and 4·6 respectively. As in both cases the results were very similar to those shown in Fig. 5 they are not reported here in detail. The optimum substrate concentration was 0·003 M-nitrocatechol sulphate with both enzyme concentrations, but the values of K_m appeared to be significantly higher than those reported above, being 12×10^{-4} M-nitrocatechol sulphate in both cases.

Effect of inhibitory substances. The following studies were carried out with an early enzyme preparation having a pH optimum of 5·0. Some of the results of the study of a large number of substances as possible inhibitors of sulphatase *A* are shown in Tables 3 and 4. Table 3 shows the effect of a number of compounds on the enzymic activity. In confirmation of the results of Tanaka (1938) for molluscan phenol sulphatase, sodium sulphate was found to be a powerful inhibitor of sulphatase *A*, which therefore differs in this respect from the phenol sulphatase of Taka diastase (Taka sulphatase) as the latter is not inhibited by sodium sulphate (Robinson, Smith, Spencer & Williams, 1952). Like Taka sulphatase, however, sulphatase *A* is strongly inhibited by sodium sulphite. Fig. 6 shows that the inhibition by both these substances is competitive and allows the calculation of the respective values of K_i by the method of Hunter & Downs (1945). These values are $7{\cdot}5 \times 10^{-4}$ M-sodium sulphate and $2{\cdot}0 \times 10^{-6}$ M-sodium sulphite respectively.

The other results listed in Table 3 show little of interest, except that 0·05 M-potassium cyanide does not inhibit sulphatase *A*, whereas Taka sulphatase is completely inhibited by this concentration of cyanide (Robinson *et al.* 1952). Like Taka sulphatase, sulphatase *A* is strongly inhibited by phosphate. Again, the activity of sulphatase *A* is uninfluenced by sodium chloride, thus distinguishing it from the phenol sulphatase of the limpet (*Patella vulgata*) which is activated by this substance (Dodgson & Spencer, 1952). The activation of rat-liver sulphatase by potassium chloride reported by Dodgson, Spencer & Thomas (1953) is probably not a true activation, but rather an apparent one brought about by a change in the state of dispersion of the enzyme studied by these authors. Potassium chloride is without effect on sulphatase *A*.

Table 3. *Effect of various compounds on the activity of sulphatase* A

(Final volume of reaction mixture 0·8 ml. containing 0·2 ml. 0·5 M-acetate buffer, pH 4·9, 0·4 ml. 0·006 M-nitrocatechol sulphate and 0·2 ml. enzyme solution. The inhibitor was dissolved in the acetate buffer to give the required concentration.)

Compound	Final concentration (M)	Inhibition (%)
Na_2SO_4	0·025	85
	0·0025	40
Na_2SO_3	$0{\cdot}25 \times 10^{-3}$	100
	$0{\cdot}25 \times 10^{-3}$	90
	$0{\cdot}0025 \times 10^{-3}$	5
NaCl	0·05	0
KCl	0·05	0
$MgCl_2$	0·1	15
	0·01	0
$BaCl_2$	0·025	40
KCN	0·01	2
NaF	0·01	95
KH_2PO_4	0·025	100
$C_6H_5PO_4Na_2$	0·025	80
$C_6H_5SO_3K$	0·05	10
$C_6H_5SO_2K$	0·05	0

Table 4. *Effect of various sulphuric acid esters on the activity of sulphatase* A

(Experimental conditions as in Table 3.)

Compound	Concentration (M)	Inhibition (%)	K_i
Potassium methyl sulphate	0·05	0	—
Potassium benzyl sulphate	0·05	10	—
Potassium *cyclo*hexyl sulphate	0·05	3	—
Potassium phenyl sulphate	0·05	10	3×10^{-1}
Potassium *m*-tolyl sulphate	0·05	30	1×10^{-1}
Potassium 1-naphthyl sulphate	0·01	18	4×10^{-2}
Potassium 2-naphthyl sulphate	0·01	30	3×10^{-2}
Potassium 2-phenanthryl sulphate	0·002	30	4×10^{-3}
Potassium androsterone sulphate	0·005	30	1×10^{-2}
Sodium glucose 6-sulphate	0·025	0	—
Sodium glucose 3-sulphate	0·025	0	—
Sodium chondroitin sulphate	10 mg./ml.	0	—
Heparin	10 mg./ml.	0	—

Table 4 shows the effect of several sulphuric acid esters on the activity of sulphatase *A*. It is immediately obvious that only the esters of the more complex compounds exert any significant inhibitory action, there being a definite decrease in the value of K_i and therefore an increase in the affinity of the enzyme for the inhibitor with an increase in complexity towards a polycyclic alkyl or aryl sulphate. None of the carbohydrate sulphates so far studied exhibited any inhibitory effect on the enzyme.

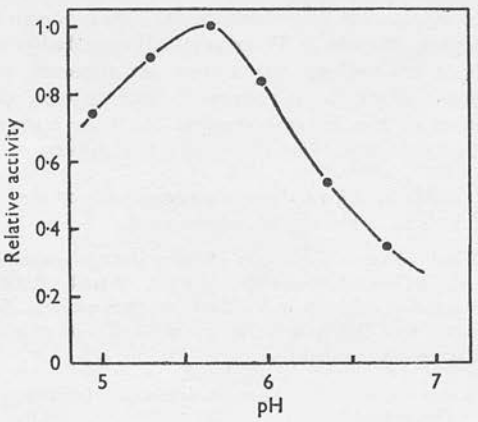

Fig. 7. Effect of pH on the hydrolysis of *m*-tolyl sulphate. General conditions as in Fig. 3 except that the substrate concentration was 0·1 M *m*-tolyl sulphate.

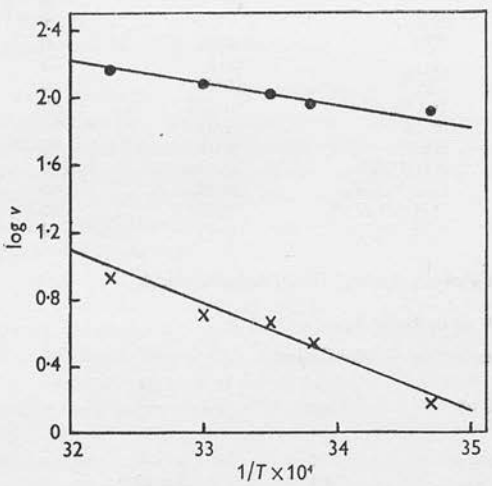

Fig. 8. Plot of log reaction rate (log *v*) against reciprocal of absolute temperature ($1/T$) for varying concentrations of sulphatase *A*. Final volume of reaction mixture 0·8 ml. containing: 0·2 ml. enzyme solution, 0·2 ml. acetate buffer, pH 5·2 and 4·6 in the high and low enzyme concentrations respectively; and 0·4 ml. 0·006 M-nitrocatechol sulphate. Incubated for 1 hr. ●—●, relative enzyme concentration 6; ×—×, relative enzyme concentration 1.

The degree of inhibition is independent of the concentration of the substrate for any given inhibitor concentration, at least over the relatively small range of substrate concentration (0·00075 M to 0·003 M-nitrocatechol sulphate) so far studied. This may be taken as an indication that the inhibition by these sulphuric acid esters is non-competitive. This inhibition cannot, however, be completely non-competitive because, as described below, at least some of these aryl sulphates are slowly hydrolysed by the enzyme.

Hydrolysis of aryl sulphates. This problem has not yet been studied exhaustively, but the results indicate that sulphatase *A* hydrolyses the simpler aryl sulphates so far studied at a much lower rate than it does nitrocatechol sulphate. Also the affinity of the enzyme for these sulphates is low: in the case of *p*-nitrophenyl sulphate and *m*-tolyl sulphate the values of K_m were 0·04 and 0·2 M respectively, compared with a value of 0·0008 M for nitrocatechol sulphate. Again, the optimum pH for the hydrolysis of these sulphates is at approximately pH 5·7 in 0·13 M-acetate buffer, compared with the optimum at pH 4·9 for the hydrolysis of nitrocatechol sulphate. The pH-activity curve for the hydrolysis of *m*-tolyl sulphate is shown in Fig. 7.

As with nitrocatechol sulphate, early enzyme preparations gave a simple pH curve for the hydrolysis of the above sulphates, while more recent preparations have given multiple optima, the major optimum, however, being at pH 5·7. Relative rates of hydrolysis have not been accurately determined, but it appears that both *m*-tolyl and *p*-nitrophenyl sulphates are hydrolysed from 10 to 20 times more slowly than nitrocatechol sulphate by sulphatase *A*. Pilot experiments have indicated that the rates of hydrolysis of 1- and 2-naphthyl sulphates and 2-phenanthryl sulphate are of the same order as that for the hydrolysis of *m*-tolyl sulphate.

Energy of activation of sulphatase A. The energy of activation was determined at several enzyme concentrations, in each case the enzyme activity being assayed at the optimum pH for the appropriate enzyme concentration. Assays were carried out in 0·003 M-nitrocatechol sulphate at various temperatures between 15 and 37°. Fig. 8 shows the results of a typical experiment plotted according to the Arrhenius equation (Arrhenius, 1889)

$$\ln \frac{k_2}{k_1} = \frac{A}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right),$$

where k is the reaction rate at absolute temperature T, R is the gas constant and A the energy of activation. From the data of Fig. 8 the energy of activation may be calculated, and in the experiment shown the values were 6500 cal./mole and 14 000 cal./mole

at high and low enzyme concentrations respectively (relative enzyme concentrations 6 and 1, corresponding to the upper and lower curves of Fig. 4).

DISCUSSION

The reported method of purification gives a very considerable concentration of the enzyme, as is indicated in Table 1; the final yield at stage *A*-4 is approximately 20 % of the enzyme present at the stage of the unfractionated extract and represents 1000-fold concentration of the enzyme with respect to protein nitrogen. Even at stage *A*-4, however, the enzyme is not purè, as the electrophoretic pattern of a typical preparation determined at pH 6·5 in the Hilger Tiselius apparatus indicates the presence of at least four components. In order to determine which of the components represented sulphatase *A*, electrophoresis was carried out on filter-paper strips, using an apparatus essentially similar to that described by Latner (1952). The bulk of the enzymic activity did not appear to be associated with the major component. It may therefore be concluded that, although a considerable degree of purification has been attained, stage *A*-4 does not represent a pure enzyme.

It should be noted that the method of preparation shows that sulphatase *A* is a globulin, as it is almost quantitatively precipitated by 30 % saturated ammonium sulphate and is insoluble in distilled water. Sulphatase *A* therefore differs markedly from Taka sulphatase which Dzialoszynski (1951) has shown to be an albumin.

The anomalous relationship between enzyme concentration and reaction velocity, shown in Fig. 1 and Table 3, is very unusual. It is difficult to explain this effect but the following mechanism seems a probable one, the more so as it has a bearing on the multiple nature of the pH curve. The basic postulates are two in number:

(1) That the enzyme molecules (E) are capable of associating reversibly with one another to form a dimeric enzyme (E_2)

$$2E \rightleftharpoons E_2$$

The dissociation constant of this reaction is given by

$$K = \frac{n_2}{n_1^2} \qquad (1)$$

where n_1 and n_2 are the concentrations of E and E_2 respectively.

(2) That the dimeric form of the enzyme is a more efficient catalyst than is the monomeric form, i.e. $k_2 > k_1$ in the following reactions:

$$S + E \xrightarrow{k_1} E + \text{products}$$

$$S + E_2 \xrightarrow{k_2} E_2 + \text{products}.$$

If the above postulates are valid, then the velocity of the enzyme reaction is given by

$$-\frac{dn_s}{dt} = (k_1 n_1 + k_2 n_2)\, n_s$$
$$= (k_1 n_1 + k_2 K n_1^2)\, n_s \qquad (2)$$

where n_s is the concentration of the substrate. Or, in terms of a total enzyme concentration, n, reckoned as monomeric units, and α, the fraction of the total amount of enzyme which is present in the dimeric form

$$n_1 = (1-\alpha)\, n, \qquad n_2 = \tfrac{1}{2}\alpha n.$$

Substituting the above in (2)

$$-\frac{dn_s}{dt} = [k_1(1-\alpha)\, n + k_2 K(1-\alpha)^2\, n^2]\, n_s \qquad (3)$$

where the value of α is given by the expression

$$\alpha = 1 + \frac{1}{4Kn} - \sqrt{\left[\left(1+\frac{1}{4Kn}\right)^2 - 1\right]} \qquad (4)$$

obtained by substituting the above expressions for n_1 and n_2 in terms of n and α into equation (1) and solving the quadratic so obtained for α.

It is obvious from equations (3) and (4) that under such conditions the reaction velocity would be proportional not to the enzyme concentration, but to some power, intermediate between the first and second, of the enzyme concentration. Further, if the enzyme were capable of polymerizing in several stages —E, E_2, E_3, ..., E_n—the relationship shown in equation (3) would be of the same general form, provided that the second of the above postulates remained valid.

It must be stressed that direct evidence for the above theory will be difficult to obtain until the enzyme is obtained in a pure state, and the above explanation must be regarded as tentative, the more so as no explanation can be offered as to why the original preparations gave a simple pH curve with a single optimum at pH 5, while more recent preparations have shown multiple pH optima.

The first of the above postulates, however, is by no means unlikely as many examples of protein-protein interactions have now been described. The most studied example is that of insulin which has definitely been shown (Gutfreund, 1948, 1952) to exist in various polymerized forms of a simple subunit; likewise Pedersen (1950) has described similar phenomena in bovine CO-haemoglobin.

The second postulate is at first sight less possible, as polymerization of the simple molecules must decrease the absolute number of enzymically active particles present, which must in turn decrease the chance of combination between the enzyme and its substrate, and so might be expected to reduce the enzymic activity, rather than to increase it. That this is apparently not the case is indicated by the

results shown in Fig. 8 and the values of the activation energies calculated therefrom. At high enzyme concentrations the activation energy is around 7000 cal./mole whereas at low concentrations the activation energy rises to 14 000 cal./mole. This implies that at high concentrations the enzyme is more catalytically active than at low concentrations, and so supports the second of the above two postulates. It must be stressed that the above values of the activation energy have little absolute significance as intermediate values may be obtained, presumably due to the fact that it is impossible to obtain an enzyme solution completely homogeneous with respect to any given polymeric form and therefore the values refer to solutions containing two or more molecular species of enzyme.

Further evidence for the probable truth of the above hypothesis is given by a consideration of the pH-activity curves shown in Fig. 4 which indicate a definite shift in the pH optimum with changes in enzyme concentration. If the enzyme can exist in a monomeric form and one or more polymerized forms then it is not unlikely that each of the forms would have a definite pH optimum. From the results shown in Fig. 4 it seems justifiable to conclude that the pH optimum of the simple, unpolymerized form is approximately pH 4·6 and with increasing degrees of polymerization of the enzyme the pH optimum moves to pH 4·9 and then to pH 5·2.

From the inhibitory action of citrate and fluoride on the enzyme it seems probable that sulphatase *A* requires magnesium ions for its complete activity. This conclusion would not be expected from the findings of Hommerberg (1931) and of Tanaka (1938), both of whom claimed that magnesium chloride inhibited sulphatases from a number of animal tissues: this inhibition is, however, confirmed by the results shown in Table 3 which indicate that 0·1 M-magnesium chloride inhibits sulphatase *A* slightly. Lower concentrations were without effect, although some preparations were activated to a slight extent (not more than 10 %) by 0·005 M-magnesium chloride. This effect was unfortunately variable. On the other hand, dialysis of stage *A*-3 enzyme for 48 hr. against several changes of glass-distilled water did not significantly depress the enzymic activity, although the enzyme was precipitated during the dialysis. In this connexion, Seligman, Chauncey & Nachlas (1951) claimed that rat-liver sulphatase was activated by magnesium chloride: it seems, however, that these claims are of doubtful validity. The enzyme used by these workers was a 'homogenate' of formalin-fixed tissues, and the so-called activation was obtained by steeping the blocks of tissue in a solution of magnesium chloride before grinding: it seems probable that the effect can be explained by the steeping in the aqueous solution removing traces of formalin. This latter view is supported by the fact that Seligman *et al.* (1951) also claimed that $Fe_3(SO_4)_2$, $(NH_4)_2SO_4$ was an activator of the enzyme.

Only preliminary studies of the specificity of sulphatase *A* have as yet been carried out, but it is obvious from the data of Table 4 that the enzyme has very little affinity for the simpler aryl sulphates, and apparently none for the few carbohydrate sulphates so far studied. Also, the simpler aryl sulphates are hydrolysed much more slowly than is nitrocatechol sulphate. The considerable increase in the affinity of the enzyme for sulphates containing a polycyclic ring system is of interest as it suggests a possible role for the enzyme in steroid metabolism. Unfortunately, it has not been possible to investigate the action of the enzyme on oestrone sulphate, as no pure specimens of this substance were available, all samples being badly contaminated with inorganic sulphate, nor was it possible to synthesize oestrone sulphate in a sufficient state of purity by the methods of Butenandt & Hofstetter (1939) or Grant & Glen (1949). In this connexion, it is of interest that oestrone sulphate administered to rats is apparently very rapidly hydrolysed to oestrone (Hanahan & Everett, 1950), whereas similarly administered phenyl sulphate is excreted unchanged (Garton & Williams, 1949). Although sulphatase *A* is strongly inhibited by androsterone sulphate, and has a high affinity for that substance (Table 4) no hydrolysis of androsterone sulphate could be detected, even after 18 hr. incubation with a con-

Table 5. *Comparison of Taka sulphatase and sulphatase* A

(In both cases the substrate used was nitrocatechol sulphate.)

	Sulphatase *A*	Taka sulphatase
Type of protein	Globulin	Albumin*
pH optimum (acetate)	4·9	5·9
Optimum substrate concn.	0·003 M	0·003 M
K_m	8×10^{-4} M	$3{\cdot}5 \times 10^{-4}$ M
Effect of 0·02 M-KCN	No effect	100 % Inhibition
Effect of 0·02 M-Na_2SO_4	85 % Inhibition	No effect
Relative rate of hydrolysis of *p*-nitrophenol sulphate	1/20	2

* Dzialoszynski (1951).

centrated enzyme solution. This is in accordance with the view (Fromageot, 1938) that the sulphatases so far described are aryl sulphatases, specifically hydrolysing phenolic sulphates and having no action on alcoholic sulphates, and contrasts sharply with the recent claim of Henry, Thevenet & Jarrige (1952) that a sulphatase in the gut of *Helix pomatia* readily hydrolyses dehydro*epi*androsterone sulphate.

The above results stress the great differences between sulphatase *A* and any previously described sulphatase, especially Taka sulphatase. Table 5 summarizes the principal differences between sulphatase *A* and Taka sulphatase, the data for the latter being those of Robinson *et al.* (1952).

SUMMARY

1. A method is described for the purification of ox-liver sulphatase *A*. A 1000-fold purification is achieved.

2. Differences between the original and recent enzyme preparations are described. The original preparations had a pH optimum of 4·9–5·0 in 0·13 M-acetate buffer; more recent preparations have shown multiple optima at pH 4·6, 4·9 and 5·2.

3. In the recent preparations the pH optimum varies with the enzyme concentration. In high enzyme concentrations the major optimum is at pH 5·2 and in low enzyme concentrations at pH 4·6.

4. The enzymic activity is not directly proportional to the enzyme concentration, but to the enzyme concentration raised to some power intermediate between the first and second (approximately the power $\frac{3}{2}$). It is suggested that this effect can be explained by polymerization of the enzyme molecules to give complexes which are more active enzymically than are the unpolymerized molecules.

5. The variation in pH optimum with enzyme concentration can also be explained on the above basis.

6. The optimum substrate concentration is 0·003 M-nitrocatechol sulphate and the K_m is 8×10^{-4} M-nitrocatechol sulphate.

7. Sulphatase *A* is competitively inhibited by sodium sulphate and sodium sulphite ($K_i = 7 \times 10^{-4}$ M and 2×10^{-6} M respectively).

8. Sulphatase *A* hydrolyses the simpler aryl sulphates at a much lower rate than it does nitrocatechol sulphate.

9. Sulphatase *A* is distinguished from the aryl sulphatase of Taka diastase.

The author wishes to express his thanks to Dr E. A. Moelwyn-Hughes for his help in suggesting the interpretation of some of the data given in this paper. He also wishes to thank Prof. G. F. Marrian, F.R.S., for samples of a number of steroids, and Dr R. B. Duff for a number of polysaccharide sulphates. The electrophoretic analysis in the Tiselius apparatus was kindly performed by Mr H. J. Cruft. Thanks are due to Mr D. Love for carrying out the protein nitrogen estimations and to Mr R. Watt for skilled technical assistance. The author is also deeply indebted to Messrs Frigidaire for their assistance in designing and manufacturing the low-temperature bath used in these investigations.

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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*

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(*Received* 1 *January* 1954)

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 μg. 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. 0·5 M sodium acetate, pH 6·5, and 4·5 ml. 0·3 M-$CaCl_2$. 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, 1953*a*). 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-Na_2CO_3 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-Na_2CO_3 to develop a yellow colour in these tubes containing liberated *p*-nitrophenol.

Esterase. This was detected by the method of Huggins & Lapides (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-

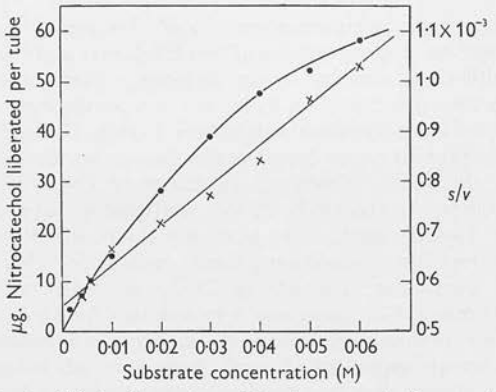

Fig. 1. Effect of varying substrate concentration on the reaction velocity. Incubated 1 hr. at 37°, with varying concentrations of nitrocatechol sulphate in 0·15M acetate buffer, pH 5·7. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution; ●—●, plot of reaction velocity against 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 $2{\cdot}5 \times 10^{-2}$ 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 concentration 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.

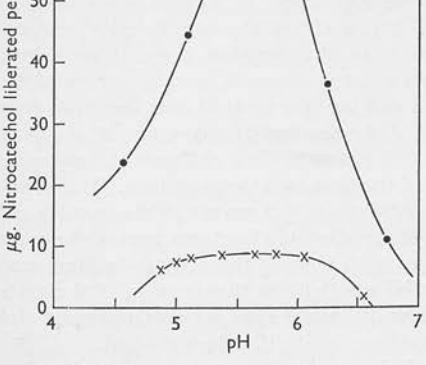

Fig. 2. Effect of pH on reaction velocity. Incubated 1 hr. at 37° in 0·15 M acetate or citrate buffers. Substrate concentration 0·06 M nitrocatechol sulphate. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution; ●—●, acetate buffer; ×—×, citrate buffer.

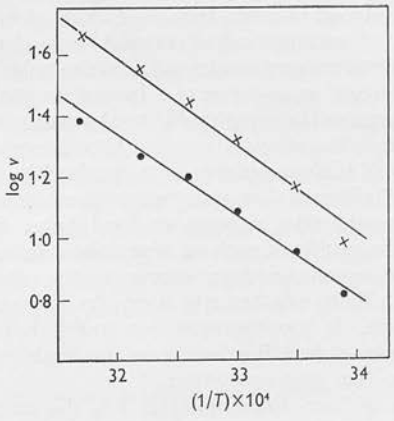

Fig. 3. Plot of log reaction velocity (log v) against the reciprocal of absolute temperature ($1/T$) for two concentrations of enzyme. Substrate concentration 0·06 M nitrocatechol sulphate, pH 5·7 in 0·15 M acetate buffers, final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme. Incubated 1 hr.

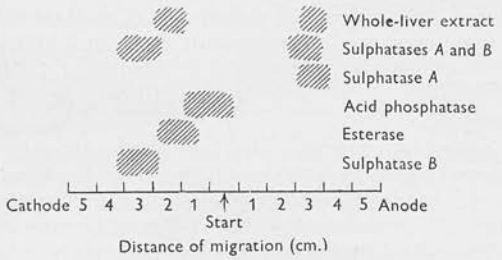

Fig. 4. Typical results of electrophoretic investigations of sulphatase preparations. Run 16 hr. at 200 v; pH 7·1 in veronal buffer.

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, 1953*a*) 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 0·15 M acetate buffers and at substrate concentrations of 0·003 M and 0·06 M 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 Na_2CO_3, giving the 'acetate-treated' enzyme. A similar sample was

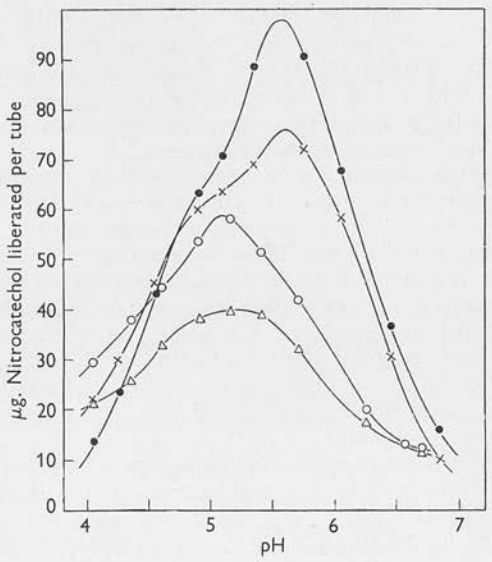

Fig. 5. Effect of pH on reaction velocity of an unfractionated extract of an acetone powder of ox liver (●—● and ○—○, at substrate concentrations of 0·06 M and 0·003 M nitrocatechol sulphate, respectively) and of the combined fractions *A* and *B* isolated therefrom (×—× and △—△, at substrate concentrations of 0·06 M and 0·003 M nitrocatechol sulphate, respectively). Assay in 0·15 M 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 0·15 M acetate buffers and a substrate concentration of 0·03 M 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 0·15 M 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 approximtely 20 % dispersion of fresh ox liver in water was prepared as above and centrifuged at 30000 *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).

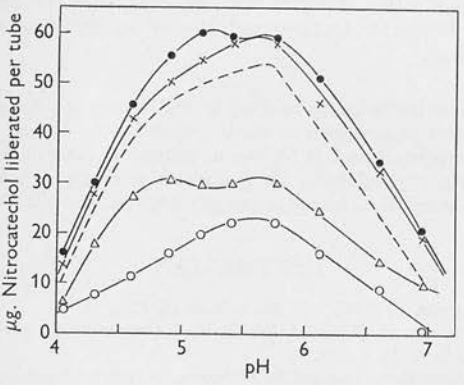

Fig. 6. Effect of pH on reaction velocity of sulphatase in a dispersion of ox liver before and after various treatments; for details of which see text. Assay in 0·15 M acetate buffers, substrate concentration 0·03 M nitrocatechol sulphate; general conditions as in Fig. 2; ●—●, whole dispersion of ox liver; ×—×, 'acetate-treated'; △—△, 'acetate-insoluble'; ○—○, 'acetate-soluble'; – – –, summation of 'acetate-soluble' and 'acetate-insoluble' curves.

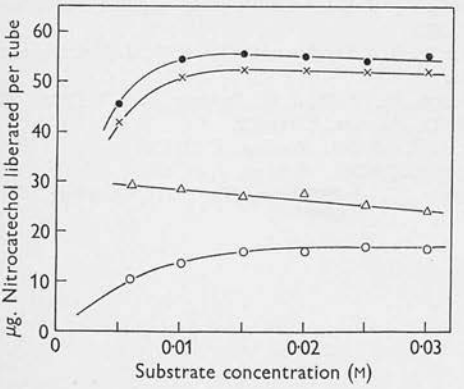

Fig. 7. Effect of varying substrate concentration on the reaction velocity of sulphatase in a dispersion of ox liver before and after various treatments. Assay in 0·15 M acetate buffer, pH 5·5, general conditions as in Fig. 1; ●—●, whole dispersion; ×—×, 'acetate-treated'; △—△, 'acetate-insoluble'; ○—○, 'acetate-soluble'.

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 *A*	Sulphatase *B*
pH optimum (in acetate)	4·9	5·7
Optimum substrate concn.	0·003 M	>0·10 M
K_m	8×10^{-4} M	7×10^{-2} M
$K_i\ SO_4^{2-}$	7×10^{-4} M	7×10^{-2} M
$K_i\ SO_3^{2-}$	2×10^{-6} M	5×10^{-4} M

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.

The author is deeply indebted to Drs Dodgson and Spencer for their co-operation in kindly checking the activity of sulphatases *A* and *B* for the hydrolysis of potassium *p*-acetylphenyl sulphate. He also wishes to thank Dr Edgar Stedman, F.R.S., for a generous gift of the esterase inhibitor.

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THE INTRACELLULAR DISTRIBUTION OF SULPHATASE

by

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In a recent publication DODGSON, SPENCER AND THOMAS[1] have reported that the bulk of the arylsulphatase activity of rat liver is present in the microsomes, some 70% of the total recovered sulphatase activity being present therein after their isolation in isotonic sucrose[2]. This is at variance with preliminary qualitative results from this laboratory[3] which indicated that in the case of mouse liver the bulk of the sulphatase was of mitochondrial origin. Because of this discrepancy it appeared necessary to reinvestigate the problem and the results reported below confirm the mitochondrial origin of the enzyme.

To avoid possible species differences rat liver was used in the present investigation. Cell fractionation was carried out according to SCHNEIDER AND HOGEBOOM[4], the nuclear, mitochondrial, and microsomal fractions being defined as those sedimenting at 700 *g*, 5500 *g*, and 25,000 *g* respectively. Sulphatase activity was estimated by the method already described[3] using dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) as substrate. Preliminary investigation had shown that the optimal conditions for the assay of sulphatase in a whole homogenate of rat liver were a substrate concentration of 0.03 *M* nitrocatechol sulphate and a pH of 5.9 in 0.15 *M* acetate buffer. These conditions were therefore used in the present study with an incubation period of 1 h at 37°.

The results are given in Table I which clearly shows that the bulk of the sulphatase is present in the mitochondrial fraction of rat liver although considerable amounts also occur in the microsomal fraction. Table I also shows that the recovery of added nitrocatechol is virtually quantitative from all fractions under the above assay conditions. The total recovery of sulphatase activity was approximately 80%: this rather low value is comparable to that of DODGSON *et al.*[1], but the reason for the loss of enzyme is not clear. The loss may, however, be apparent rather than real as it has already been shown[5] that the activity of ox liver sulphatase A is not linearly related to the enzyme concentration and that an enzyme fraction with electrophoretic properties similar to those of sulphatase A exists in rat liver[6].

The above results therefore confirm the earlier observation[3] that liver sulphatase is predominantly of mitochondrial origin and do not support the view of DODGSON *et al.*[1] that the enzyme has its origin in the microsomes. The reason for this discrepancy is not yet obvious but it may be due, in part at least, to the use of a different substrate, potassium β-acetylphenyl sulphate, by the latter workers as DODGSON *et al.* (private communication) have recently confirmed the results from this laboratory when using nitrocatechol sulphate as the substrate in their assays.

TABLE I

INTRACELLULAR DISTRIBUTION OF SULPHATASE IN RAT LIVER

The results are expressed as percentages of the total sulphatase recovered and are the values for seven animals. The recovery of added nitrocatechol is quoted as a percentage.

	Sulphatase activity		*Recovery of 36 µg added nitrocatechol %*
	Range %	*Mean %*	
Nuclei	9–20	15	98
Mitochondria	43–62	50	98
Microsomes	20–23	22	99
Soluble fraction	11–21	14	99

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The Sulphatase of Ox Liver

4. A NOTE ON THE INHIBITION OF SULPHATASES *A* AND *B*

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(*Received* 8 *June* 1954)

The literature contains many conflicting reports on the influence of electrolytes on the activity of sulphatases, the early work being particularly difficult to interpret, as in many cases clear distinctions were not drawn between the enzymes obtained from different sources, such as mammalian liver, molluscan viscera, and *Aspergillus oryzae*. Recent work on mammalian liver sulphatase has also been unsatisfactory, as in all cases except that of previous work from this laboratory the enzymes used were unpurified preparations containing a number of different sulphatases, or even homogenates of tissues which had been treated with histological fixatives (Seligman, Chauncey & Nachlas, 1951). The present situation is extremely confused: sodium chloride has been reported to inhibit sul-

phatase in tissue sections, but to be without action on homogenates (Rutenburg, Cohen & Seligman, 1952) yet potassium chloride has been claimed (Dodgson, Spencer & Thomas, 1954*a*) to activate the sulphatase in homogenates. Magnesium chloride has similarly been claimed both as an inhibitor (Hommerberg, 1931) and an activator (Seligman *et al.* 1951). It has previously been shown that both sulphatases *A* and *B* are inhibited by sulphate ions (Roy, 1953*b*, 1954*a*), yet both Seligman *et al.* (1951) and Dodgson *et al.* (1954*a*) report the activation of crude sulphatase preparations by sulphate ions. This effect would appear very improbable on theoretical grounds alone.

The present paper describes an investigation of the effect of electrolytes on both crude ox-liver sulphatase and partly purified preparations of sulphatases *A* and *B*. Various other possible enzyme inhibitors have also been studied, and the results are reported below.

EXPERIMENTAL

The general experimental methods have already been described (Roy, 1953*a*, *b*, 1954*a*), the enzymic activity being followed by the colorimetric estimation of the 4-nitrocatechol liberated on hydrolysis of dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate). Sulphatases *A* and *B* were prepared as before, and in the present investigation were purified to stages *A*-3 and *B*-2 respectively.

The 5% (w/v) dispersion of ox liver in water used as the source of unfractionated enzyme was prepared by treating the tissue for 30 sec. in an Atomix blender at room temperature. The liver was used as soon as possible (usually about 1 hr.) after the slaughter of the animal.

Both in the case of the unfractionated enzyme and of sulphatases *A* and *B* an enzyme concentration was chosen such that approximately 100 μg. nitrocatechol were liberated under the experimental conditions described below.

Sulphatase *A* activity was estimated at pH 5·0 and a substrate concentration of 0·003 M nitrocatechol sulphate, and sulphatase *B* at pH 5·9 and 0·06 M nitrocatechol sulphate. The buffers used were sodium acetate, final concentration 0·15 M. The electrolytes were dissolved in the appropriate buffer and mixed with the substrate before the addition of the enzyme, so that the enzyme was treated simultaneously with the substrate and the electrolyte under investigation. Routine adjustments of pH were made with N acetic acid, not HCl as in previous work, since chloride ions have been found to have an influence on the activity of sulphatase *B*.

Since some of the other types of inhibitor were known to react only slowly with proteins, the buffered enzyme (0·25 M acetate, pH 5·0 and 5·9 for sulphatases *A* and *B* respectively) was treated with the appropriate concentration of inhibitor for 1 hr. at room temperature; then the inhibitor–enzyme solution was mixed with the appropriate substrate solution. Attempts to reverse the inhibition were carried out by adding the appropriate reagent to the enzyme–inhibitor mixture 10 min. before adding the substrate.

RESULTS

Influence of electrolytes

Unfractionated enzyme. Fig. 1 shows the effect of 0·025 M sodium sulphate and 0·08 M potassium chloride on the sulphatase activity of a 5% (w/v) dispersion of ox liver. In confirmation of the results of Dodgson *et al.* (1954*a*) it is obvious that at certain pH values both these compounds considerably enhanced the sulphatase activity of such a preparation. At other pH values an inhibition was noted in both cases. This effect was not shown by 0·001 M sodium sulphite, which inhibited strongly at all pH values. As these results were at variance with those previously reported (Roy, 1953*b*, 1954*a*) an investigation of the action of electrolytes on the activity of sulphatases *A* and *B* was undertaken.

Dodgson, Spencer & Thomas (1953), in their study of unfractionated rat-liver sulphatase, reported that the use of 0·2 M phosphate buffers in place of 0·5 M acetate buffers caused a shift in the pH optimum for the hydrolysis of *p*-acetylphenyl sulphate from pH 7·0 to 7·75. When phosphate buffers were used in the present study of the unfractionated ox enzymes no activity could be detected between pH 6·5 and 8·0. This discrepancy is resolved by the fact that the assay method used by Dodgson *et al.* (1953) estimates only the insoluble or microsomal enzyme (Dodgson, Spencer & Thomas, 1954*b*) which is not inhibited by phosphate (Dodgson & Spencer, private communication) and does not estimate the soluble enzymes derived from mitochondria (Dodgson *et al.* 1954*b*) which are inhibited by phosphate and which are essentially the enzymes assayed in the methods used in this

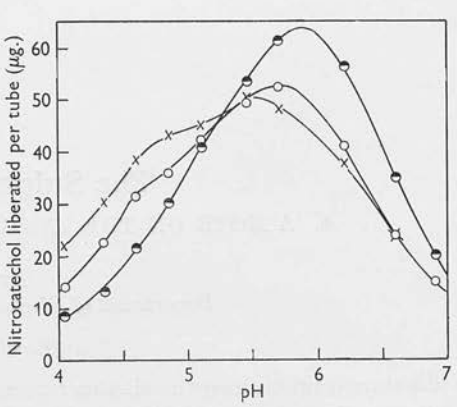

Fig. 1. Effect of electrolytes on the sulphatase activity of an untreated dispersion of ox liver. Assays in 0·15 M acetate buffers, substrate concentration 0·03 M nitrocatechol sulphate, total volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution. Incubated 1 hr. at 37°. ○—○, control; ●—●, 0·025 M-Na_2SO_4; ×—×, 0·08 M-KCl.

laboratory (Dodgson *et al.* 1954*b*; Roy, 1954*b*). Although this microsomal enzyme occurs in ox liver (Dodgson *et al.* 1954*b*) the amounts present would be too small to be detected by the present method so that no activity would be expected in the presence of phosphate. It is now proposed to refer to this microsomal sulphatase as sulphatase *C* until it is possible to name the various sulphatase fractions systematically: this decision has been made in agreement with Drs Dodgson and Spencer. The different effect of phosphate obtained in the two laboratories is therefore due to the use of different substrates and to the substrate specificity of the sulphatases (Dodgson *et al.* 1954*b*).

Sulphatase A. The effect of various electrolytes on the activity of sulphatase *A* is shown in Fig. 2. It should be noted that in these experiments a high concentration of enzyme was used so that the pH optimum was approximately pH 5·1 (Roy, 1953*b*). The most striking effect is obviously that of sulphate ions: in their presence the pH optimum is shifted from pH 5·1 to 6·2. Because of this shift in the position of the optimum there is an apparent activation of sulphatase *A* by sulphate ions on the alkaline side of the pH optimum of the untreated enzyme. It must be stressed that this phenomenon is not an activation in the true sense, as the activity of the sulphate-treated enzyme at the appropriate pH optimum is always less than that of the untreated enzyme under its optimal conditions (Fig. 2).

No comparable effect was noted with any of the other ions studied, suggesting that the mechanism of inhibition by sulphate is different from that of the other anions shown in Fig. 2. It is of interest that 0·08 M potassium chloride causes a slight (10 %) inhibition of sulphatase *A* at the pH optimum and on the alkaline side thereof: 0·005 M magnesium acetate caused a 10 % activation throughout the entire pH range studied. Previously reported experiments using high concentrations of magnesium chloride were presumably complicated by the simultaneous and opposing effects of magnesium and chloride ions.

Sulphatase B. In this case no comparable activation by sulphate ions was noted (Fig. 3), but chloride ions had a definite activating effect. It was reported previously (Roy, 1954*a*) that the pH optimum of sulphatase *B* was pH 5·7–5·8; these experiments were unfortunately carried out in the presence of variable amounts of chloride, and it has now been found that in the absence of chloride ions the pH optimum is in the region of pH 6·0 (Fig. 3). It can be shown by the use of chloride-free substrate solutions that chloride ions cause a pronounced activation on the acid side of the pH optimum with the net result of a broadening of the pH optimum into the acid region.

The influence of a number of other ions on sulphatase *B* activity is also indicated in Fig. 3. In contrast to sulphatase *A*, sulphatase *B* was uninfluenced by 0·005 M magnesium acetate.

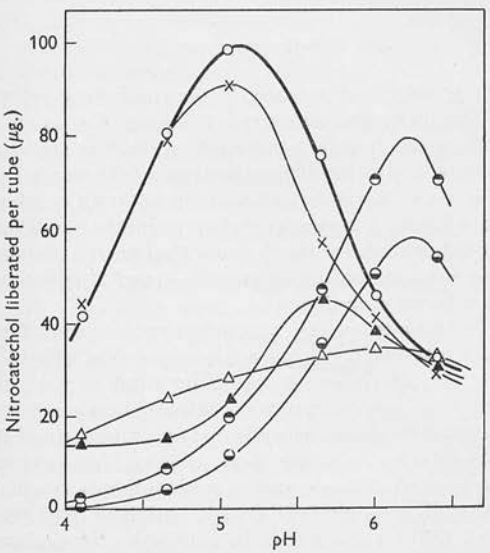

Fig. 2. Effect of electrolytes on the activity of purified sulphatase *A*. Conditions as in Fig. 1, except that the substrate concentration was 0·003 M nitrocatechol sulphate. ○—○, control; ×—×, 0·08 M-KCl; △—△, 0·0005 M-NaH_2PO_4; ▲—▲, 0·00001 M-Na_2SO_3; ◓—◓, 0·025 M-Na_2SO_4 or K_2SO_4; ◒—◒, 0·05 M-Na_2SO_4.

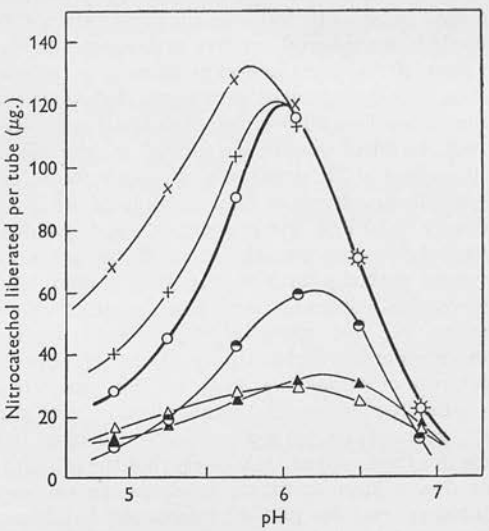

Fig. 3. Effect of electrolytes on the activity of purified sulphatase *B*. Conditions as in Fig. 1, except that the substrate concentration was 0·05 M nitrocatechol sulphate. ○—○, control; ×—×, 0·08 M-KCl; +—+, 0·01 M-KCl; △—△, 0·0025 M-NaH_2PO_4; ▲—▲, 0·0001 M-Na_2SO_3; ◓—◓, 0·1 M-Na_2SO_4.

Table 1. *The action of sulphydryl compounds and of* SH *reagents on sulphatases* A *and* B

Enzyme solutions treated for 1 hr. at room temp. with the inhibitors in 0·25 M acetate buffers, pH 5·0 and 5·9 for sulphatases *A* and *B* respectively. Reversal of inhibition by SH reagents attempted by addition of sulphydryl compound 10 min. before addition of the substrate.

Results of two experiments given, each being the mean of duplicate estimations, with an accuracy of ±2%.

Reagent	Concn. (M)	Activity (relative to control = 1) Sulphatase *A*		Sulphatase *B*	
Iodoacetate	10^{-2}	0·27	0·30	0·05	—
p-Chloromercuribenzoate	10^{-4}	0·31	0·33	1·09	1·07
Cysteine	5×10^{-3}	1·01	1·08	1·25	1·19
Glutathione	5×10^{-3}	0·91	0·88	1·02	0·97
p-Chloromercuribenzoate + cysteine	10^{-4} + 5×10^{-3}	0·98	1·05	1·25	1·19
p-Chloromercuribenzoate + glutathione	10^{-4} + 5×10^{-3}	0·73	0·81	1·01	0·99

Miscellaneous inhibitors

There has been no report in the literature of any compound which might be regarded as a specific inhibitor of mammalian sulphatases, nor indeed of any sulphatase, except for the report by Robinson, Smith, Spencer & Williams (1952) that taka-sulphatase is inhibited by potassium 4-hydroxy-3-nitrophenyl sulphate. This ester is, however, hydrolysed by both sulphatases *A* and *B*. Torda (1943) claimed that the sulphatase of cat muscle was inhibited by 5% (w/v) cocaine and activated by 5% (w/v) yohimbine.

No result of any interest was obtained in the study of various alkaloids on sulphatases *A* and *B*, neither of which was affected by yohimbine (10^{-2} M), eserine (10^{-3} M), nor the methiodide of *m*-dimethylaminophenyl methyl carbamate (10^{-3} M). Cocaine (10^{-2} M) had likewise no effect on sulphatase *B*, but slightly inhibited sulphatase *A*; higher concentrations of cocaine strongly inhibited sulphatase *A*, but this effect would seem to be of no significance.

The effect of SH reagents is of some interest, and the results are shown in Table 1. Sulphatase *A* was strongly inhibited by iodoacetate and *p*-chloromercuribenzoate: the inhibition of the latter was reversed partially by glutathione and completely by cysteine. Cysteine alone had no effect on sulphatase *A*, but glutathione inhibited slightly. Some specimens of glutathione were very powerful inhibitors of sulphatase *A*: this effect varied from specimen to specimen, although all were more than 90% reduced, as judged by iodine titration, and were free from copper. It is likely that the inhibition was due to decomposition products, as the most inhibitory samples had a pronounced sulphurous smell. Sulphatase *B* was likewise inhibited by iodoacetate, but was slightly activated by *p*-chloromercuribenzoate; a sample of glutathione giving a 10% inhibition of sulphatase *A* was without action on sulphatase *B*, while cysteine activated strongly. The activation by *p*-chloromercuribenzoate was unexpected, as the other results in Table 1 would suggest that sulphatase *B*, like sulphatase *A*, was an SH enzyme. The explanation may be that the *p*-chloromercuribenzoate inhibited some agent in the enzyme preparation which tended to inactivate the sulphatase. In this connexion it should be noted that the available preparations of sulphatase *B* are probably much less pure than those of sulphatase *A*, the protein concentration in the reaction mixture being approximately 250 μg./ml. in the case of *B* and 20 μg./ml. in *A*.

The results, however, indicate that sulphatase *A* is an SH enzyme and are not incompatible with the view that sulphatase *B* is also an SH enzyme, although they do not prove the latter point.

DISCUSSION

The results resolve many of the conflicting reports in the literature and stress the need for using at least partly purified preparations in the study of enzyme inhibition. The activation of the sulphatase of a whole dispersion of ox liver by both sulphate and chloride ions under the appropriate conditions is further evidence for the view that such a preparation contains both sulphatases *A* and *B* which are therefore not artifacts.

In the case of the purified enzymes, one of the most interesting effects is the very powerful inhibition of sulphatases *A* and *B* by sulphite ions, the affinity of both enzymes for sulphite being approximately 100 times their affinity for sulphate and for nitrocatechol sulphate (Roy, 1954*a*). This perhaps reaches the extreme case in taka-sulphatase which, although strongly inhibited by sulphite (Robinson *et al.* 1952) is unaffected by sulphate. No explanation of this can be offered, but it is unlikely that the effect is due simply to the reducing power of sulphite ions, as cysteine is not an inhibitor. Neither does it seem probable, owing to the reversible nature of the inhibition by sulphite, that it is caused by the sulphite reacting with an aldehyde

grouping in the enzyme, as postulated by Rosenfeld & Ruchelman (1940) in the case of taka-sulphatase. The striking shift in the pH optimum of sulphatase *A* in the presence of sulphate is very interesting: it would seem that this must be due to combination of the sulphate with the enzyme, probably the active centre, as the inhibition of sulphatase *A* by sulphate is competitive (Roy, 1953*b*). This might be taken as further evidence that sulphatase *A* contains a positively charged grouping in its active centre (Roy, 1954*a*).

The general problem of the function of these enzymes remains obscure. The possibility that sulphatase might play a part in the *in vivo* synthesis of sulphuric acid esters by acting as a transferase with the 'activated sulphate' of Bernstein & McGilvery (1952) as substrate has been considered briefly by Dodgson *et al.* (1954*a*). The possibility of the occurrence of this reaction *in vivo* cannot be assessed, but it is clear that it could not be occurring in the *in vitro* experiments of Bernstein *et al.* (1952), as these workers used isotonic phosphate buffers, pH 6·5, under which conditions both sulphatases *A* and *B* are completely inactive, and sulphatase *C* only slightly active. It is likewise impossible to assess the possible hydrolytic activities of sulphatases *in vivo* as, according to Gamble (1947), the intracellular fluid contains 100 m-equiv./l. of phosphate, mostly organic. *In vitro* experiments indicate that sulphatases *A* and *B* would be strongly inhibited in such a medium, both enzymes being inhibited by phosphate esters. It is not possible to give quantitative figures owing to the difficulty of obtaining such esters completely free from inorganic phosphate, a very powerful inhibitor.

The multiplicity of sulphatases (Roy, 1953*a*; Dodgson *et al.* 1954*b*) and their widespread distribution throughout animal tissues (Rutenburg *et al.* 1952) might be taken to indicate that these enzymes are fulfilling some important metabolic role, Haldane (1954) even suggesting that such a multiplicity of enzymes might be an evolutionary adaptation of some advantage to the organism concerned. No such metabolic function is as yet obvious, the bulk of the evidence indicating a surprising inertness of sulphatases *in vivo* (Hawkins & Young, 1954). This situation invites comparison with that of β-glucuronidase which also exhibits this multiplicity (Mills, Paul & Smith, 1953) and apparent inertness *in vivo* (Garton & Williams, 1949). It may well be that both groups of enzymes play related roles in the metabolism of mucopolysaccharides, as suggested by the work of Meyer, Linker & Rapport (1951) for β-glucuronidase, and that their respective activities *in vivo* are only distantly related to the commonly used assay methods. In the case of sulphatases at least this would be borne out by the peculiar affinity of these enzymes for highly unphysiological substrates.

SUMMARY

1. Unfractionated preparations of ox-liver sulphatase are activated both by chloride and sulphate ions.

2. Sulphatase *A* is activated by sulphate ions on the alkaline side of the pH optimum owing to a shift of the position of the pH optimum in the presence of sulphate ions. A slight inhibition of sulphatase *A* is brought about by chloride ions at and above the pH optimum.

3. Sulphatase *B* is activated by chloride ions on the acid side of the pH optimum.

4. The effects of various other anions on sulphatases *A* and *B* are given.

5. Sulphatases *A* and *B* are uninfluenced by sodium and potassium ions; magnesium ions are without action on sulphatase *B*, but activate sulphatase *A* to a small extent.

6. Sulphatase *A* is shown to be an SH enzyme. In the case of sulphatase *B* the evidence is conflicting.

7. The significance of the influence of ions on sulphatases is discussed in relation to the possible activity of these enzymes *in vivo*.

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The Sulphatase of Ox Liver

5. SULPHATASE *C**

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The previous papers of this series have dealt with those sulphatases which can readily be extracted by water from an acetone-dried powder of ox liver (Roy, 1953*a*, *b*; 1954*a*) or which can be obtained in true solution by extraction of the fresh tissue with water (Roy, 1954*a*). These sulphatases, named sulphatases *A* and *B* (Roy, 1953*a*), are apparently predominantly localized in the mitochondria, at least in the liver of the mouse (Roy, 1953*a*) and of the rat (Roy, 1954*b*). They rapidly hydrolyse dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate), but attack simpler compounds such as *m*-tolyl, *p*-nitrophenyl and *p*-acetylphenyl sulphates relatively slowly. Previously unpublished work in this laboratory indicated the presence in mouse liver of another sulphatase which was localized in the microsomes, which rapidly hydrolysed *m*-tolyl and *p*-nitrophenyl sulphates, and which could not be obtained in true solution by the usual methods. Dodgson, Spencer & Thomas (1955) and Dodgson, Spencer & Wynn (1956) have shown the occurrence of comparable enzymes in rat and human tissues respectively.

The present paper describes the preparation and properties of this insoluble sulphatase, sulphatase *C*, of ox liver.

* Part 4: Roy (1955).

EXPERIMENTAL

Preparation of sulphatase C

Ox liver (200 g.) was cut into small pieces and treated in an Atomix blender (Measuring and Scientific Equipment Co. Ltd.) for 2 min. with 300 ml. of ice-cold water. All the following stages were carried out at 0° unless otherwise stated. The suspension was centrifuged for 5 min. at 2000 rev./min. and the residue of connective tissue, etc., discarded. The supernatant was then centrifuged for 30 min. at 20 000 *g* and the loosely packed sediment collected. This sediment was washed twice by suspending it in water and recentrifuging.

The washed sediment was suspended in about 100 ml. of water and treated with 0·2 vol. of 10% Lissapol NX (I.C.I. Ltd.). After standing overnight the debris was centrifuged down at 35 000 *g* and discarded, giving a clear solution of sulphatase *C*. The enzyme was precipitated by pouring its solution into 4 vol. of acetone at −20° and was centrifuged down at −15°: the sediment was washed successively with acetone and ether at −20° and finally dried rapidly *in vacuo* over P_2O_5. This yielded sulphatase *C* as a red-brown insoluble powder. Last traces of soluble enzymes were removed by suspending the enzyme in water, by the use of a loose-fitting glass homogenizer to obtain an even suspension, and sedimenting sulphatase *C* at 35 000 *g*. The residue was washed twice with water, suspended in a convenient volume of water and freeze-dried, yielding approximately 1 g. of a buff-coloured powder which could readily be suspended in water.

The yield of sulphatase *C* activity was about 40% of that present in the original liver dispersion and represented a 40-fold concentration of the enzyme with respect to dry wt. For use in the assay described below the powder was suspended in water to give a concentration of 10 mg./ml.

Assay of sulphatase C

The method used depended upon the colorimetric determination of the *p*-nitrophenol liberated by the enzymic hydrolysis of potassium *p*-nitrophenyl sulphate, prepared by the method of Burkhardt & Lapworth (1926). To 0·2 ml. of 0·5 M 2-amino-2-hydroxymethyl-1:3-propanediol (aminotrishydroxymethylmethane)–acetic acid buffer (tris–acetate buffer), pH 8·0, was added 0·2 ml. of 0·04 M potassium *p*-nitrophenyl sulphate, followed by 0·4 ml. of the enzyme suspension. The mixture was incubated for 30 min. at 37°, with occasional shaking to keep the enzyme in suspension, and the reaction was then stopped by the addition of 3 ml. of 2% phosphotungstic acid in 0·1 N-HCl. Precipitated proteins were removed by centrifuging, and the colour was developed by pipetting 3 ml. of the clear supernatant into 5 ml. of M-Na_2CO_3. The intensity of the yellow colour was read in a Spekker absorptiometer against a suitable blank, with Ilford filter no. 601 (maximum transmission at 425 mμ.). Assays were performed in duplicate and the appropriate enzyme and substrate blanks were always included.

A few experiments were carried out with nitrocatechol sulphate as substrate. In these determinations the reaction mixture was the same as that described above except that the substrate solution was 0·08 M dipotassium nitrocatechol sulphate. The liberated 4-nitrocatechol was determined by means of the red colour developed in alkaline quinol solution (Roy, 1953*a*).

When simple aryl sulphates, such as *m*-tolyl sulphate or the naphthyl sulphates, were used as substrates the liberated phenols were determined with Folin & Ciocalteu's reagent as previously described (Roy, 1953*b*).

RESULTS

The enzymic activity was firmly bound to the cell particles and was completely insoluble in dispersions of ox liver in aqueous media. Under similar conditions at least 75% of the sulphatase *A* and *B* activity of ox liver is soluble (Roy, 1954*a*). Treatment of the first washed suspension of the enzyme with a number of detergents gave a 'solution' of sulphatase *C*. Of the detergents tested the non-ionic Lissapol NX (I.C.I. Ltd.) and Triton X-100 (Rohm & Haas Inc.) were the most effective in solubilizing the enzyme. The cationic Cetavlon (I.C.I. Ltd.) was considerably less effective and also caused an appreciable inhibition of the enzymic activity. As suggested by Spencer, Dodgson, Rose & Thomas (1955), the solubilization of the enzyme is probably due to its incorporation into the micelles of the detergent, as the enzyme is rendered completely insoluble on removal of the surface-active agent by precipitation with acetone. The sulphatase *C* of ox liver apparently resembles that of human liver (Dodgson *et al.* 1956) more than that of rat liver (Spencer *et al.* 1955) as it could not be obtained in true solution by treatment of the solubilized enzyme with crude preparations of lipase (L. Light and Co., Colnbrook, Bucks). It was also impossible to obtain a true solution of the enzyme by treatment of the insoluble preparations with *n*-butanol under a variety of conditions as described by Morton (1950).

Properties of sulphatase C

Under the conditions specified above, the hydrolysis of *p*-nitrophenyl sulphate was of zero order for times not exceeding 30 min., and with this time of incubation the reaction velocity was directly related to the concentration of enzyme. With longer times of incubation the reaction velocity decreased rapidly, apparently through denaturation of the enzyme.

The optimum substrate concentration determined at pH 8·0 was approximately 0·008–0·010 M *p*-nitrophenyl sulphate (Fig. 1) and at higher substrate concentrations the reaction velocity decreased rapidly. K_m, calculated by the method of Lineweaver & Burk (1934), was 0·002 M *p*-nitrophenyl sulphate. Both the value of K_m and the degree of substrate inhibition were very considerably influenced by changes in pH. In an attempt to obtain some information about the nature of the groups in the active centre of the molecule the variation of K_m with pH was investigated. Fig. 2 shows the results plotted according to the equations developed by Dixon (1953). The pK_m/pH plot

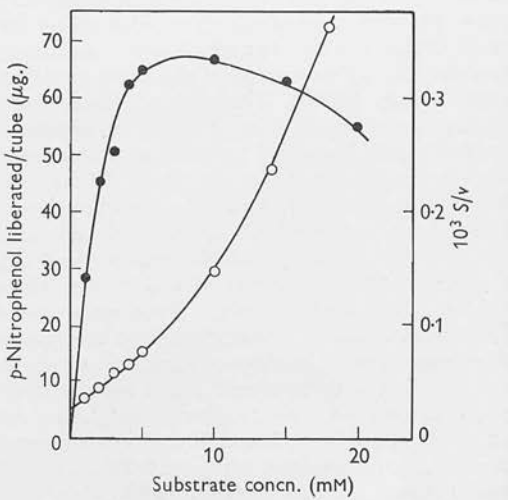

Fig. 1. Influence of substrate concentration on the reaction velocity. Volume of reaction mixture, 0·8 ml., containing 0·2 ml. of tris–acetate buffer, pH 8·0, and 0·4 ml. of sulphatase *C* suspension. Incubated for 30 min. at 37°. ●, Plot of reaction velocity (v) against substrate concentration (S); ○, plot of S/v against S.

showed a discontinuity at a pH of approximately 7·8, and according to the interpretation of these plots given by Dixon (1953) this must represent the pK of a dissociating group present either in the substrate or in the active centre of the enzyme. As *p*-nitrophenyl sulphate does not have a group which could dissociate in this region, the discontinuity must represent the pK of some group in the active centre of the enzyme. No indication of the nature of this group can be given. The fact that the slope of the pK_m/pH plot in the region above pH 7·8 is -1 indicates that in this pH range there is a change in charge of $+1$ when the enzyme combines with the substrate.

The optimum pH, determined in tris–acetate buffers at a substrate concentration of 0·01 M *p*-nitrophenyl sulphate, was 7·9–8·0 (Fig. 3). When the pH values of the buffer solutions were adjusted with HCl or H_2SO_4 no change in the position of the pH optimum was noted, and the presence of 0·08 M-NaCl or -KCl, or 0·1 M-K_2SO_4 in the reaction mixtures did not alter the position of the optimum. In phosphate buffers, on the other hand, the optimum was displaced to about pH 8·5.

With a view to distinguishing sulphatase *C* from sulphatases *A* and *B*, the action of a number of possible inhibitors was investigated. The results are shown in Table 1 and Fig. 4. In all these experiments the inhibitor was dissolved in the buffer so that the enzyme was added to the previously mixed substrate and inhibitor. The most interesting results are those summarized in Fig. 4, which shows that the inhibition of sulphatase *C* by any of a number of

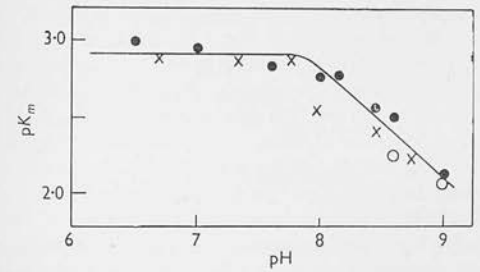

Fig. 2. Plot of pK_m against pH. Conditions as in Fig. 1. The different symbols refer to three independent experiments.

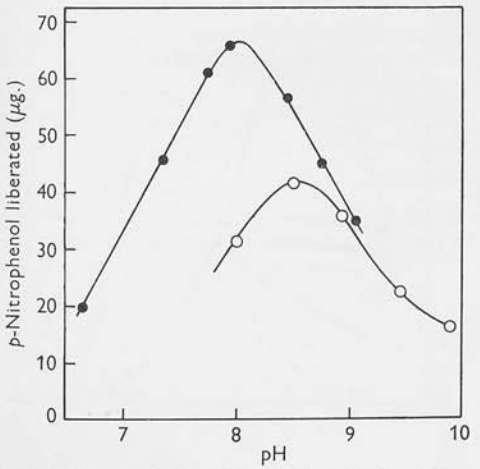

Fig. 3. Influence of pH on the reaction velocity. Volume of reaction mixture, 0·8 ml., containing 0·2 ml. of 0·04 M *p*-nitrophenyl sulphate and 0·4 ml. of sulphatase *C* suspension. Incubated for 30 min. at 37°. ●, 0·15 M tris–acetate buffers; ○, 0·01 M tris–phosphate buffers.

Table 1. *Action of possible inhibitors on sulphatase* C *activity*

The reaction mixture contained 0·2 ml. of buffer, pH 8, 0·2 ml. of 0·04 M *p*-nitrophenyl sulphate and 0·4 ml. of enzyme preparation. The inhibitors were dissolved in the buffer before use. The activity is expressed relative to control determinations with activity 1·00.

Inhibitor	Concn. (M)	Activity
Na_2SO_3	0·0001	0·45
NH_4Cl	0·05	0·80
$(NH_4)_2SO_4$	0·025	0·79
Magnesium acetate	0·05	0·64
$BaCl_2$	0·025	1·15
Barium acetate	0·025	1·10
NaF	0·01	0·55
NH_4F	0·01	0·68
KCN	0·01	0·43
Iodoacetate	0·01	0·25

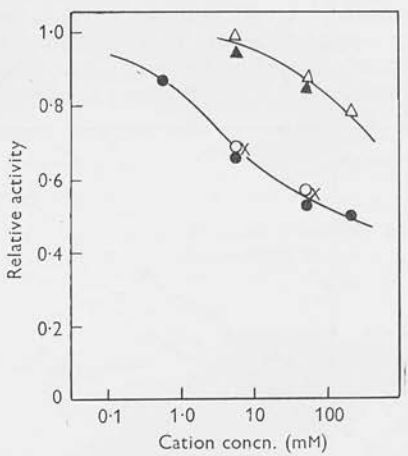

Fig. 4. Influence of various concentrations of electrolytes on the reaction velocity. Volume of reaction mixture, 0·8 ml., containing 0·2 ml. of 0·04 M *p*-nitrophenyl sulphate, 0·2 ml. of tris–acetate buffer, pH 8·0, and 0·4 ml. of sulphatase *C* suspension. ●, NaCl; ○, Na_2SO_4; ×, sodium acetate; △, KCl; ▲, K_2SO_4.

salts is due to the cation, not to the anion, Na^+ ions being considerably stronger inhibitors than K^+ ions. It should be pointed out that in the experiments with sodium salts the reaction mixtures also contained 0·01 M-K^+ ions derived from the substrate. This inhibition of sulphatase *C* by cations makes it difficult to interpret with certainty any possible action of anions, but from the results shown in Fig. 4 it seems to be clear that Cl^- and SO_4^{2-} ions can have little, if any, action on the enzyme.

The kinetics of the inhibition of sulphatase *C* by Na^+ and K^+ ions were investigated and the results are shown in Fig. 5. The inhibition by K^+ ions was non-competitive and the value of K_i was 0·7 M-K^+, as determined by the method of Lineweaver & Burk (1934). The inhibition by Na^+ ions did not correspond exactly with any of the described types of inhibition but it was clearly of the uncompetitive type. It was not, however, strictly uncompetitive as the plot of $1/v$ against $1/S$ did not give a line parallel to that of the uninhibited reaction, but parallel to that of the reaction inhibited by K^+ ions (Fig. 5). The reason for this is not clear, but it may be due to the presence of K^+ ions from the substrate in the reaction mixture. If the inhibition is assumed to be uncompetitive the value of K_i was calculated to be 0·2 M-Na^+.

The results of the study of other possible inhibitors showed little of note and are summarized in Table 1. Like all sulphatases, sulphatase *C* was strongly inhibited by SO_3^{2-} ions. It was also inhibited by cyanide but was relatively insensitive to fluoride. The inhibition by Mg^{2+} confirms the early observations of Hommerberg (1931), who was almost certainly studying the sulphatase *C* present in the unfractionated-enzyme preparations then available. The activation by Ba^{2+} ions is worthy of note. This activation cannot be due to removal of SO_4^{2-} or HPO_4^{2-} ions, which, even if present in the washed enzyme preparations used, are not inhibitors of sulphatase *C* activity. The study of inhibitors reacting with specific groups in the enzyme molecule was not undertaken, as any negative results would have had little significance in view of the very crude nature of the enzyme preparations available. Sulphatase *C* activity was, however, inhibited about 40 % by 0·01 M *p*-chloromercuribenzoic acid, and this inhibition was reversed by cysteine, which was itself without action on the enzyme. These experiments were carried out under the general conditions already described (Roy, 1955) and showed that sulphatase *C*, like sulphatase *A*, is an SH enzyme.

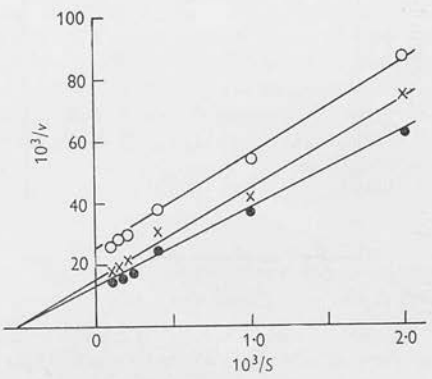

Fig. 5. Effect of NaCl and KCl on the reaction velocity. Conditions as in Fig. 4. Plotted as $1/v$ against $1/S$. ●, Control; ×, 0·1 M-KCl; ○, 0·1 M-NaCl.

Hydrolysis of other substrates

The effect of pH on rate of hydrolysis of nitrocatechol sulphate by preparations of sulphatase *C* is shown in Fig. 6. The curve shows two peaks, one at pH 5·7, the other at pH 7·5. This suggested that the preparation contained appreciable amounts of sulphatases *A* and *B*, as the pH optimum for the hydrolysis of nitrocatechol sulphate by a mixture of these enzymes is in the region of 5·7 (Roy, 1954*a*). The optimum at pH 7·5 was therefore presumably due to sulphatase *C*. This interpretation of the pH curve is supported by the results of a study of the influence of inhibitors. In the presence of fluoride, which inhibits sulphatases *A* and *B* but not *C*, the peak at pH 5·7 was suppressed, and in the presence of cyanide, which inhibits sulphatase *C* but not *A* or *B*, the peak at 7·5 was suppressed, as shown in Fig. 6.

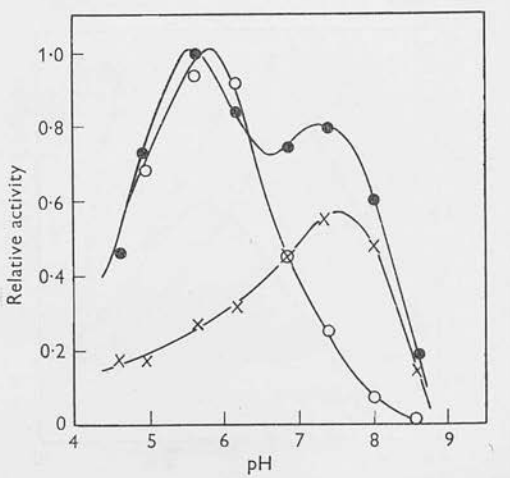

Fig. 6. Effect of pH on the hydrolysis of nitrocatechol sulphate by sulphatase *C* preparations. Conditions as in Fig. 3 except that the substrate solution was 0·08 M nitrocatechol sulphate. ●, Control; ○, 0·02 M-KCN; ×, 0·01 M-NaF.

Because of the presence of a mixture of sulphatases, all capable of hydrolysing nitrocatechol sulphate, kinetic studies on sulphatase *C* with this substrate were not carried out. The effect of substrate concentration was, however, studied at pH 5·7 and at pH 7·5. At pH 7·5 the optimum substrate concentration was 0·02 M nitrocatechol sulphate and the K_m approximately 0·008 M. At pH 5·7 the effect of varying substrate concentrations was comparable with the effect on a mixture of sulphatases *A* and *B* (Roy, 1954*a*).

The rate of hydrolysis of nitrocatechol sulphate at pH 7·5, at which pH sulphatases *A* and *B* are virtually inactive (Fig. 6), was approximately half that of *p*-nitrophenyl sulphate, both rates being determined at the appropriate optimum substrate concentrations. This fact readily distinguishes sulphatase *C* from sulphatases *A* or *B*, which hydrolyse nitrocatechol sulphate very much more rapidly than they do *p*-nitrophenyl sulphate (Table 3). A number of experiments suggested that the rates of hydrolysis of *m*-tolyl, 1-naphthyl and 2-naphthyl sulphates were of the same order as that of nitrocatechol sulphate, the determinations being carried out under the arbitary conditions of pH 8·0 and a substrate concentration of 0·02 M.

Rat-liver sulphatase C

In view of the strong inhibition of ox-liver sulphatase *C* by Na^+ ions it seemed desirable to study the action of these ions on similar preparations of rat-liver sulphatase *C*, as Dodgson, Spencer & Thomas (1955) did not detect any inhibition of their preparations of sulphatase *C* by such cations. The enzyme was prepared from rat liver exactly as described above for ox liver.

According to Dodgson, Spencer & Thomas (1955) the optimum conditions for the assay of rat-liver sulphatase *C* are pH 8·5 with a substrate concentration of 0·006 M *p*-nitrophenyl sulphate. In the present work the assays were carried out with 0·005 M *p*-nitrophenyl sulphate at pH 8·0. Under these conditions no inhibition of rat-liver sulphatase *C* was caused by either Na^+ or K^+ ions, as shown in Table 2. On the contrary, in confirmation of the results of Dodgson, Spencer & Thomas (1954), a slight activation by NaCl and KCl was noted. In this respect at least, therefore, rat-liver sulphatase *C* differs slightly from the corresponding ox enzyme.

DISCUSSION

The results described above demonstrate conclusively the presence in ox liver of a third sulphatase, sulphatase *C*, strikingly different in properties from sulphatases *A* and *B*, previously described (Roy, 1953*b*, 1954*a*). These differences are summarized in Table 3. Although kinetic studies on enzyme preparations such as those described above must be interpreted with caution, as the enzyme is not in solution, it is unlikely that these differences in properties are caused by this insolubility. Apart from the fact that the properties of sulphatase *C* are not significantly altered when the enzyme is solubilized by detergents, the properties of the sulphatases *A* and *B* in these insoluble preparations are apparently no different from those of the soluble enzyme previously described. Studies on the intracellular localization of the enzyme have

Table 2. *Comparison of the influence of salts on the activity of rat-liver and ox-liver sulphatase* C

Conditions as in Table 1.

Salt	Concn. (M)	Activity: Rat	Activity: Ox
NaCl	0·05	1·20	0·56
Na_2SO_4	0·025	1·05	0·53
KCl	0·05	1·20	0·85
K_2SO_4	0·025	1·03	0·76

Table 3. *Properties of sulphatases from various sources*

The data for ox-liver sulphatases *A* and *B* are taken from Roy (1953*b*, 1954*a*), for sulphatase *C* from the present investigation, for *Aspergillus oryzae* from Robinson *et al.* (1952), and for *Alcaligenes metalcaligenes* from Dodgson, Spencer & Williams (1955). NCS, Nitrocatechol sulphate; NPS, *p*-nitrophenyl sulphate.

	Ox liver			*Aspergillus oryzae*	*Alcaligenes metalcaligenes*
	A	*B*	*C*		
K_m, NCS (mM)	0·8	70	8·0	0·35	0·22
K_m, NPS (mM)	40	25	2·0	0·17	0·47
K_i, SO_4^{2-} (mM)	0·7	70	∞	∞	∞
K_i, SO_3^{2-} (mM)	0·002	0·5	0·1	—	—
Optimum pH, NCS	4·9	6·0	7·5	5·9	8·0
Optimum pH, NPS	5·7	—	8·0	6·2	8·8
Inhibition by 0·01 M-KCN (%)	2	0	60	100	69
Inhibition by 0·01 M-NaF (%)	95	—	40	50*	0
Ratio of rates of hydrolysis, NCS:NPS	20	>50	0·5	0·5	0·2

* Morimoto (1938).

not been carried out because of practical difficulties in working with ox liver, and because of the fact that the above method of assay is not suited for use in the study of unfractionated-tissue preparations which reduce the liberated *p*-nitrophenol. On the basis of previous observations on the mouse, and the observations of Dodgson *et al.* (1954) on the rat, it seems that sulphatase *C* is localized entirely in the microsomes, and its extreme insolubility suggests that it must be associated with the microsomal membrane postulated by Pallade & Siekevitz (1955). It is interesting to compare this purely microsomal localization of sulphatase *C* with that of sulphatases *A* and *B*, which apparently occur in both the mitochondria and microsomes (Roy, 1954*b*, Dodgson *et al.* 1954).

The properties of sulphatase *C* are summarized in Table 3 along with those of the only other aryl sulphatases which have been studied in detail. It is clear from the data of Table 3 that sulphatase *C* is more closely related to the fungal and bacterial enzymes than to sulphatases *A* and *B*. This confirms the suggestion of Roy (1953*a*, *b*) that there are at least two groups of sulphatases in nature, and it has been suggested (Dodgson & Spencer, private communication) that the fungal and bacterial enzymes along with sulphatase *C* be called group 1 sulphatases, and the very different sulphatases *A* and *B* be called group 2 sulphatases.

The most characteristic property of sulphatase *C* is its inhibition by Na^+ ions and, to a lesser extent, by K^+ ions. This inhibition apparently distinguishes the sulphatase *C* of ox liver from that of the rat (Dodgson, Spencer & Thomas, 1953) and probably also from that of man (Dodgson *et al.* 1956). No corresponding inhibition has been noted with any soluble mammalian sulphatase (Roy, 1955) but the routine use of acetate buffers in their assay may have masked such an effect. Fromageot (1938) quotes Neuberg & Lindhardt (1923) and Neuberg & Wagner (1925) as reporting the inhibition of the sulphatase of *Aspergillus oryzae* (Taka sulphatase) by acetate buffers, although the experimental results presented in the latter two papers hardly support this statement. Dzialoszynski (1951) showed that the activity of Taka sulphatase was depressed in acetate buffers. These effects might well have been due to the cations of the acetate buffer. On the other hand, Robinson, Smith, Spencer & Williams (1952) found no inhibition of Taka sulphatase by Na^+ ions, but again this might have been due to the routine use of acetate buffers by these workers. It would therefore appear that a re-investigation of the effect of cations on the various sulphatases might be of considerable interest.

This work stresses again the danger of assaying sulphatases in unfractionated-tissue preparations. It has been stated (Dodgson, Spencer & Thomas, 1955) that the assay of sulphatase *C* in such preparations is justifiable when substrates such as *p*-acetylphenyl sulphate or *p*-nitrophenyl sulphate are used, as with these substrates competition by sulphatases *A* and *B* is negligible. The present work shows, however, that at least with ox liver, assays in such unfractionated preparations would be valueless owing to the presence of variable amounts of Na^+ ions in the preparations. Before such assays of sulphatase *C* can justifiably be carried out it is necessary to demonstrate that in the species under investigation the sulphatase *C* is uninfluenced by cations, as is apparently the case in the rat. The doubtful validity of the assay of sulphatases *A* and *B* in unfractionated-tissue preparations has already been discussed (Roy, 1954*a*; Dodgson, Spencer & Thomas, 1955).

SUMMARY

1. A method is described for the purification and assay of the sulphatase *C* of ox liver.

2. Sulphatase *C* is an extremely insoluble enzyme which has not been obtained in solution. Its properties are described. The optimum conditions for the assay of sulphatase *C* are a substrate concentration of 0·01 M *p*-nitrophenyl sulphate at pH 8·0.

3. Sulphatase *C* hydrolyses nitrocatechol sulphate at approximately half the rate of *p*-nitrophenyl sulphate.

4. Sulphatase *C* is inhibited by Na^+ ions and to a lesser extent by K^+ ions. A number of other inhibitors have been studied and it is concluded that sulphatase *C* is an SH enzyme.

5. The properties of sulphatase *C* are compared with those of other aryl sulphatases.

6. The problem of assaying sulphatases in unfractionated-tissue preparations is discussed.

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THE SULPHATASE OF OX LIVER.

6. Steroid Sulphatase.

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Gibian & Bratfisch (1956) have recently described the occurrence in rat and ox liver of a steroid sulphatase hydrolysing dehydroepiandrosterone sulphate (DHAS). This report is of considerable interest as it had previously been believed (Roy, 1956a) that such an enzyme was lacking from mammalian tissues. Work from this laboratory (Roy, 1953, 1954a, 1956c) has already shown the occurrence of three arylsulphatases in ox liver and it was of obvious interest to study the relationship of steroid sulphatase to those enzymes. From the work of Gibian & Bratfisch (1956) it seemed likely that steroid sulphatase was associated with arylsulphatase C (sulphatase C), as their preparations were insoluble in the usual protein solvents and showed their optimal activity in the region of pH 8. Their preparations of steroid sulphatase hydrolysed

oestrone sulphate which would suggest that an aryl-sulphatase was present despite the observation that phenolphthalein disulphate was not hydrolysed. This latter might simply be a reflexion of the specificity of the arylsulphatase as it had been noted that this substrate is not readily hydrolysed by mammalian arylsulphatases (unpublished observations).

The present paper extends the observations of Gibian & Bratfisch (1956) on the properties of mammalian steroid sulphatase, especially with regard to its relationship with sulphatase C and to its specificity.

EXPERIMENTAL.

The steroid sulphates used as substrates for the enzyme were synthesised as previously described (Roy, 1956a) and the enzyme was obtained from ox liver exactly as described for the preparation of sulphatase C (Roy, 1956c). The insoluble enzyme preparation was used at a concentration of 2-3 mg./ml. in the assay described below.

Steroid sulphatase activity was assayed by suitable modifications of the methods previously described for the determination of steroid sulphates

(Roy, 1956a, b). To 0.2 ml. of 0.5 M 2-amino-2-hydroxy-methylpropane-1,2-diol - acetic acid buffer (tris buffer), pH 7.8, was added 0.4 ml. of 0.4 mM DHAS followed by 0.2 ml. of enzyme suspension. The mixture was incubated with occasional shaking for 1 hr. at 37°, the reaction then stopped by the addition of 5 ml. of ethanol and the precipitated proteins removed by centrifuging. A 5 ml. sample of the clear supernatant was taken to dryness on the water bath, the residue taken up in 2 ml. of diluted methylene blue reagent (Roy, 1956b) and the methylene blue salt of the DHAS extracted into 5 ml. of chloroform. The amount of DHAS present was then determined colorimetrically as previously described (Roy, 1956,a).

Control determinations were always carried out in which the enzyme was incubated separately from the remainder of the reaction mixture and was added only immediately before the ethanol. These control values were identical to those obtained when the substrate was added to the separately incubated buffer-enzyme mixture. The enzymic activity was therefore proportional to the difference between the amounts of DHAS remaining after incubation with and without the enzyme. As already stressed (Roy, 1956a) this method cannot be used for accurate kinetic studies as the difference between these

two values is normally small, corresponding to about 25% hydrolysis of the substrate.

Recovery of added DHAS from the unincubated reaction mixture was between 85% and 90% of the theoretical, a value considered satisfactory for the investigations described below. The degree of hydrolysis of the substrate on incubation with the enzyme was directly related to the enzyme concentration (Fig. 1).

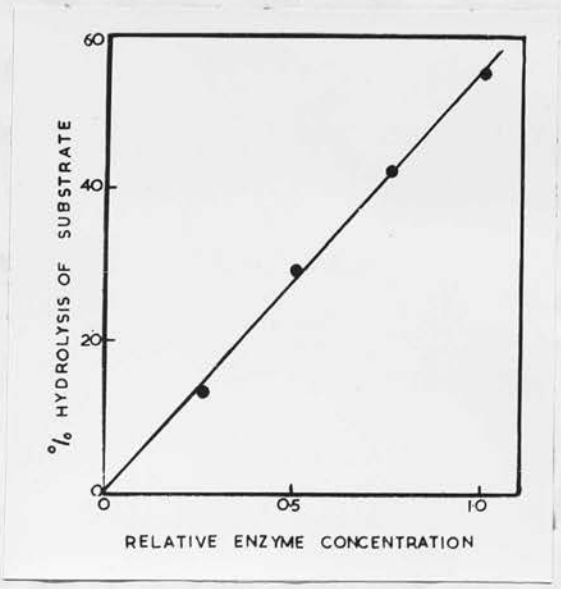

Fig. 1. Effect of enzyme concentration on the reaction velocity. Volume of reaction mixture 0.8 ml. Substrate concentration 0.2 mM DHAS at pH 7.8 in 0.13 M tris buffer. Incubated for 1 hr. at 37°.

Cell fractionation and assay of arylsulphatases. Cell fractionation was carried out in 0.25 M sucrose by the standard method of Hogeboom (1955), using a glass homogeniser fitted with a nylon pestle. The aryl-sulphatase activities of the fractions were determined as previously described using 0.03 M nitrocatechol sulphate at pH 6.0 for the assay of sulphatases A + B (Roy, 1954b) and 0.004 M p-nitrophenyl sulphate at pH 7.8 for sulphatase C (Roy, 1956c). In both cases the time of incubation was 15 mins. Steroid sulphatase was assayed as above, but with an incubation time of 3 hr. The sum of the activities of the various fractions was taken as representing the sulphatase content of the whole tissue.

RESULTS.

The optimum substrate concentration for steroid sulphatase was 0.2 mM DHAS, as shown in Fig. 2. Although the method was not suitable for the accurate determination of K_m the results shown in Fig. 2 gave a good fit of the Lineweaver & Burk (1934) equations and gave a value of 0.04 mM DHAS for the K_m, suggesting a high affinity of the enzyme for its substrate. The optimum pH in 0.13 M tris buffers was

in the region of 7.7 - 7.9 (Fig. 3).

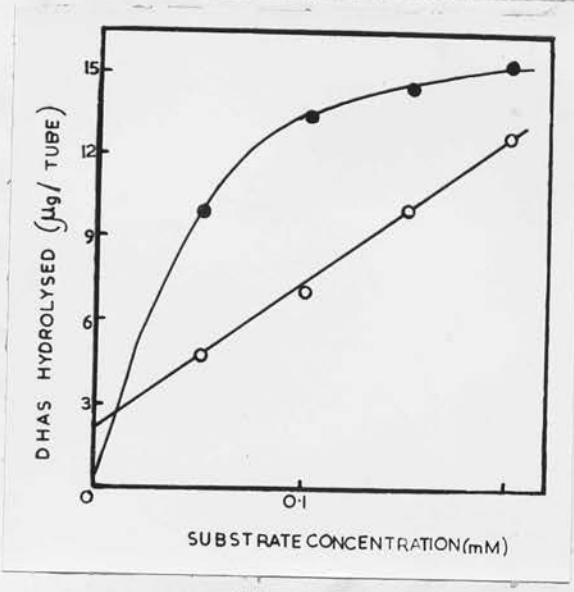

Fig. 2.

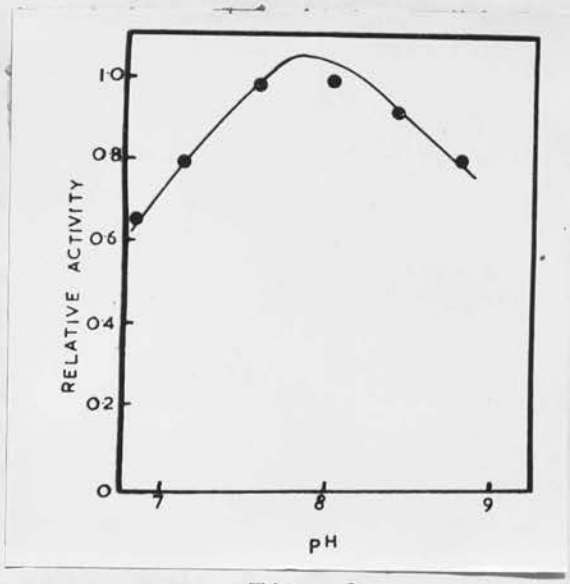

Fig. 3.

Fig. 2. Effect of substrate concentration (s) on reaction velocity (v). Volume of reaction mixture 0.8 ml. containing various concentrations of DHAS at pH 7.8 in 0.13 M tris buffers. Incubated for 1 hr. at 37^{o}. ● , plot of v against s; ○ , plot of s/v x 10^{6} against s.

Fig. 3. Effect of varying pH on the reaction velocity. Conditions as in Fig. 2 but the substrate concentration held at 0.2 mM DHAS and the pH of the buffers varied.

The high specificity of the steroid sulphatase of Patella vulgata (Roy, 1956a) made this aspect of the

mammalian enzyme of considerable interest, the more so as the preliminary results of Gibian & Bratfisch (1956) suggested that a similar specificity might be expected in the case of the mammalian enzyme. The results of this investigation are shown in Table 1: the determinations reported therein were carried out at a pH of 7.8 and a substrate concentration of 0.2 mM steroid sulphate, conditions not necessarily optimal for all the possible substrates. The results show that mammalian steroid sulphatase has a high specificity, exactly comparable to that of the steroid sulphatase of Patella vulgata, as only the 3β-sulphates of the 5α or Δ^5 series of steroids were hydrolysed at a significant rate by the enzyme. Cortisone-21-sulphate was not hydrolysed.

The relationship of steroid sulphatase to sulphatase C was difficult to evaluate directly as both enzymes were associated with the insoluble fraction of the cell and so were difficult to purify to any significant extent. With a number of different enzyme preparations p-nitrophenyl sulphate, a substrate for sulphatase C, was hydrolysed at rates varying from 5 - 15 times greater than DHAS: this variation in the ratio of the rates of hydrolysis of the two substrates suggested that two separate enzymes

TABLE 1. The specificity of steroid sulphatase.

All the assays were carried out at a substrate concentration of 0.2 mM and at a pH of 7.8 in 0.13 M tris buffer. Incubation was for the time indicated at 37°. The substrates were the potassium salts of the sulphates of the steroids listed below. In the case of cortisone the substrate was the 21-sulphate.

	Percentage hydrolysis after 1 hr.	17 hr.
3α-hydroxy-5α-androstan-17-one	-	0
3β-hydroxy-5α-androstan-17-one	12	45
3α-hydroxy-5β-androstan-17-one	-	0
3β-hydroxy-5β-androstan-17-one	-	0
3β-hydroxyandrost-5-ene-17-one	31	79
17α-hydroxyandrost-4-ene-3-one	-	0
17β-hydroxyandrost-4-ene-3-one	-	0
3α-hydroxy-5α-pregnan-20-one	-	0
3β-hydroxy-5α-pregnan-20-one	14	79
3α-hydroxy-5β-pregnan-20-one	-	0
3β-hydroxy-5β-pregnan-20-one	-	3
3β-hydroxypregn-5-ene-20-one	28	76
20α-hydroxypregnane	-	0
20β-hydroxypregnane	-	0
Cortisone	-	0

were involved. It is of interest that phenolphthalein disulphate (a gift from Professor L. Young) was not hydrolysed under comparable conditions. As sulphatase C was greatly influenced by the presence of certain cations in the reaction mixture (Roy, 1956c) the effect of a number of electrolytes on steroid sulphatase activity was investigated. The results are given in Table 2 which also shows, for comparison, the effect of these substances on sulphatase C. It is obvious that these electrolytes have very different effects on the two activities and it would therefore seem justifiable to conclude that steroid sulphatase and sulphatase C are different enzymes.

Rat liver sulphatases. Some experiments were carried out using rat liver as the enzyme source. This tissue was chosen for cell fractionation studies as it, unlike ox liver, can be obtained immediately after the death of the animal. Cell fractionation experiments indicated that, within the limits of the method, all the steroid sulphatase activity was localised in the microsomal fraction, again showing the close relationship of steroid sulphatase to sulphatase C which has a similar intracellular distribution (Dodgson, Spencer & Thomas, 1954).

The relative amounts of the various sulphatases,

Table 2. The influence of electrolytes on the activities of steroid sulphatase and sulphatase C.

Steroid sulphatase activity was determined at pH 7.8 and at a substrate concentration of 0.2 mM DHAS; sulphatase C activity was determined at pH 8.0 and at a substrate concentration of 0.01 M p-nitrophenyl sulphate. The activities are expressed relative to controls, activity 1.0, containing no added electrolyte.

	Concentration. (M)	Relative activity. Steroid sulphatase	Sulphatase C
NaCl	0.05	1.05	0.43
KCl	0.05	0.98	0.77
Na_2SO_4	0.025	0.97	0.41
K_2SO_4	0.025	1.06	0.87
Na_2SO_3	0.0001	0.30	0.45
Na_2HPO_4	0.02	0.55	0.60
KCN	0.01	1.02	0.43
NH_4F	0.01	0.95	0.68

present in rat liver are shown in Table 3. The amounts of the steroid sulphate synthesising system (Roy, 1956b) present are also shown in the table. These values must be considered tentative owing to the limitations of the method used. This method is, however, preferable to an

attempt to assay the enzymes in unfractionated tissue preparations, a procedure which cannot give satisfactory results with the presently available methods (Dodgson, Spencer & Wynn, 1956). It is obvious from the values reported in Table 3 that in rat liver there is present considerably less steroid sulphatase than either of the arylsulphatases.

TABLE 3. Relative activities of the sulphate enzymes in rat liver. Enzymic activities expressed as μM sulphate ester hydrolysed or synthesised per hour per gm. wet weight of liver under the conditions specified in the text

Sex	Sulphatases. A + B	C	Steroid Sulphatase.	DHAS Synthesis.
Female	170	16	0.17	0.17
Male	160	43	0.45	0.07

DISCUSSION.

The results given above show conclusively the occurrence in ox and in rat liver of an enzyme capable of hydrolysing DHAS, or in general the 3β-sulphates of the 5α or Δ^5 series of steroids, the latter

compounds being hydrolysed considerably more rapidly. The enzyme involved is conveniently called steroid sulphatase, although this obviously does not fully describe its specificity as shown in Table 1. It is of interest that mammalian steroid sulphatase preparations will not hydrolyse cortisone-21-sulphate: this is in support of the suggestion (Roy, 1956a) that the hydrolysis of this compound by enzyme preparations from Patella vulgata is due not to steroid sulphatase but to some other enzyme.

In most respects the mammalian and molluscan steroid sulphatases are very similar. Apart from the difference in solubility the only striking distinction between the two enzymes is the position of their pH optima. With molluscan steroid sulphatase the pH optimum is at pH 4.5 while with the mammalian enzyme the pH optimum is at 7.8. These differences explain why previous attempts to detect a mammalian steroid sulphatase have not been successful.

The steroid sulphatase of ox and rat livers is closely associated with sulphatase C, in the latter species at least both the enzymes occurring exclusively in the microsomes, but the evidence presented above would seem to indicate conclusively that steroid sulphatase and sulphatase C are different enzymes.

The results in Table 3 show that from a purely quantitative point of view the amount of steroid sulphatase present in rat liver is negligible compared with the amounts of arylsulphatases present, but on the other hand the steroid sulphatase activity is as great as, or greater than, the ability to synthesise DHAS. The livers of male rats have a considerably higher steroid sulphatase activity than do the livers of female animals. This sex difference also occurs with sulphatase C, as shown in Table 3 and as has previously been noted by Dodgson, Spencer & Thomas (1953). It is of interest to compare these figures for the potential sulphatase activity of rat liver with the ability to synthesise SO_4^{2-} from cysteine, via cysteine sulphinic acid. According to Awapara (1955) rat liver can form approximately 3 μM SO_4^{2-}/gm./hr. through this pathway. If this be the case then it is obvious from the values in Table 3 that the rate of production of SO_4^{2-} by sulphatase activity could be much greater than its rate of production from cysteine. No information is available on the actual activities <u>in vivo</u>.

From a practical point of view mammalian steroid sulphatase is probably of little interest. Although it has been used to hydrolyse urinary steroid sulphates (Langecker, 1956) its use for this purpose must be

limited by the specificity of the enzyme, as is the case with the steroid sulphatase of Patella vulgata. Further, the specific activity of the available preparations of mammalian steroid sulphatase is very much less than that of the molluscan enzyme - in the assay described above the ox enzyme is used in a concentration of 2-3 mg./ml. whereas the molluscan enzyme is used in a concentration of only about 0.1-0.2 mg./ml. For many purposes, therefore, the molluscan enzyme would be more suitable as, for instance, the recovery of steroids is less satisfactory in the presence of large amounts of protein. The further purification of the mammalian enzyme is made difficult by its insoluble nature.

SUMMARY.

1. The presence of a steroid sulphatase in ox and rat liver has been confirmed. The enzyme is localised in the microsomes and has not been obtained in a soluble form.

2. Steroid sulphatase hydrolyses the 3β-sulphates of the 5α and Δ^5 series of steroids.

3. The pH optimum is at 7.8 in 2-amino-2-hydroxymethyl-propane-1,3-diol - acetic acid buffers. The optimum substrate concentration is 0.2 mM dehydroepiandrosterone sulphate.

4. Steroid sulphatase is distinct from arylsulphatase C although the two enzymes are closely associated.

5. Quantitatively the amount of steroid sulphatase in rat liver is negligible compared with the amounts of arylsulphatases present. The enzyme is present in amounts comparable with those of the steroid sulphate synthesising system.

The author is indebted to Miss Isla Sharp for her skilled technical assistance during this work.

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Biochem. J., 1956., Vol. 62, Part 3, 35P.]

The Kinetics of Sulphatase A. By A. B. ROY. (*Department of Biochemistry, University of Edinburgh*)

Roy (1953*a*) described a method for the determination of aryl sulphatases using dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) as substrate and demonstrated the occurrence in ox liver of two sulphatases capable of hydrolysing this substrate. One of these enzymes, sulphatase A, was shown to exhibit anomalous kinetics for the hydrolysis of nitrocatechol sulphate. The reaction velocity was not directly related to the enzyme concentration but apparently to the enzyme concentration raised to the power of $\frac{3}{2}$ (Roy, 1953*b*). An explanation of this anomaly was suggested on the assumption that the enzyme could exist in a number of polymeric forms. Recently Dodgson & Spencer (private communication) pointed out that this explanation was incorrect and that the fundamental anomaly was that the reaction was not of zero order at low enzyme concentrations.

Reinvestigation of this problem has confirmed these observations of Dodgson & Spencer. A study of the hydrolysis of potassium *p*-nitrophenyl sulphate by sulphatase A suggested that the kinetics of this reaction were normal. Unfortunately the low rate of this hydrolysis made the interpretation of these experiments difficult, but the results suggested that the anomaly in the hydrolysis of nitrocatechol sulphate might not be due to the enzyme but to the substrate. It has now been shown that the anomalous kinetics of the hydrolysis of nitrocatechol sulphate are caused by the presence of some impurity in normal preparations of this substance. Purified specimens of nitrocatechol sulphate have been prepared through the chloroform-soluble methylene blue salt which was reconverted to the sodium salt by treatment with Zeo-Carb 225 (Permutite Co. Ltd, London). With these purified preparations of nitrocatechol sulphate as substrate the kinetics of sulphatase A activity were normal, the reaction being of zero order at all enzyme concentrations studied and the reaction velocity being directly related to the enzyme concentration.

It is therefore concluded that the previously suggested explanation for the anomalous kinetics of sulphatase A is wrong and that the effect is due to the presence of some impurity in normal preparations of nitrocatechol sulphate. No suggestion of the nature of this contaminant has been obtained.

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(Reprinted from Nature, Vol. 178, p. 376 only, August 18, 1956)

Occurrence of Nitropyrogallol Disulphate in Preparations of Nitrocatechol Sulphate

DURING an investigation of ox-liver sulphatase *A* [1,2] it became apparent that the substrate, nitrocatechol sulphate, was impure. As this was prepared by persulphate oxidation of *p*-nitrophenol[3,4] it seemed that a likely contaminant might be nitropyrogallol disulphate, formed by the oxidation of the phenol in both *ortho* positions.

When samples of nitrocatechol sulphate were submitted to paper electrophoresis at *p*H 4·2 the presence of two components was obvious, the yellow nitro compounds being readily seen after drying at 110°. In 4 hr. at a potential difference of 250 volts the major and minor components moved 4 cm. and 9 cm. respectively towards the cathode. The two bands were eluted with water and the phenols liberated from the sulphate esters by heating for 1 hr. at 100° in *N* hydrochloric acid. The phenol from the major component had an absorption spectrum in alkali identical with that of 4-nitrocatechol[5]. That of the minor component had a single sharp maximum and 430 mμ and was identical with the absorption spectrum of authentic 5-nitropyrogallol, kindly provided by Dr. T. S. Gardner. Partition chromatography on paper[6] confirmed the identity of the two phenols with 4-nitrocatechol and 5-nitropyrogallol. The major component of the substrate was therefore nitrocatechol sulphate.

The sulphate contents of samples of the acid hydrolysate of the minor component were determined by Dr. J. W. Minnis. The samples contained 318 μgm. nitropyrogallol and yielded 1,017 μgm. barium sulphate. These results were consistent with the view that the minor component of the substrate was nitropyrogallol disulphate.

Colorimetric determinations of the two components following electrophoretic separation showed that of three routine preparations of nitrocatechol sulphate two contained approximately 10 per cent, and one about 0·1 per cent of nitropyrogallol disulphate. A preparation by Dr. Brian Spencer contained approximately 2 per cent of the disulphate. The effect of nitropyrogallol disulphate on the kinetics of nitrocatechol sulphate hydrolysis by sulphatase *A* is under investigation; but electro-

phoretically purified samples of nitrocatechol sulphate exhibit the same anomalous kinetics as do unpurified preparations.

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Vol. XIII/1, 1957 – pag. 32

10.

The Kinetics of Sulphatase A

The anomalous kinetics of the hydrolysis of dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) by ox liver aryl sulphatase A (sulphatase A) were first noted in this laboratory[1] and similar anomalies have since been detected in the corresponding enzymes of rat[2] and human[3] liver. The reaction velocity is not directly proportional to the enzyme concentration but with incubation times of 1 h it is linearly related to the enzyme concentration raised to the power of 3/2[4]. It was suggested[4] that this effect might be due to a polymerisation of the enzyme. DODGSON and SPENCER[5] showed the fundamental anomaly to be that the reaction was not of zero order at low concentrations of enzyme and suggested an explanation for this effect in a series of competing reactions involving the enzyme, substrate and reaction products. Simultaneously, ROY[6] showed that nitrocatechol sulphate used in these studies was impure, containing up to 10% of nitropyrogallol disulphate[7] which could be removed by paper electrophoresis. It was also shown[6] that preparations of nitrocatechol sulphate obtained through its methylene blue salt did not exhibit these anomalous kinetics, the reaction being of zero order and the velocity directly proportional to the enzyme concentration. This seemed excellent evidence for the suggestion[6] that the anomalies might be due to

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the use of impure substrate preparations but more recent work has cast some doubt on this interpretation as specimens of nitrocatechol sulphate free from nitropyrogallol sulphate still give anomalous kinetics[8]. In view of the increasing use of nitrocatechol sulphate as a substrate in sulphatase assays it seems that a more detailed report of the observations might be of value.

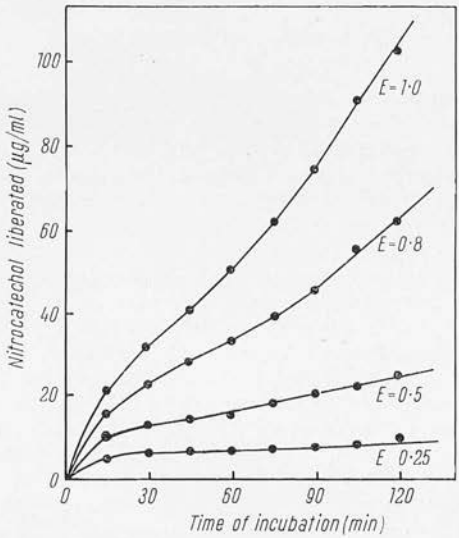

Fig. 1. – Progress curves for the hydrolysis of nitrocatechol sulphate (0·003 *M*) at pH 5·0 by sulphatase A. The relative enzyme concentrations are indicated on the appropriate curves.

In the present studies the nitrocatechol sulphate was free from nitropyrogallol disulphate and the experimental procedures were those already described in detail[9]. The enzyme was ox liver sulphatase A–3[4]. Assays were carried out at 37° in 0·15 *M* acetate buffer, pH 5·0, and at a substrate concentration of 0·003 *M* nitrocatechol sulphate.

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Typical progress curves for the hydrolysis of nitrocatechol sulphate by sulphatase A are shown in Figure 1 and in Figure 2 is shown the relationship between enzyme concentration and reaction velocity. The progress curves consist typically of three parts: stage 1, in which the initial velocity decreases rapidly; stage 2, during which the velocity may fall to almost zero; and stage 3 when the velocity rises again to an almost constant value which is considerably less than the initial velocity.

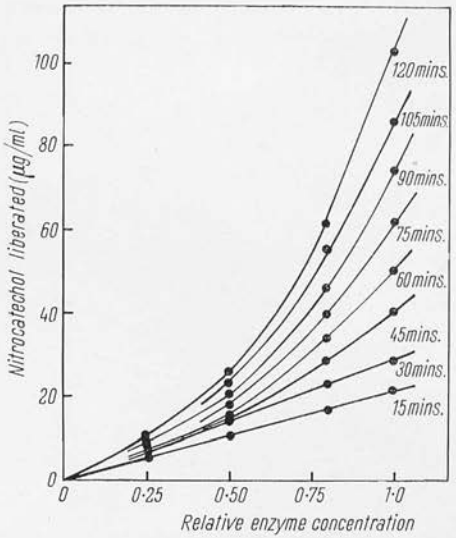

Fig. 2.—Effect of varying times of incubation on the relation between the concentration of sulphatase A and the velocity of the hydrolysis of nitrocatechol sulphate.

The relative proportions of these three stages vary considerably with changes in enzyme concentration and with those used in the original work[1] stage 2 disappears so that the reaction approximates to one of zero order. Preincubation of the enzyme in the absence of substrate did not significantly alter the shapes of these progress curves. Increasing the substrate concentration to 0·01 *M* caused an increased velocity during stage 1 and a prolongation of stage 2 but the velocity in stage 3 was ap-

parently unaltered. Lowering the pH to 4·5 made no significant difference to the progress curves but at pH 6·0 the length of stage 2 was greatly prolonged, becoming important even at high concentrations of enzyme. The most interesting effect was that of SO_4^{2-}ions: in the presence of 0·001 *M* SO_4^{2-}ions stage 2 virtually disappeared, even at low enzyme concentrations, as shown in Figure 3. This effect was still obvious at pH 6·0 and is therefore presumably responsible for the pronounced activation of sulphatase A by SO_4^{2-}ions at that pH[10].

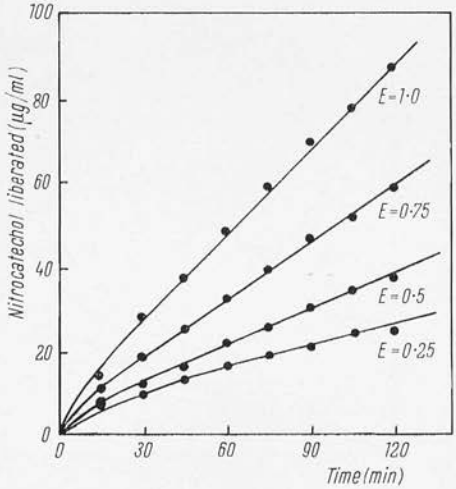

Fig. 3.—Effect of 0·001 *M* K_2SO_2 on the progress curves of the hydrolysis of nitrocatechol sulphate by sulphatase A.

Preincubation of the enzyme with SO_4^{2-}ions in the absence of substrate did not alter the progress curves from those in Figure 3. The addition of nitrocatechol (0·1 *mM*) to the reaction mixture caused an overall increase in the reaction velocity. When both nitrocatechol and SO_4^{2-}ions were added in equivalent amounts their effects were additive. For comparison with these results Figure 4 shows curves obtained using substrate preparations purified via the chloroform-soluble methylene blue

[10] A. B. Roy, Biochem. J. *59*, 8 (1955).

salt: the completely normal kinetics of this hydrolysis are obvious.

One point of considerable importance was whether these anomalous kinetics were shown for the hydrolysis of substrates other than nitrocatechol sulphate. The high specificity[4] of sulphatase A made detailed study of this point difficult as to obtain results of immediate significance it was essential to use the enzyme under the same conditions for the hydrolysis of all the substrates. Figure 5 illustrates the hydrolysis of 0·05 *M* *p*-nitrophenyl sul-

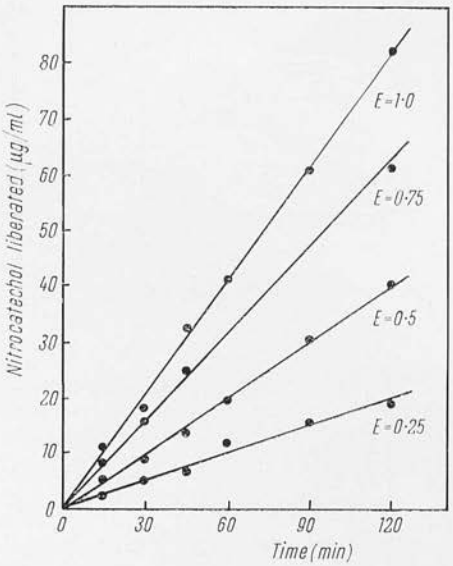

Fig. 4. — Progress curves for the hydrolysis by sulphatase A of nitrocatechol sulphate obtained via the methylene blue salt.

phate by sulphatase A. It is obvious that the reaction does not show the anomalies characteristic of the hydrolysis of nitrocatechol sulphate, the progress curves being simply those to be expected if the enzyme were unstable under the experimental conditions. It can also be seen that the reaction velocity is not directly related to the enzyme concentration. The addition of 0·001 *M* SO_4^{2-}-ions did not alter the progress curves although it caused con-

siderable inhibition. These results are not in agreement with those obtained for human sulphatase A by DODGSON and SPENCER[11] who state that the hydrolysis of *p*-nitrophenyl sulphate exhibits the same anomalies as that of nitrocatechol sulphate.

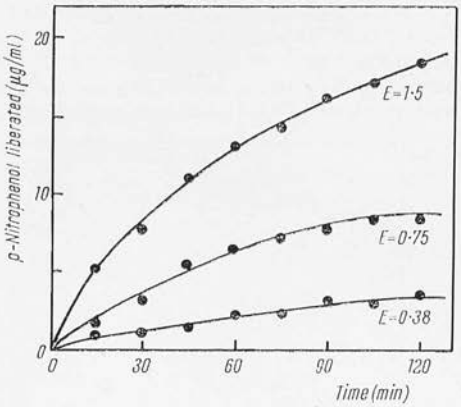

Fig. 5.—Progress curves for the hydrolysis of *p*-nitrophenyl sulphate (0·05 *M*) at pH 5·0 by sulphatase A.

These results are extremely difficult to interpret and any explanation must account for the following facts:

(1) During the hydrolysis of nitrocatechol sulphate the reaction velocity rapidly falls and then rises again. Under identical conditions with *p*-nitrophenyl sulphate the velocity falls but does not subsequently rise.

(2) The rise in velocity of the hydrolysis of nitrocatechol sulphate is greatly enhanced by 0·001 *M* SO_4^{2-} ions although higher concentrations are inhibitory. The hydrolysis of *p*-nitrophenyl sulphate is not activated.

(3) Nitrocatechol increases the rate of hydrolysis of its sulphate.

(4) Preincubation of the enzyme in the absence of substrate, with or without nitrocatechol or SO_4^{2-} ions, makes no difference to the progress curves.

(5) Purification of the nitrocatecholsulphate through its methylene blue salt gives preparations which do not exhibit anomalous kinetics.

[11] K. S. DODGSON and B. SPENCER, Biochim. biophys. Acta *21*, 175 (1956).

From these data it is impossible to decide whether or not the anomalies are due to some peculiarity of the nitrocatechol sulphate or to some inherent property of the enzyme. The differences in behaviours between nitrocatechol sulphate and nitrophenyl sulphate suggests the former, but on the other hand this difference might simply be due to the much lower affinity and rate of hydrolysis of nitrophenyl sulphate[4]. As preincubation of the enzyme in the absence of substrate does not affect the progress curves it seems that the fall in velocity in stage 1 of the hydrolysis is due not to a simple inactivation of the enzyme, but to some complex reaction involving the substrate. Further, as the reaction velocity increases again in stage 3 it must be presumed that the inactivation in stage 1 is reversible. This increase in velocity in stage 3 cannot be directly due to the action of nitrocatechol and SO_4^{2-}ions liberated by the hydrolysis as if this were so then a similar effect would be expected on adding SO_4^{2-}ions to the system hydrolysing nitrophenyl sulphate.

Dodgson and Spencer[5] proposed an explanation based on the substrate inhibition occurring with nitrocatechol sulphate. They suggested that this substrate inhibition differed from the normal type in that it was a slow reaction, so accounting for the drop in velocity during stage 1. They then suggested that the later rise in velocity was due to a reversal of the substrate inhibition by the reaction products. Such an explanation seems improbable as it is difficult to see how the reaction products could reverse the substrate inhibition.

The following explanation seems a possible alternative. If it is supposed that the enzyme-substrate complex (ES) can react in two ways, one reforming the active enzyme (E) and the other giving an inactive enzyme (E′), then the following equilibria must be considered during the reaction:

$$E + S \rightleftharpoons ES \begin{cases} \rightarrow E + \text{Products} \\ \rightarrow E' + \text{Products.} \end{cases}$$

The rate at which the inactivation of the enzyme will occur during the hydrolysis of nitrocatechol sulphate will therefore be governed by the kinetics of the various reactions. Further, as the reaction products accumulate, or are added, it is possible that the active form of the

enzyme, E, could be reformed from the inactive one, E′, if the appropriate equilibrium constants were of the correct magnitude. In the case of the hydrolysis of nitrophenyl sulphate, on the other hand, the equilibrium

$$ES \rightleftharpoons E' + \text{Products}$$

may lie so far to the right that the addition of the reaction products might make relatively little difference to the rate of formation of E′. Not all the experimental findings can be explained on this basis. It might be expected from the above that the addition of SO_4^{2-}ions at the beginning of the reaction would prevent the initial rapid formation of E′ but as seen from Figure 3 this does not occur. Again the results using the substrate obtained via the methylene blue salt cannot be explained on the above basis.

These points suffice to show the extremely complex nature of the kinetics of sulphatase A and although the hypothesis proposed may be incorrect it provides a basis for further investigation. They also show the dangers inherent in the use of nitrocatechol sulphate in the assay of sulphatases of the type of sulphatase A: other sulphatases apparently do not show these anomalies[12] so that the use of this substrate in their assay is straighforward.

A. B. Roy

Department of Biochemistry, University New Buildings, Edinburgh, July 17, 1956.

Résumé

Les cinétiques de l'hydrolyse du sulfate de nitrocatéchol par la sulfatase A sont très anormaux parce qu'il n'y a aucune relation linéaire entre la vitesse de la réaction et la concentration de l'enzyme. La réaction tient trois phases: dans la première, la vitesse décroît rapidement, dans la deuxième, elle diminue de plus en plus tandis qu'elle s'accroît dans la troisième. La réaction est très sensible à la composition du mélange réactionel, et l'addition des produits de la réaction, particulièrement le sulfate inorganique, augmente la vitesse de l'hydrolyse. En utilisant comme substrat le *p*-nitrophén ylsulfate, ces irrégularités ne sont pas constatées. Une interprétation de ces résultats est présentée!

[12] K. S. Dodgson, B. Spencer, and K. Williams, Biochem. J. *61*, 374 (1955). – D. Robinson, J. N. Smith, B. Spencer, and R. T. Williams, Biochem. J. *51*, 202 (1952).

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THE STEROID SULPHATASE OF *PATELLA VULGATA*

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Recently several groups of workers[1,2,3,4] have reported the use of crude sulphatase preparations from molluscan viscera for the hydrolysis of urinary steroid sulphates, but no investigation of the enzyme involved has so far been undertaken. The enzyme, which has been named "alkyl sulphatase"[4] and "steroid sulphatase"[5] has been assayed by its ability to hydrolyse sodium dehydroepiandrosterone sulphate (DHAS)[2,3,4], and it has apparently been assumed, without evidence, that it is capable of hydrolysing all steroidal alkyl sulphates. The error of this assumption is shown by the work of SAVARD, BAGNOLI AND DORFMAN[5] who have demonstrated that although the steroid sulphatase of *Otala punctata* will hydrolyse DHAS, it will not attack androsterone sulphate. The present paper describes preliminary findings with the corresponding enzyme of *Patella vulgata* which hydrolyses DHAS and which has been used[3,4] as a means of hydrolysing urinary conjugated steroids prior to their estimation.

The enzyme was obtained by extracting with 0.1 *M* KCl an acetone powder of the visceral hump of *Patella vulgata*, and was purified by fractional precipitation between 30 % and 50 % saturated ammonium sulphate and between 33 % and 50 % acetone at −9° and pH 6.5 in 0.05 *M* acetate buffer. A ten-fold purification of the enzyme has so far been achieved, and a typical preparation from 50 g acetone powder gave 30 ml of a solution of steroid sulphatase containing 3000 s.u./ml, 400 s.u./mg protein; 1 s.u. being defined as the amount of steroid sulphatase hydrolysing 1 μg DHAS in 1 h under the standard conditions described below. The term steroid sulphatase is preferable to alkyl sulphatase as there is no evidence that the enzyme will hydrolyse alkyl sulphates in general, the above preparation not hydrolysing dichlorophenoxyethyl sulphate under either the conditions described by VLITOS[6] or those optimal for the hydrolysis of DHAS. It would therefore seem that the term alkyl sulphatase should be restricted to the enzyme shown by VLITOS[6] to be present in *Bacillus cereus var. mycoides*.

DHAS was estimated by a micro-modification of the method developed by VLITOS[6] for the assay of sodium dichlorophenoxyethyl sulphate, making use of the solubility in chloroform of the complexes between methylene blue and sulphuric acid esters. This blue complex was estimated colorimetrically. For the estimation of enzymic activity 0.25 ml enzyme was added to 0.25 ml 0.5 *M* acetate buffer, pH 4.5, and 0.5 ml 0.4 m*M* DHAS: the mixture was incubated for 1 h at 37° and the unhydrolysed DHAS estimated by the above method. This method was not directly applicable to accurate kinetic studies, depending as it did on the relatively small difference (10 %–20 %) between the amounts of DHAS present after incubation in the presence of and in the absence of enzyme, but a great advantage of the method for preliminary work was its direct applicability to the estimation of any steroid sulphate.

Using these methods the presence of an enzyme hydrolysing DHAS in the visceral hump of *Patella vulgata* was readily confirmed. This enzyme, steroid sulphatase, had a very flat pH optimum around pH 4.5 in 0.15 *M* acetate buffers and had an optimum substrate concentration of approximately 0.3 m*M* DHAS. Epiandrosterone sulphate was hydrolysed at approximately 25 % the rate of DHAS and androsterone sulphate was not hydrolysed to any detectable extent. Steroid sulphatase thus shows a very high degree of stereochemical specificity. The effect of a number of inhibitors on the activity of the enzyme is shown in Table I.

TABLE I

THE INFLUENCE OF INHIBITORS ON THE ACTIVITY OF STEROID SULPHATASE AND OF GLUCOSULPHATASE, THE DATA FOR THE LATTER BEING THAT OF SODA[9]

		Percentage inhibition	
		steroid sulphatase	*glucosulphatase*
Glucose	0.5 *M*	15	95
SO_4^{2-}	0.005 *M*	65	35
$H_2PO_4^-$	0.005 *M*	98	73
F^-	0.005 *M*	90	79
H_3BO_3	0.005 *M*	83	65
Cl^-	0.05 *M*	30	0

Previous workers have demonstrated that molluscan tissues in general are rich sources of a number of sulphatases, a phenol sulphatase, a glucosulphatase and a chondrosulphatase having been described[7] as well as a steroid sulphatase[2,3,5]. The relationship of steroid sulphatase to these other sulphatases is not yet fully understood as the purest preparations available exhibit both phenol sulphatase and glucosulphatase activity, these two enzymes being detected by their ability to hydrolyse under arbitary conditions nitrocatechol sulphate and glucose-3-sulphate respectively. Using the methods of paper electrophoresis already described[8] it was shown that steroid sulphatase was not identical with phenol sulphatase as the two enzymes were readily separable by electrophoresis in veronal buffer, pH 7.4. The relationship to glucosulphatase is not so clear: the glucosulphatase activity closely follows the steroid sulphatase through the purification of the latter, but an insufficient degree of purification has been achieved to allow any significant conclusions to be drawn from this fact. On the other hand, as shown in Table I, the responses of steroid sulphatase and glucosulphatase to inhibitors are quantitatively very different, suggesting that the two enzymes are not identical. The data in Table I referring to glucosulphatase is that of SODA[9] for the glucosulphatase of *Charonia lampas*.

These preliminary results show the need for a thorough investigation of steroid sulphatase, especially its specificity, before any reliance can be placed on results obtained through using it as a means of hydrolysing urinary steroid conjugates. Furthermore, the inhibition of steroid sulphatase by many of the ions present in urine must restrict the use of the enzyme to the hydrolysis of either a desalted urine or partially purified urine extracts.

The author wishes to express his thanks to Professor G. F. MARRIAN, F.R.S., for generous gifts of steroids and for his interest in this work. He also wishes to thank Messrs. Gemec Ltd. for a gift of sodium dichlorophenoxyethyl sulphate.

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The Steroid Sulphatase of *Patella vulgata*

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Molluscan tissues have been known for many years to be a rich source of a number of sulphatases (Soda, 1936). Recently considerable interest has been shown in these enzymes as a means of hydrolysing urinary steroid sulphates (Henry, Thevenet & Jarrige, 1952; Stitch & Halkerston, 1953; Jayle & Beaulieu, 1954). To date, three sulphatases have been detected in extracts of *Patella vulgata*: aryl sulphatase, hydrolysing aryl sulphates (Dodgson & Spencer, 1953); glycosulphatase, hydrolysing glucose sulphates (Dodgson & Spencer, 1954); and steroid sulphatase, hydrolysing dehydro*epi*androsterone sulphate (Stitch & Halkerston, 1953; Roy, 1954*b*). A fourth enzyme, chondrosulphatase, hydrolysing chondroitin sulphate, has been detected in related molluscs by Soda, Katsura & Yoda (1940) and is probably also present in *Patella*. None of these enzymes has been obtained free from the others, nor even purified to any extent, so that the specificity of the various enzymes is obscure.

An enzyme hydrolysing dehydro*epi*androsterone sulphate has been detected in *Helix pomatia* (Henry *et al.* 1952) and in *Otala punctata* (Savard, Bagnoli & Dorfman, 1954). The relation of these enzymes to that of *Patella* has not yet been investigated, but they appear to be similar in their general properties, although none of these has been studied in detail.

The present paper gives the results of an investigation into the purification and properties of steroid sulphatase carried out with the aim of obtaining a preparation suitable for use in the assay of urinary steroid conjugates. A preliminary account of some aspects of this work has already appeared (Roy, 1954*b*).

EXPERIMENTAL

Preparation of substrates

Dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) was prepared by the method already described (Roy, 1953*a*). Potassium glucose-6-sulphate was prepared from the barium salt synthesized by the method of Duff (1949). It should be noted that this material is not a pure compound (Dodgson & Spencer, 1954). Chondroitin sulphate was prepared from human costal cartilage by the method of Einbinder & Schubert, 1950).

Steroid sulphuric acid esters were synthesized by a modification of the method of Paterson & Klyne (1948). The steroid alcohol (50 mg.) was dissolved in 3 ml. of dry benzene and an equal weight of pyridine-SO_3 was added. The mixture was boiled under reflux on the water bath for 1·5 hr., then cooled and diluted with 30 ml. of light petroleum (b.p. 60–80°). After standing for 1 hr. at 0°, the precipitated pyridinium salt of the steroid sulphate was filtered off and washed with light petroleum. The residue was extracted 5 times with 2 ml. portions of boiling chloroform in order to separate the soluble pyridinium salt from the excess pyridine-SO_3, and the chloroform solution so obtained was taken to dryness *in vacuo*. The residue of the pyridinium salt was dissolved in the minimum of boiling 70% aqueous ethanol and an equal volume of hot cold-saturated K_2SO_4 was added. Water was added dropwise to the boiling mixture until complete solution was effected. On cooling, the potassium salt of the steroid sulphate crystallized out and was purified by recrystallization from water. In the case of the sulphates of the Δ^5 unsaturated steroids, treatment with boiling water was kept as brief as possible to prevent decomposition owing to the hydrolysis of these compounds in hot aqueous solution (Figs. 7, 8). The yield of the recrystallized material was normally between 50% and 70% of the theoretical.

In the case of highly polar, labile steroids such as cortisone, the above method was modified by using pyridine as the solvent and allowing the sulphation to proceed at room temperature for 60 hr.

Potassium cholesteryl sulphate was prepared by the method of Sobel & Spoerri (1941). Sodium cortisone sulphate was a preparation of Merck and Co. Inc.

In the following, when the term 'steroid sulphate' is used, the compound referred to is the salt, usually the potassium salt, of the sulphuric acid ester of the steroid in question.

Estimation of steroid sulphates

The determination of steroid sulphates was carried out by a technique developed from that of Vlitos (1953) for the estimation of sodium dichlorophenoxyethyl sulphate in soil; this makes use of the solubility in chloroform of the methylene blue complexes of sulphuric acid esters. The methylene blue reagent was similar to that of Vlitos (1953) and was prepared by dissolving 250 mg. of methylene blue chloride in water, adding 50 g. of Na_2SO_4 and 10 ml. of H_2SO_4, and making up to 1 l. with water.

To 1 ml. of an aqueous solution of the steroid sulphate, containing from 25 to 100 μg. of steroid sulphate, was added 1 ml. of methylene blue reagent. The mixture was then extracted with 5 ml. of chloroform by shaking vigorously in a stoppered tube for 30 sec. Any emulsion was broken by centrifuging and the aqueous layer was sucked off, after which 2 ml. of the clear chloroform layer was pipetted into 10 ml. of 75% aqueous ethanol. The intensity of the resulting blue solution was read in the Spekker absorptiometer with Ilford filter no. 608 (700 mμ

against a reagent blank prepared as above. Smaller amounts of steroid sulphates could be determined by reading the intensity of the chloroform solution without diluting and using the micro-cells.

If the solution of the steroid sulphate contained protein the recovery of the sulphate tended to be low and erratic. This was overcome by heating the solution in a boiling-water bath for 5 min. and cooling in running water before adding the methylene blue reagent. With this slight modification the recovery of dehydro*epi*androsterone sulphate (DHAS) from protein solutions was constant at 95%, as shown by the results in Table 1. That this slightly low recovery is due to the hydrolysis of DHAS at 100° can be seen from the data of Fig. 7 and from the fact that the recovery of the more stable *epi*androsterone sulphate was 100% under the same conditions. The above method was therefore suitable for the assay of steroid sulphatase. The slight inaccuracy of the method was more than countered by its general applicability, there being no significant difference between calibration curves prepared from DHAS, *epi*androsterone sulphate or pregnenolone sulphate.

Table 1. *Recovery of DHAS added to enzyme solutions without incubation*

Protein concentration 250 μg./ml. at pH 4·5 in 0·15 M acetate.

Added DHAS (μg.)	Recovery (%)	
	Range	Mean
96·0	93–96	95
76·8	94–97	95
57·6	94–95	94
38·4	95–98	96
19·2	91–100	93

The partition of the steroid sulphate–methylene blue complex between the aqueous and chloroform phases was influenced to only a slight extent by the presence of electrolytes in concentrations normally encountered in enzyme work. A concentration of 0·1 M-NaCl in the aqueous phase increased the partition in favour of the chloroform phase by only 10%, while similar concentrations of acetate, sulphate, and phosphate were without effect.

Measurement of enzyme activity

Aryl sulphatase. This was assayed by a simplification of the technique developed by Roy (1953*a*) for the assay of ox-liver sulphatases, using nitrocatechol sulphate as substrate and determining colorimetrically the liberated 4-nitrocatechol. Because of the very high activity of the enzyme preparations used, the protein concentration in the reaction mixture was so low that deproteinizing was not necessary.

To 0·25 ml. of 0·5 M acetate buffer, pH 5·6, and 0·5 ml. of 0·01 M nitrocatechol sulphate, previously adjusted to pH 5·6 with acetic acid, was added 0·25 ml. of enzyme solution to give a final substrate concentration of 0·005 M nitrocatechol sulphate at pH 5·6, the optimum conditions for the assay of aryl sulphatase. After incubation for 1 hr. at 37° the reaction was stopped and the colour was developed by the addition of 8 ml. of 1·5 N-NaOH. The intensity of the red colour was read in the Spekker absorptiometer with Ilford filter 604 (520 mμ.) against a blank in which the enzyme and substrate had been incubated separately and mixed only immediately before the addition of the alkali.

Steroid sulphatase. To 0·25 ml. of 0·5 M acetate buffer, pH 4·5, were added 0·5 ml. of 0·4 mM DHAS and 0·25 ml. of enzyme solution giving a final substrate concentration of 0·2 mM DHAS at pH 4·5. After incubation at 37° for 1 hr. the reaction was stopped by heating the tubes in a boiling-water bath for 5 min. After cooling, the amount of DHAS remaining was determined by the method described above. Simultaneous control experiments were always carried out, in which the enzyme and substrate were incubated separately, being mixed only immediately before heating in the water bath. The amount of DHAS in these control tubes was also estimated as above.

The enzymic activity was therefore proportional to the difference between the amounts of DHAS remaining after incubation in the presence of and in the absence of the enzyme. This difference was normally small, about 15%, so that the method was not suited for accurate kinetic studies, but it was adequate for the experiments described below.

A few experiments were carried out in which the above reaction mixtures were extracted with carbon tetrachloride in order to separate the dehydro*epi*androsterone from the DHAS. Samples of these extracts were taken to dryness and the amounts of dehydro*epi*androsterone present were determined by the usual Zimmerman technique. The results obtained were in agreement with those obtained by the methylene blue technique, but, as no greater accuracy could readily be attained, the method was not used as a routine.

Preparation of steroid sulphatase

Preliminary experiments showed that the aryl sulphatase and steroid sulphatase of *Patella* extracts closely paralleled one another during acetone or ammonium sulphate fractionation procedures, so that the following method, although designed primarily for the purification of steroid sulphatase, gives a considerable purification of both enzymes. The final stage, stage 3, is a convenient one for the further purification of these two enzymes.

The starting material was an acetone powder of the visceral hump of *Patella vulgata*, the common limpet. The animals were collected locally and the visceral humps dissected out as soon as possible after collection. An acetone powder was prepared from this material in the usual manner (Dodgson & Spencer, 1953) and was kept *in vacuo* over P_2O_5 until required. The acetone powder showed no diminution in activity when stored thus for 6 months.

Stage 1. 50 g. of acetone powder were extracted for 1 hr. at room temperature with 500 ml. of 0·1 N-KCl and the extract was separated by centrifuging. The residue was re-extracted with a further 250 ml. of KCl solution, and the combined extracts were chilled to 0°. The pH of the solution was carefully lowered to 2·5 with N-HCl, allowed to remain there for 2 min. and then taken to 5 with M sodium acetate. This destroyed the very large amounts of β-glucuronidase present without significantly affecting the sulphatases (Dodgson & Spencer, 1953) and also eliminated a factor, presumably a cellulase, which, if this procedure were omitted, weakened the dialysis tubing used in the later stages. The mixture was then made 0·3 saturated with respect to $(NH_4)_2SO_4$ by the addition of the requisite

amount of a saturated solution. After standing for 3 hr. at 0°, the precipitate was centrifuged off and discarded. The supernatant was made 0·7 saturated with respect to $(NH_4)_2SO_4$ and the heavy precipitate was allowed to settle out overnight at 0°. The precipitate was centrifuged off, dissolved in 50 ml. of water and dialysed overnight against running tap water at room temperature, giving 160 ml.

Stage 2. To 140 ml. from stage 1 were added 20 ml. of 0·5 M sodium acetate, pH 6·5, and 2 ml. of 0·3 M-$CaCl_2$. The volume was made up to 200 ml. and the mixture precipitated with 100 ml. of acetone at −9° as previously described (Roy, 1953*b*). The precipitate was centrifuged off and discarded, while the supernatant, kept at −9°, was precipitated with a further 100 ml. of acetone. The supernatant obtained on centrifuging was discarded and the precipitate was dissolved in 20 ml. of water and dialysed overnight to give 80 ml.

Stage 3. 50 ml. from stage 2 were brought to pH 5 with sodium acetate and the solution made 0·4 saturated with respect to $(NH_4)_2SO_4$ as before. After standing for some hours at 0°, the precipitate was centrifuged off and discarded. The supernatant was made 0·7 saturated with respect to $(NH_4)_2SO_4$ and, after standing overnight at 0°, the precipitate was separated, dissolved in 20 ml. of water and dialysed overnight to give 50 ml.

As a routine this preparation was freeze-dried, giving 450 mg. of a bulky, readily soluble powder; the dry material was stored at −10°, under which conditions it was apparently stable indefinitely. For the assay of steroid sulphatase this dry preparation was used in a final concentration of 150 μg./ml., and for aryl sulphatase in a concentration of 4 μg./ml.

In a typical preparation the recovery of the steroid sulphatase at stage 3 was about 50% of that present in the KCl extract and represented a 50-fold concentration of the enzyme with respect to the protein content of the solutions.

Paper electrophoresis

Electrophoresis on paper was carried out as previously described (Roy, 1954*a*), at pH 7·4 in 0·03 M veronal buffer on Whatman no. 100 filter paper. The time of running was 6 hr. at 500 v. Aryl sulphatase and steroid sulphatase activities were localized by cutting the paper into 1 cm. strips and incubating portions of these strips in the reaction mixtures described above.

RESULTS

Aryl sulphatase

As the properties of this enzyme have already been investigated by Dodgson & Spencer (1953), the results obtained in the present investigation are only briefly reported in so far as they have a bearing on the relationship of this enzyme to steroid sulphatase. The results obtained were in general agreement with those obtained by the above workers who used *p*-acetylphenyl sulphate as the substrate in their investigations, not nitrocatechol sulphate.

The pH optimum for the hydrolysis of nitrocatechol sulphate was 5·4–5·6 in acetate buffers (Fig. 1), and the optimum substrate concentration at that pH was 0·005 M (Fig. 2). These latter results fitted the Lineweaver & Burk (1934) equations and showed K_m to be 0·0007 M nitrocatechol sulphate. The reaction velocity determined at pH 5·6 and at a substrate concentration of 0·005 M nitrocatechol sulphate was directly proportional to the enzyme concentration and remained constant for at least 2 hr., apart from a slight decrease in the reaction velocity after the first 5 min.

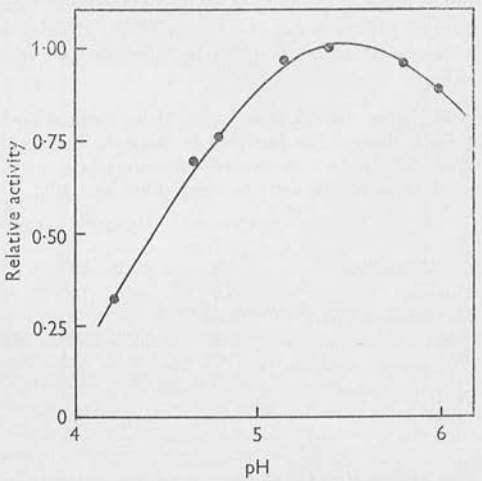

Fig. 1. Effect of pH on aryl sulphatase activity. Volume of reaction mixture 1·0 ml., containing 0·5 ml. 0·01 M nitrocatechol sulphate, 0·25 ml. enzyme and 0·25 ml. 0·5 M acetate buffers of varying pH. Incubated for 1 hr. at 37°.

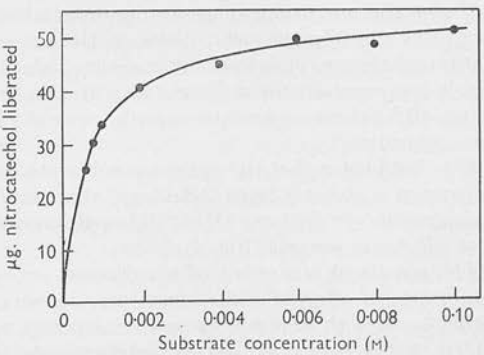

Fig. 2. Effect of varying substrate concentrations on aryl sulphatase activity. Volume of reaction mixture 1·0 ml., containing 0·25 ml. 0·5 M acetate buffer, pH 5·6, 0·25 ml. enzyme solution and 0·5 ml. nitrocatechol sulphate solutions of varying concentrations. Incubated for 1 hr. at 37°.

The effect of a number of inhibitors was studied and the results are summarized in Table 2. In every case the inhibitor was dissolved in the acetate buffer so that the enzyme was added to the previously mixed substrate and inhibitor. The results shown in Table 2 confirm those obtained by Dodgson & Spencer (1953), the most important observation being the activation of aryl sulphatase by Cl^- ions. The inhibition by SO_4^{2-} was not due to a shift in the position of the pH optimum, as was the case with ox-liver sulphatase A (Roy, 1955).

Table 2. *Effect of various substances on the hydrolysis of nitrocatechol sulphate (NCS), DHAS and cortisone-21-sulphate (CS) by extracts of* Patella vulgata

In each case the conditions were those optimal for the enzymic hydrolysis, as described in the text. The activity is expressed relative to control determinations with no added substance, this activity being taken as 100%.

Compound	Concentration (M)	Relative activity NCS	DHAS	CS
Glucose	0·5	80	100	50
Glucose-6-sulphate	0·025	—	70	25
NaCl	0·05	118	75	96
KCl	0·05	—	74	—
Na_2SO_4	0·005	95	25	28
NaH_2PO_4	0·005	16	2	7
NaF	0·005	26	10	—

The above kinetic studies gave no indication of the presence of more than one aryl sulphatase in extracts of *Patella vulgata*, but, as reported below, the results of the electrophoresis studies showed the presence of two aryl sulphatases.

Steroid sulphatase

Under the conditions of assay described above, there was direct relationship between the concentration of the enzyme and the reaction velocity, which was constant for at least 2 hr.; by this time about 40% of the substrate initially present had been hydrolysed.

Fig. 3 indicates that the optimum substrate concentration is about 0·2 mM DHAS and that the K_m is approximately 0·04 mM DHAS. The pH optimum is at pH 4·5 in acetate (Fig. 4).

The results of a number of experiments on the inhibition of steroid sulphatase are shown in Table 2. As with aryl sulphatase, the enzyme was added to the previously mixed substrate and inhibitor. The most important result is the inhibition of steroid sulphatase by Cl^- ions under conditions which give an activation of aryl sulphatase. As previously reported (Roy, 1954*b*), steroid sulphatase will not hydrolyse androsterone sulphate. It has now been shown that this compound has little inhibitory action on the hydrolysis of DHAS by steroid sulphatase, a concentration of 0·2 mM androsterone sulphate inhibiting the hydrolysis of DHAS by only about 10%, showing that steroid sulphatase has very little affinity indeed for androsterone sulphate.

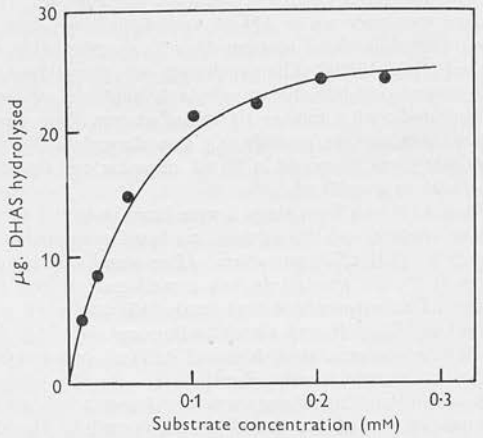

Fig. 3. Effect of varying substrate concentrations on steroid sulphatase activity. Volume of reaction mixture 1·0 ml., containing 0·25 ml. 0·5 M acetate buffer, pH 4·5, 0·25 ml. enzyme, and 0·5 ml. DHAS solutions of varying concentrations. Incubated for 1 hr. at 37°.

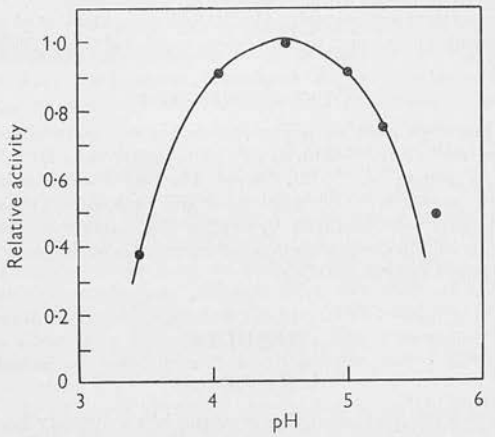

Fig. 4. Effect of pH on steroid sulphatase activity. Volume of reaction mixture 1 ml., containing 0·5 ml. 0·4 mM DHAS, 0·25 ml. enzyme solution and 0·25 ml. 0·5 M acetate buffers of varying pH values. Incubated for 1 hr. at 37°.

Specificity of steroid sulphatase. These experiments (Table 3) were carried out under conditions optimal for the hydrolysis of DHAS, pH 4·5 and a substrate concentration of 0·2 mM. It is obvious that steroid sulphatase has a very high degree of

stereochemical specificity hydrolysing only the 3-sulphates of the 5α-3β-hydroxy- or of the Δ^5-3β-hydroxy-steroids. The compounds studied have been mainly the 3-sulphates as these are the only steroid sulphates which have been shown to occur naturally in urine, and also as complete series of other hydroxy steroids are very difficult to obtain.

Table 3. *Specificity of steroid sulphatase*

All the assays were carried out at a final substrate concentration of 0·2 mM and a pH of 4·5 in 0·15M acetate. Incubated at 37° for the time indicated. The substrates were the potassium salts of the sulphates of the steroids listed below, except in the case of cortisone sulphate which was the sodium salt.

Steroid	Percentage hydrolysis after 1 hr.	17 hr.
3α-Hydroxy-5α-androstan-17-one (Androsterone)	0	1
3β-Hydroxy-5α-androstan-17-one (*epi*Androsterone)	26	96
3α-Hydroxy-5β-androstan-17-one	2	0
3β-Hydroxy-5β-androstan-17-one	0	1
3β-Hydroxyandrost-5-ene-17-one (Dehydro*epi*androsterone)	59	96
17β-Hydroxyandrost-4-ene-3-one (Testosterone)	0	0
3α-Hydroxy-5α-pregnan-20-one	0	1
3β-Hydroxy-5α-pregnan-20-one	5	78
3α-Hydroxy-5β-pregnan-20-one	2	0
3β-Hydroxy-5β-pregnan-20-one	0	1
3β-Hydroxypregnan-5-ene-20-one (Pregnenolone)	56	94
3β-Hydroxycholest-5-ene* (Cholesterol)	—	25
Cortisone (21 sulphate)	2	33

* This compound was used in suspension, not in solution, owing to its extreme insolubility.

The optimum conditions for the hydrolysis of *epi*androsterone sulphate and of pregnenolone sulphate were determined. In both cases the optimum pH was from 4·5 to 4·6 and the shape of the pH-activity curve was in neither case significantly different from that of DHAS; this was to be expected, since there is no change in the ionization of any of the substrates in the pH range involved. The optimum substrate concentration for *epi*androsterone sulphate was apparently considerably greater than 0·2 mM, outside the useful range of the method of assay. In the case of pregnenolone sulphate the optimum substrate concentration was in the region 0·06–0·08 mM and above this concentration substrate inhibition occurred to a slight, but variable, extent. The appropriate curves are shown in Fig. 5, but that of pregnenolone sulphate must be regarded as provisional as, for a reason at present unknown, the recovery of pregnenolone sulphate was less constant than that of DHAS under identical conditions. This may be due in part to the greater lability of pregnenolone sulphate in hot aqueous solution (Figs. 7, 8).

It was not possible to determine the action of steroid sulphatase on non-steroid sulphates owing to the crude nature of the enzyme preparation available, but the preparation used in the above experiments would not hydrolyse at a detectable rate the sulphates of *cyclo*hexanol, benzyl alcohol,

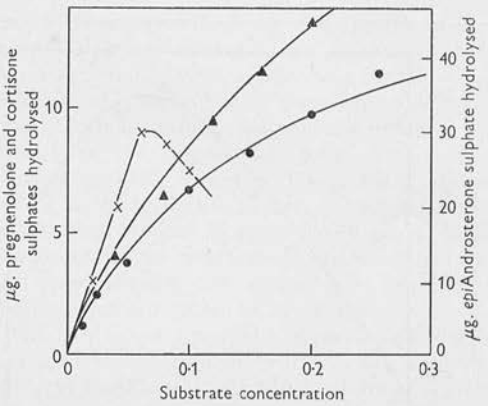

Fig. 5. Effect of varying substrate concentrations on the hydrolysis of *epi*androsterone sulphate, pregnenolone sulphate and cortisone sulphate by extracts of *Patella*. Conditions as in Fig. 3, except in the case of cortisone sulphate which was incubated for 17 hr. *epi*Androsterone sulphate, ●; pregnenolone sulphate, ×; cortisone sulphate, ▲.

or of dichlorophenoxyethanol, suggesting that steroid sulphatase was not a general alkyl sulphatase. Although direct proof is not at present possible, it may be inferred from the electrophoresis experiments described below that steroid sulphatase will not hydrolyse nitrocatechol sulphate, nor presumably aryl sulphates in general.

Hydrolysis of cortisone sulphate. The hydrolysis of cortisone-21-sulphate by the enzyme preparation used above was very surprising in view of the otherwise high specificity of steroid sulphatase. It seemed not improbable that this hydrolysis was due, not to steroid sulphatase, but to some other sulphatase present in the preparation. This view is supported by the inhibition experiments reported in Table 2, and by the fact that the pH optimum for this hydrolysis is considerably higher than that for the hydrolysis of DHAS, being in the region of pH 5·3, as shown in Fig. 6. The affinity of the enzyme for cortisone sulphate is also rather low, as shown in Fig. 5. The evidence is therefore not inconsistent with the view that the hydrolysis of cortisone sulphate was due to an enzyme other than steroid sulphatase. Unfortunately the above results are all complicated by the fact that the

time of hydrolysis required in the case of the cortisone sulphate experiments was 17 hr. (Table 3), as against 1 hr. in the case of DHAS, so that the differing effects of the various inhibitors in the two cases might be due, not to different enzymes being involved, but simply to the introduction of complicating effects by the prolonged incubation time. (See also the results of electrophoresis studies described below.)

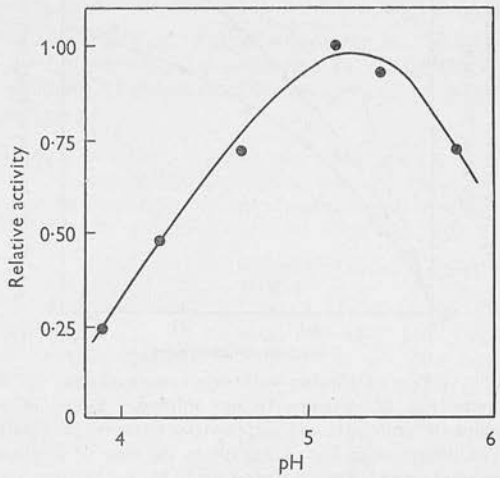

Fig. 6. Effect of pH on the hydrolysis of cortisone sulphate by extracts of *Patella*. Conditions as in Fig. 4 except that incubation time was 17 hr.

Non-enzymic hydrolysis of steroid sulphates. There appears to have been no systematic study of the rates of hydrolysis of steroid sulphates in aqueous solution, although it is generally held that DHAS is much more labile in hot solutions than the corresponding saturated compound (Munson, Gallagher & Koch, 1944). A few experiments on the non-enzymic hydrolysis of steroid sulphates were therefore carried out and the results are reported to allow comparison with the results of the enzyme experiments reported in Table 3.

The results of these experiments are given in Figs. 7 and 8. In both cases the great lability of the 3-sulphates of the Δ^5-unsaturated steroids was obvious. When the experiments reported in Fig. 8 were repeated using 0·1 N-HCl in place of H_2SO_4, the rate of hydrolysis of the sulphates was even greater, as would be expected.

It should be noted that under the conditions used in the assay of steroid sulphatase the steroid sulphates were completely stable, no significant hydrolysis occurring even after incubation for 17 hr. in acetate buffer, pH 4·5, at 37°.

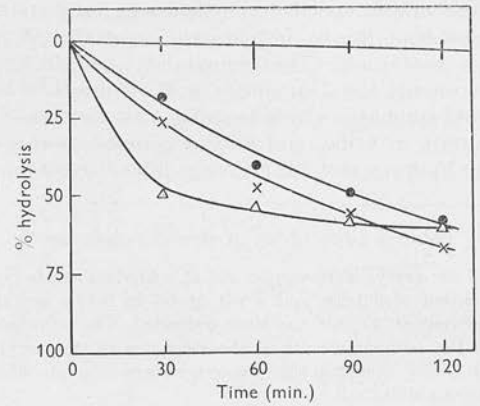

Fig. 7. Hydrolysis of steroid sulphates at pH 7 and 100°. Reaction mixture: 4 ml. 0·4 mM steroid sulphate and 4 ml. 0·02 M phosphate buffer, pH 7. DHAS, ●; pregnenolone sulphate, ×; cholesteryl sulphate, △. The corresponding points for androsterone, *epi*androsterone, 3β-hydroxy-5α-pregnan-20-one and 3β-hydroxy-5β-pregnan-20-one sulphates fall within the limits indicated by the vertical lines.

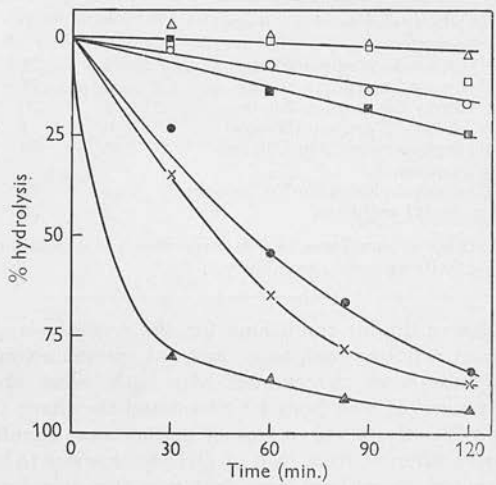

Fig. 8. Hydrolysis of steroid sulphates at pH 1 and 100°. Reaction mixture: 4 ml. 0·4 mM steroid sulphate and 4 ml. 0·2 N-H_2SO_4. DHAS, ●; pregnenolone sulphate, ×; cholesteryl sulphate, ▲; androsterone sulphate, ○; *epi*androsterone sulphate, △; 5α-pregnanolone sulphate, □; 5β-pregnanolone sulphate, ■.

Paper electrophoresis

Although the inhibition studies reported in Table 2 indicated that aryl sulphatase and steroid sulphatase were different enzymes, the possibility could not be discounted that the different responses to inhibitors might be due to a single enzyme

attacking two different substrates under different conditions. As the two enzymes closely followed one another through the usual fractionation procedures, it was considered advisable to attempt the separation of the two enzymes by paper electrophoresis.

The results of a typical experiment are shown in Fig. 9. Although a complete separation of aryl sulphatase and steroid sulphatase was not achieved, the two enzymes separated to a sufficient extent to show their separate identity. The pattern shown in Fig. 9 was obtained in six consecutive experiments

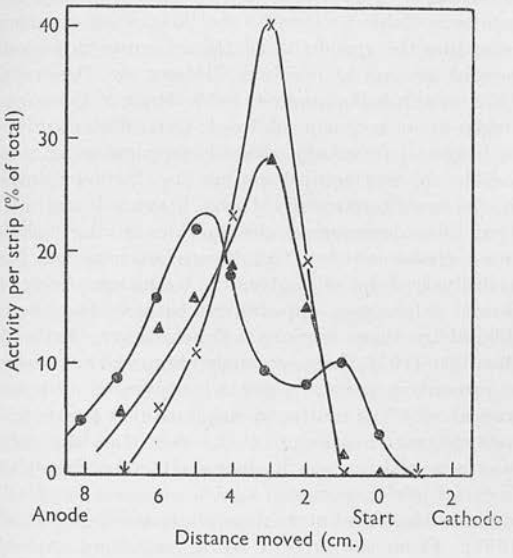

Fig. 9. Paper electrophoresis of an extract of *Patella*. 2 mg. stage 3, run 6 hr. at 500 v on Whatman no. 100 paper in 0·03 M veronal buffer, pH 7·4. Paper cut into 1 cm. strips and portions assayed with the following substrates. Nitrocatechol sulphate, ●; DHAS, ×; cortisone sulphate, ▲.

and is quite outside any possible experimental error. In an attempt to decide whether the hydrolysis of cortisone sulphate was due to steroid sulphatase, the enzyme responsible for this hydrolysis was also localized. As shown in Fig. 9, the hydrolysis of cortisone sulphate closely followed that of DHAS and no separation of the two activities was possible. This might be taken to indicate that only one enzyme is involved in the hydrolysis of cortisone sulphate and of DHAS, despite the data of Table 2.

Fig. 9 also shows that there appeared to be two aryl sulphatases present in the preparation investigated (stage 3), although once again no clear-cut separation was achieved. Attempts to bring about a further separation of the two aryl sulphatases by increasing the time of the run to 16 hr. were not successful, as the increased time of running apparently caused a considerable destruction of the faster-moving enzyme, so that the relative amount of the slower component was greatly increased without a much greater separation of the two components being achieved.

DISCUSSION

The method described for the preparation of steroid sulphates is apparently a general one and is capable of giving good yields even on the 15 mg. scale. In this respect it is more convenient than those methods which use chlorosulphonic acid directly as the sulphating agent (Butenandt & Hofstetter, 1939; Grant & Glen, 1949; Holden, Levi & Bromley, 1949; Holden & Bromley, 1950), as the final product is more easily purified. The only steroid so far investigated which will not react with pyridine-SO_3 is oestrone, in which the hydroxyl group is phenolic in nature.

The procedure described gives a considerable degree of purification of both steroid sulphatase and aryl sulphatase, and the final stage is a convenient one for the further purification of these enzymes. It is obvious from the data shown in Table 2 and in Fig. 9 that aryl sulphatase and steroid sulphatase are different enzymes. The results of the investigation of aryl sulphatase are in agreement with those previously obtained by Dodgson & Spencer (1953) using a different method. The behaviour of the aryl sulphatase activity on electrophoresis suggests that more than one such enzyme might be present in extracts of *Patella*. Kinetic studies gave no indication of this, but, as previously discussed (Roy, 1954*a*), this does not of necessity indicate that only one enzyme is present. Indeed, the very flat shape of the pH–activity curve (Fig. 1) might well indicate that more than one enzyme was present. Furthermore, Dodgson, Lewis & Spencer (1953) found that some 20% of the aryl sulphatase of *Patella* homogenates was insoluble in acetate buffer, pH 5·5: this might also indicate the presence of two enzymes, although this was not suggested by these workers.

Regarding the results of the investigation of steroid sulphatase, the most important finding is undoubtedly the very high specificity of the enzyme. This specificity was unexpected, considering the relatively low specificity requirements of the other sulphatases so far described. As shown by the results in Table 3, steroid sulphatase hydrolyses only the 3β-sulphates of the 5α or Δ^5 series of steroids, those of the latter group being hydrolysed at a considerably greater rate than the former. These findings are in agreement with those of Savard *et al.* (1954), who found that the steroid

sulphatase of *Otala punctata* (a tropical terrestial gastropod) would hydrolyse DHAS, but not the sulphates of androsterone or testosterone. Further, steroid sulphatase does not appear to hydrolyse alkyl sulphates in general, so that the term 'alkyl sulphatase' used by Stitch & Halkerston (1953) to describe the enzyme present in extracts of *Patella* which hydrolyses DHAS, is a misnomer. The term alkyl sulphatase should be restricted to enzymes of the type described by Vlitos (1953) in *Bacillus cereus mycoides* which hydrolyses dichlorophenoxyethyl sulphate. It should be noted in this connexion that Soda (1936) has shown that molluscs apparently do not contain a general alkyl sulphatase. It would seem that the enzyme hydrolysing DHAS is better described as 3β-steroid sulphatase, as this indicates the specificity of the enzyme as known at present.

The specificity of steroid sulphatase for the hydrolysis of the 3β-sulphates of the 5α or Δ^5 steroids is of interest with regard to the conformations of the substrates. The 3β:5α steroids are those in which the molecule attains its most planar form, the 3β substituent being in the equatorial, and therefore least hindered, position (Klyne, 1955). The other isomers have molecules which depart more or less extensively from the planar form. Unfortunately, the conformation of the Δ^5 steroids does not appear to be known. There is also a negative side-chain effect, as the nature of the substituent at position 17 does not appear significantly to influence the rates of hydrolysis of the various substrates. It is of interest that there is an exact parallelism between the ability of a 3-sulphate to act as a substrate for steroid sulphatase and the ease of precipitation of the corresponding hydroxy compound with digitonin (Haslam & Klyne, 1953).

The hydrolysis of cortisone-21-sulphate by extracts of *Patella* was very surprising in view of the otherwise high specificity of the enzyme. Savard *et al.* (1954) have shown that extracts of *Otala* will also hydrolyse cortisone sulphate. The results can be explained on the assumption that cortisone sulphate is being hydrolysed, not by steroid sulphatase, but by some other enzyme present in the crude extracts available. Should this view be correct, it would seem that a very likely enzyme is glycosulphatase, which is present in small amounts in the preparations used (Roy, 1954*b*), as there is a close structural relationship between cortisone-21-sulphate and glucose-6-sulphate, a normal substrate for glycosulphatase. This point can be decided only when a further purification of the enzymes involved has been achieved.

The outstanding problem at present is therefore the complete separation of the various sulphatases present in extracts of *Patella*, namely, aryl sulphatase and steroid sulphatase along with considerably smaller amounts of glycosulphatase.

Steroid sulphatase in the assay of urinary steroids

The only specificity studies on steroid sulphatase previously reported are those of Savard *et al.* (1954) for the enzyme present in extracts of *Otala punctata*. Other workers have not studied the specificity of steroid sulphatases and have apparently assumed that, because they hydrolyse DHAS, they will hydrolyse all related steroid sulphates. The error of this assumption is clearly demonstrated by the results in Table 3. Despite the lack of information regarding the specificity of the enzymes involved, several groups of workers (Henry & Thevenet, 1952; Stitch & Halkerston, 1953; Jayle & Beaulieu, 1954) have recently claimed that the enzymic hydrolysis of urinary steroid conjugates is preferable to acid hydrolysis, as the former causes much less destruction of the liberated steroids. That this decrease in destruction is more than counterbalanced by the incompleteness of the hydrolysis of the sulphates, due to the specificity of steroid sulphatase, appears not to have been considered by these workers. For instance, Jayle & Beaulieu (1954) have recently claimed that there is present in normal urine a conjugated 17-ketosteroid which is neither a sulphate nor a glucuronide, basing this claim on the fact that the conjugate is resistant to hydrolysis by the digestive juice of *Helix pomatia* which contains both β-glucuronidase and steroid sulphatase (Henry *et al.* 1952). From the present work, and from that of Savard *et al.* (1954), it would seem more reasonable to assume that this resistant conjugate is androsterone sulphate which is probably present in normal male urine (Venning, Hoffman & Browne, 1942) and which is not hydrolysed by the steroid sulphatases of *Patella* or *Otala*.

A further difficulty in the use of steroid sulphatase in the hydrolysis of urinary sulphates is the inhibition of the enzyme by many of the ions present in urine (Table 2). The problem of inhibition is a minor one, although the related problem of competing substrates, as yet uninvestigated, may be of importance.

It would therefore seem that steroid sulphatase can be of little general use in the hydrolysis of urinary steroid sulphates owing to the high specificity of the enzyme. This does not detract from the value of steroid sulphatase in special cases and it seems that the enzyme may find its greatest use in the determination of the structure of isolated steroid sulphates. An example of such a use is given by the observation that ranol sulphate (Haslewood, 1952) is not hydrolysed by steroid

sulphatase, indicating that ranol sulphate is in all probability not a 3β-sulphate of a 5α or of a Δ^5 steroid.

Biological implications

The physiological significance of the very large amounts of sulphatases in molluscan tissues is obscure, but it must be remembered that at the normal body temperature of those animals the activity of these enzymes will be very much less than that determined under laboratory conditions, probably by a factor of ten or more. When this is taken into account it would seem that the aryl sulphatase activity of molluscan tissues cannot be much greater than that of mammalian tissues. In the case of steroid sulphatase and glycosulphatase, however, the interest lies in the fact that they have not so far been detected in the higher animals.

Glycosulphatase, and the closely related chondrosulphatase, could conceivably be involved in the digestion of the sulphated polysaccharides of the algae which form a large part of the diet of many marine molluscs. Unfortunately, the completely herbivorous *Patella vulgata* is comparatively poor in glycosulphatase (Dodgson & Spencer, 1954). Steroid sulphatase would also appear to be a digestive enzyme, as it is present in the intestinal juices of *Helix pomatia* (Henry *et al.* 1952), but there seems to be no known steroid sulphate which is likely to be a normal constituent of the diet of either *Helix* or of molluscs in general. The problem of aryl sulphatase is equally obscure, as this enzyme is also a constituent of the intestinal juices of *Helix* and again no naturally occurring substrate seems to be known.

Fish (1955) has recently shown that the intestinal juices of certain tropical molluscs contain very large amounts of SO_4^{2-} ions. It would be very interesting to determine whether these SO_4^{2-} ions were produced in the gut through the action of the various sulphatases on the dietary constituents or whether they were actually secreted into the gut as such. Should the latter be the case, it would be very tempting to assume that SO_4^{2-} ions play some fundamental role in the metabolism of these animals.

SUMMARY

1. A method is described for the preparation of a highly active concentrate of steroid sulphatase from an acetone powder of *Patella vulgata* by precipitation between 30 and 70% saturated ammonium sulphate and between 33 and 50% (v/v) acetone at $-9°$. The concentrate also contains large amounts of aryl sulphatase.

2. The properties of steroid sulphatase are described. It hydrolyses dehydro*epi*androsterone sulphate at an optimum substrate concentration of 0·2 mM and a pH optimum of 4·5 in acetate buffer. It is inhibited by Cl^-, SO_4^{2-} and $H_2PO_4^-$ ions.

3. Steroid sulphatase is a highly specific enzyme, hydrolysing only the 3β-sulphates of the 5α and Δ^5 steroids, having no action on the other isomeric 3-sulphates. The name 3β-steroid sulphatase is suggested for the enzyme.

4. Cortisone-21-sulphate is also hydrolysed by the enzyme preparation, but there is evidence to suggest that the enzyme responsible is not steroid sulphatase but another enzyme. It is postulated that this enzyme may be glycosulphatase.

5. It was shown by electrophoresis that steroid sulphatase is distinct from aryl sulphatase, and that the latter enzyme probably consists of two components.

6. The use of steroid sulphatase to hydrolyse urinary steroid sulphates before their assay is discussed. Various criticisms are levelled at the present tendency to use enzymic methods in the assay of urinary steroids.

The author is deeply indebted to Dr W. Klyne who provided, either personally or from the M.R.C. Steroid Reference Collection, many of the steroids, without which it would have been impossible to undertake this work. He is also grateful to Professor G. F. Marrian, F.R.S., for his continued interest and advice, and for numerous gifts of steroids. Other steroids were kindly given by Professor G. A. D. Haslewood, Dr R. I. Dorfman, and Merck and Co. Inc., to all of whom the author wishes to express his thanks. The methylene blue chloride and sodium dichlorophenoxyethyl sulphate were generously given by Messrs I.C.I. Ltd. and Gemec Ltd. respectively.

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The Enzymic Synthesis of Steroid Sulphates

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Soluble enzyme preparations from rat liver have been known for some time to be capable of synthesizing aryl sulphates from the appropriate phenol and sulphate ions in the presence of adenosine triphosphate (Bernstein & McGilvery, 1952*a*; DeMeio, 1952). There had been no investigation of the synthesis of steroid sulphates by such enzymes until very recently, when DeMeio & Lewycka (1955) were able to demonstrate the probable synthesis thereby of dehydro*epi*androsterone sulphate (DHAS), although the product was not characterized. The synthesis of such steroid sulphates is of particular interest as these compounds must play a part in the metabolism of steroids, and their synthesis is probably more representative of the true function of the enzyme system involved than is the commonly studied formation of the sulphates of toxic phenols. Furthermore, the molluscan enzyme hydrolysing these steroid sulphates, steroid sulphatase, showed

a high degree of specificity (Roy, 1955), and it was of interest to investigate the corresponding specificity of the more widely distributed synthetic system. The method of assay used in the present investigation allowed the ready investigation of a large number of different substrates and so was of more general application than the previously available methods for the assay of sulphate ester synthesis.

EXPERIMENTAL

Preparation of the enzyme. This was prepared by the method of Bernstein & McGilvery (1952*a*). Portions of rat liver were homogenized in a Potter & Elvehjem (1936) homogenizer with 3–4 vol. of 0·15 M-KCl containing 0·001 M ethylenediaminetetraacetic acid, pH 7. The temperature at this and all subsequent stages was kept at 0°. The homogenate was centrifuged for 2 hr. at 20 000 *g* and the supernatant, containing the enzyme, separated. The enzyme was then purified by fractional precipitation with ammonium sulphate, and the fraction precipitated between 1·7 M- and 2·3 M-$(NH_4)_2SO_4$ contained the bulk of the enzyme. This fraction was dissolved in water (0·7 ml./g. of liver) and the solution stored at −20°, at which temperature it was stable for at least 4 weeks. For assay by the method described below, preparations from male and female animals were diluted with 1 and 3 vol. of water respectively.

Assay of steroid sulphate synthesis. The following method was used in the assay of DHAS synthesis, but it was directly applicable to the assay of the synthesis of any steroid sulphate.

Buffered adenosine triphosphate (ATP) was prepared immediately before use by mixing equal vol. of 0·3 M-KH_2PO_4 (pH 6·8), 0·03 M-K_2SO_4, 0·005 M-$MgCl_2$, and 0·33 vol. of 0·04 M ATP, pH 6·8. The ATP was the disodium salt (L. Light and Co., Colnbrook, Bucks). To 0·5 ml. of buffered ATP was added 0·1 ml. of 0·4 mM dehydro*epi*-androsterone (DHA) in propylene glycol followed by 0·4 ml. of enzyme solution. The final concentrations of ATP and DHA were therefore 2·0 and 0·04 mM respectively. The mixture was incubated for 2 hr. at 37° and the reaction then stopped by the addition of 5 ml. of ethanol. After standing for 15 min. the tubes were capped, and the precipitated protein was removed by centrifuging. A sample of the clear supernatant (5 ml.) was taken for the assay of DHAS as described below.

Blank determinations were simultaneously carried out, in which the enzyme was incubated separately from the remainder of the reaction mixture and added after the ethanol. Assays and blanks were always performed in duplicate.

Determination of DHAS. DHAS was assayed by a micro-modification of the method already described by Roy (1956), which makes use of the solubility in chloroform of the complexes formed between methylene blue and sulphuric acid esters. This method can be applied to the determination of any steroid sulphate ester (Roy, 1956). The methylene-blue reagent used in the present method was prepared from that previously used (Roy, 1956) by diluting with an equal volume of water.

The 5 ml. sample of the protein-free solution obtained above was concentrated in a boiling-water bath for 20 min. This did not give a completely dry residue but left about 0·1 ml. of a viscous liquid, presumably propylene glycol. After cooling, the residue was taken up in 2 ml. of methylene-blue reagent, 5 ml. of chloroform was added and the mixture shaken vigorously for 20 sec. The mixture was centrifuged, the aqueous layer removed and a sample of the chloroform layer taken into a test tube and dried with Na_2SO_4. The intensity of the resulting blue solution was read in the Spekker absorptiometer with micro cells (1 cm. light path) and Ilford filter no. 608 (maximum transmission 700 mμ.) against the appropriate blank.

A calibration curve for the above method was prepared by dissolving known amounts of DHAS in a medium of composition identical with that of the assays, except that water was added in place of enzyme, followed by ethanol precipitation, etc., as described above. It is essential that the blank and calibration tubes have a composition identical with that of the assays as variations in their composition may cause variations in the shape of the calibration curve, which is not strictly linear. Ethanol precipitation minimizes these effects by the removal of the bulk of the inorganic ions which may interfere.

The recovery of known amounts of DHAS added to the incubation mixture was satisfactory, the mean recovery in the range 5–25 μg. of DHAS being 95% from solutions containing 8 mg. of protein/ml., the usual concentration in routine assays. With amounts of DHAS less than 5 μg. the recoveries were less and not so reproducible.

Isolation of DHAS. To characterize the product formed during the enzymic reaction the isolation of DHAS was attempted. To 10 ml. of buffered ATP and 1 ml. of 2 mM DHA was added 8 ml. of undiluted enzyme solution, and the mixture was incubated for 2 hr. at 37°, after which a further 1 ml. of DHA solution was added. The incubation was continued for a total of 5 hr. and the proteins were then precipitated by the addition of 100 ml. of ethanol. The mixture was filtered, and the filtrate concentrated *in vacuo* to about 2 ml. of a viscous liquid; this was taken up in 10 ml. of methylene-blue reagent and the methylene-blue complex extracted by shaking with three 50 ml. portions of chloroform. The extract was dried with Na_2SO_4 and taken to dryness *in vacuo*, and it gave a small blue residue which was dissolved in 0·5 ml. of ethanol and diluted to 5 ml. with water. To convert the methylene-blue complex into the sodium salt this solution was gently shaken for 15 min. with 200 mg. of Zeo-Karb 225 Na^+ (Permutit Co. Ltd., London), and then filtered. Traces of methylene blue were removed from the filtrate by washing twice with 2 ml. portions of chloroform; traces of chloroform were then removed from the aqueous phase by warming *in vacuo*, and the volume was made up to 10 ml. with water. The resulting solution was shown, as described below, to contain DHAS.

RESULTS

Properties of the enzyme system. Preliminary experiments indicated that the enzyme system forming DHAS had the same general properties as that forming aryl sulphates (Bernstein & McGilvery, 1952*a*, *b*; DeMeio, Wizerkaniuk & Fabiani, 1953). The synthesis of DHAS was ATP-dependent and showed its optimum activity at a concentration of 2 mM ATP (Fig. 1). Higher concentrations of ATP caused a significant inhibition of the reaction, so

that at 8 mM ATP the activity of the enzyme was only some 80% of the maximum. The optimum substrate concentration was approximately 0·04 mM DHA (Fig. 2) and substrate inhibition occurred to a considerable extent. With *epi*androsterone and androsterone the optimum substrate concentration was again 0·04 mM, as shown in Fig. 2. The enzyme system was relatively insensitive to the concentration of SO_4^{2-} ions, as the maximal activity was reached by a concentration of 0·01 M-K_2SO_4, and no decrease in activity was noted at concentrations as high as 0·1 M-K_2SO_4 (Fig. 1). In these experiments,

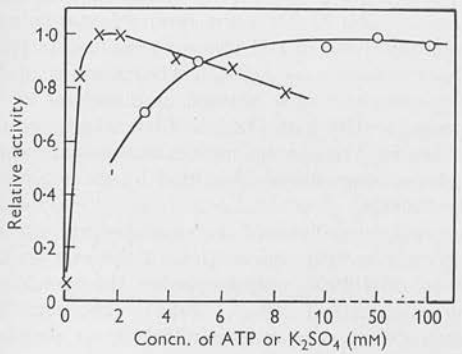

Fig. 1. Influence of different concentrations of ATP and K_2SO_4 on the synthesis of DHAS. Reaction mixture (vol. 1·0 ml.) contained 0·4 ml. of enzyme solution, 0·1 ml. o 0·4 mM DHA in propylene glycol, and 0·5 ml. of buffered ATP (see text) containing varying amounts of ATP or K_2SO_4. ×, Synthesis of DHAS with different concentrations of ATP, and ○, synthesis of DHAS with different concentrations of K_2SO_4.

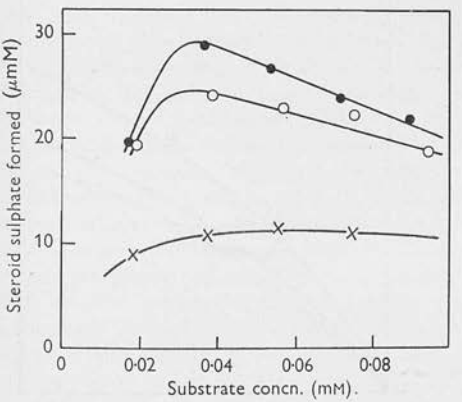

Fig. 2. Effect of substrate concentration on steroid sulphate synthesis. Reaction mixture (vol. 1·0 ml.) contained 0·5 ml. of buffered ATP (see text), 0·4 ml. of enzyme solution and 0·1 m. of solutions of varying concentration of the steroid in propylene glycol. Incubated for 2 hr. at 37°. ●, DHA; ○, *epi*androsterone; ×, androsterone.

the enzyme preparations used were dialysed against 0·2 M phosphate buffer, pH 6·9, at 0° to reduce the concentration of SO_4^{2-} ions in the enzyme to negligible amounts. Dialysis under these conditions caused about 30% inactivation of the enzyme; dialysis against distilled water caused complete inactivation. The enzyme was thus relatively insensitive to changes in the concentration of SO_4^{2-} ions, and the dialysed enzyme was very unstable. Dialysis was therefore not normally carried out.

As corticosteroids and salicylates have been reported to inhibit the synthesis of ester sulphates (Kodicek & Loewi, 1955) the effect of these substances on the synthesis of DHAS was investigated. Neither salicylic acid nor acetylsalicylic acid in mM concentrations exerted any effect on the synthesis of DHAS. Cortisone, cortisone acetate, and hydrocortisone all inhibited the formation of DHAS to approximately 20% at mM concentrations, as shown in Table 1.

During the course of the present work it was noted that enzyme preparations from the livers of female rats were consistently more active in the synthesis of DHAS than were those from the livers of male rats. That this is a true difference is readily seen from the following figures. The synthetic activity of preparations from the livers of four male rats ranged from 80 to 165 μmM of DHAS/g. of liver/2 hr., with a mean value of 130 μmM/g., while corresponding preparations from the livers of four female rats had activities ranging from 235 to 325 μmM/g., with a mean of 305 μmM/g.

Specificity of the enzyme system. This was investigated under the general conditions described above, and the results of some of the experiments are shown in Figs. 2 and 3. These figures show the synthesis of the sulphates of a number of androstan-3-ol-17-one isomers. It is obvious that although the enzyme system is relatively non-specific, in that all the isomers are conjugated with

Table 1. *Influence of inhibitors on the synthesis of DHAS*

Reaction mixture (vol. 1·0 ml.) contained 0·4 ml. of enzyme, 0·1 ml. of 0·04 mM DHA, and 0·5 ml. of buffered ATP. The water-soluble inhibitors were dissolved in the buffered ATP and the water-insoluble compounds in the substrate solution. The activity is expressed relative to the controls containing no inhibitor, activity 1·0.

Inhibitor (mM)	Activity
Sodium salicylate (1·0)	0·99
Acetylsalicylic acid (1·0)	0·98
Cortisone (1·0)	0·86
Cortisone acetate (1·0)	0·79
Hydrocortisone (1·0)	0·80
Sodium sulphite (1·0)	1·0
m-Aminophenol (1·0)	0·65
m-Aminophenol (0·5)	0·73

sulphate, there is a considerable variation in the rate of the reaction. The results presented in Fig. 3 show that when the reactions have reached their appropriate steady states the velocities differ in each case. This point is discussed below.

A number of other steroids have been investigated as possible substrates of the reaction, but the results are not quoted in detail as they were obtained under arbitrary conditions, not necessarily optimum for the substrate in question. The substrate concentration was 0·1 mM and the time of incubation 2 hr. Taking the rate of conjugation of DHA under comparable conditions to be 1·00, the rate with pregn-5-en-3β-ol-20-one or 5α-pregnan-3β-ol-20-one as substrate was 1·0, with 5β-pregnan-3β-ol-20-one, 0·6; with oestrone and *epi*testosterone, 0·4; with testosterone, 0·25; with cortisone, 0·1; and with hydrocortisone about 0·05. These results confirm that the sulphate-conjugating system shows a relatively low degree of substrate specificity.

Several compounds other than steroids were also tested as possible substrates under the same conditions, except that the substrate was dissolved in water rather than in propylene glycol. Neither *cyclo*hexanol nor β-phenylethanol nor propylene glycol was conjugated with sulphate at a detectable rate. Salicylic acid reacted very slowly, but phenol was conjugated at a rate comparable to DHA and *p*-nitrophenol at about three times the rate of DHA. When the two latter phenols were dissolved in propylene glycol rather than in water, there was a 40% diminution in the rate of the conjugation of phenol, but with *p*-nitrophenol the rate was unaltered.

Although the properties of the enzyme system forming DHAS suggest that it is identical with the system forming aryl sulphates, direct proof of this assumption has not been possible. Good evidence for the identity of the two systems is given, however, by the fact that *m*-aminophenol, which is known to be rapidly conjugated with sulphate by such preparations (Bernstein & McGilvery, 1952*a*), is a powerful inhibitor of the synthesis of DHAS, a concentration of 0·001 M *m*-aminophenol causing a 40% inhibition of DHAS synthesis (Table 1).

Characterization of DHAS. The product of the enzymic reaction was isolated as described above and was identified as DHAS. Characterization of the compound by classical means was not attempted and the substance was identified by its behaviour on hydrolysis.

Enzymic hydrolysis of the reaction product was carried out with a preparation of the steroid sulphatase of *Patella vulgata* under the conditions already described (Roy, 1954). The reaction product behaved exactly similarly to a standard solution of DHAS when submitted to this enzymic hydrolysis. A further sample was hydrolysed in 0·1 N-H_2SO_4 under the conditions described by Roy (1956), and again the behaviour was identical with that of an authentic sample of DHAS. The results of these experiments are shown in Fig. 4.

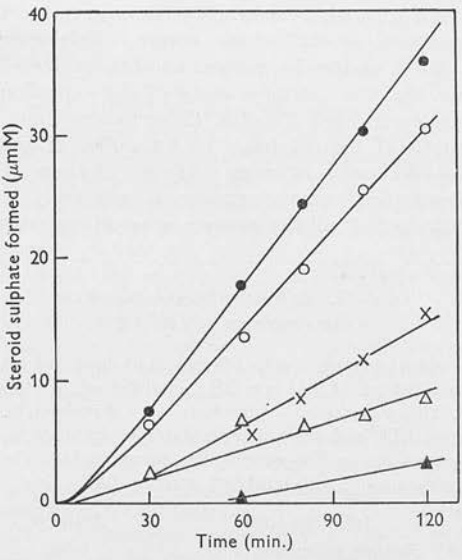

Fig. 3. Course of formation of steroid sulphates. General conditions as in Fig. 2, except that the concentration of the substrate solution was 0·4 mM, giving a final substrate concentration of 0·04 mM, and the time of incubation was varied. △, 5β-Androstan-3β-ol-17-one; ▲, 5β-androstan-3α-ol-17-one. Other symbols as in Fig. 2.

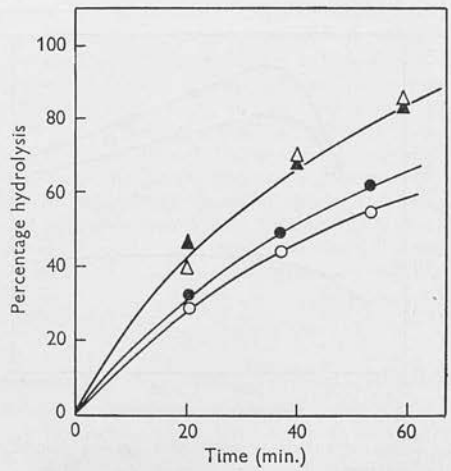

Fig. 4. Hydrolysis of authentic DHAS and DHAS prepared enzymically as described in the text. ▲, Authentic, and △, synthetic DHAS, hydrolysed by 0·1 N-H_2SO_4 at 100°. ●, Authentic, and ○, synthetic DHAS, hydrolysed by steroid sulphatase at pH 4·5 and 37°.

Further proof of the identity of the reaction product with DHAS was obtained by characterization of the steroids produced on acid hydrolysis of the reaction product. A 1 ml. sample of the solution of the product was hydrolysed for 1·5 hr. with 1 ml. of 2N-HCl at 100° and, after cooling, the steroid was extracted with two 5 ml. portions of chloroform. The extract was dried with Na_2SO_4 and taken to dryness *in vacuo*. The residue was dissolved in methanol and submitted to partition chromatography on paper, with the Bush (1952) solvent system *A*.

The steroids produced on the acid hydrolysis of the reaction product were identical with those similarly produced from authentic DHAS, namely, large amounts of chloroandrostenone and small amounts of DHA.

The above experiments were repeated on the reaction product obtained when androsterone was used as substrate. The product behaved then exactly as did authentic androsterone sulphate on hydrolysis by acid, the hydrolysis products being similar in the two cases. The reaction product was not hydrolysed by steroid sulphatase, as was expected from the specificity of that enzyme (Roy, 1956).

There is therefore no doubt that the method described above does indeed measure the rate of formation of steroid sulphates.

DISCUSSION

Although the reaction product has in no case been isolated, these experiments show that when DHA is used as substrate the reaction product is DHAS. The product is an acid-labile conjugate of DHA, which is hydrolysed both by acid and by steroid sulphatase at rates identical with those observed for the corresponding hydrolysis of authentic DHAS (Fig. 4). Further proof that the reaction product is DHAS is afforded by the fact that the reaction is dependent upon the presence of SO_4^{2-} ions in the reaction mixture. The only other product which could conceivably be formed is dehydro*epi*androsterone phosphate, but phosphate esters do not appear to be estimated by the methylene-blue technique (unpublished observations).

The general properties of the enzyme system synthesizing DHAS are similar to those of the system synthesizing *m*-aminophenyl sulphate. Both enzymes are precipitated between 1·7 and 2·3M-$(NH_4)_2SO_4$ and require the presence of 2 mM ATP and 10 mM-SO_4^{2-} ions to exhibit their maximum activity. Again, both enzyme systems are inactivated by dialysis against distilled water and both show typical lag-phase curves for the course of the reaction. It seems reasonably certain that the same enzyme system is involved in the synthesis of both types of compound, but direct evidence for this has not been obtained, except that the synthesis of DHAS is strongly inhibited by *m*-aminophenol. The enzyme system apparently shows a relatively low degree of specificity for steroid substrates, as all sterols so far tested are conjugated with sulphate. It should be pointed out, however, that one enzyme system has not yet been demonstrated to be involved in each case. The only noteworthy result is shown in Fig. 3, which demonstrates that 5β-androstan-3β-ol-17-one is conjugated more rapidly than the corresponding 3α-hydroxy isomer. This is surprising, as the 3β-hydroxyl group is in the axial, therefore hindered, position. In the 5α-androstane series the equatorial hydroxyl group of the 3β isomer reacts considerably more rapidly than the axial group of 3α isomer (Fig. 3), as would be expected.

As shown in Fig. 3, the kinetics of conjugation of the androstanolone isomers were similar to those described by Bernstein & McGilvery (1952*b*) for the conjugation of *m*-aminophenol in that in both there was a lag phase, during which the reaction velocity steadily increased to a maximum value which was maintained for a prolonged period, the linear phase. Bernstein & McGilvery (1952*b*) interpreted these results as indicating the formation of an 'active sulphate' followed by a coupling reaction between this 'active sulphate' and the hydroxyl-containing compound, this second reaction being the rate-limiting one. Segal (1955) developed this theory and provided a mathematical formulation of the results as follows. The two reactions were:

$$A + SO_4^{2-} \xrightarrow{k_1} I,$$

$$I + R.OH \xrightarrow{k_2} R.SO_4^{-},$$

the second reaction being rate-limiting. From these assumptions Segal (1955) showed that such a reaction would exhibit a lag phase, the length of which would be governed by k_2, and a linear phase when the system had reached the steady state and when the velocity would be governed solely by k_1. The equations expressing these findings were as follows (Segal, 1955). The velocity at the steady state is

$$\frac{dP}{dt} = k_1, \qquad (1)$$

and the total amount of reaction product (P) formed at any time (t) is

$$P = k_1 t - \frac{k_1}{k_2}(1 - e^{-k_2 t}). \qquad (2)$$

Equation (1) above indicates that the velocity at the steady state is independent of the nature of the substrate, R.OH, as this velocity depends only on k_1. Fig. 3 shows, on the other hand, that the

velocity at the steady state does vary with the substrate. It is therefore obvious that the theory of Segal (1955) is incorrect. The experimental results presented in Fig. 3 can, however, be explained by a very slight modification of the above theory. The modification required is the not improbable one that the first reaction, the formation of 'active sulphate', is reversible. Retaining the nomenclature of Segal (1955) and calling the velocity constant for this reverse reaction k_3, it can be shown that the velocity at the steady state is not independent of the nature of the substrate. As the velocity of the second reaction is rate-limiting, the overall reaction velocity is

$$\frac{dP}{dt} = k_2 I.$$

At the steady state

$$\frac{dI}{dt} = k_1 - k_2 I - k_3 I = 0,$$

$$I = \frac{k_1}{k_2 + k_3},$$

$$\frac{dP}{dt} = \frac{k_1 k_2}{k_2 + k_3}. \qquad (3)$$

This equation (3) shows that the velocity at the steady state depends not only upon k_1 but also upon k_2 and k_3. As k_2 must vary with the substrate used, so also must the velocity at the steady state. This does not alter the rest of the argument of Segal (1955) but only makes the equation describing the total amount of reaction product formed at time t rather more complex, as follows:

$$P = k_1 t - \frac{k_1}{k_2 + k_3}(1 - e^{-(k_2+k_3)t}). \qquad (4)$$

The form of equation (4) is identical with that of equation (2), but it does not allow the evaluation of k_1 and k_2, as does equation (2), because of the occurrence of a third variable, k_3. None of the constants k_1, k_2 and k_3 is a true velocity constant as they must contain various concentration terms which are not varied under the experimental conditions used. This theory would therefore seem to explain the experimental results more correctly than that of Segal (1955).

There have been many reports in the literature of the inhibition of sulphate-ester synthesis by corticosteroids and by salicylates, but most of these apply to the synthesis of mucopolysaccharides by tissue slices and so are difficult to interpret, as has been stressed by Kodicek & Loewi (1955). The results described above confirm the inhibition of sulphate-ester synthesis by corticosteroids, but show that salicylates have no effect on this process, at least in DHAS synthesis. It must therefore be presumed that previous reports (DeMeio & Tkacz, 1952) of the inhibition by salicylates of aryl sulphate synthesis in liver slices were based on the inhibition of some energy-yielding process involved in the much more complex systems studied by these authors.

One of the most interesting results of the present investigation is the demonstration in rat liver of an enzyme system capable of forming the sulphate esters of a number of steroid alcohols. This shows to be incorrect the statement of Anderton, Smith & Williams (1948) that before a hydroxyl group can be conjugated with sulphate it must have a pK in the range 7–10 and must occur in an enolizing system such as –C:C(OH).C. Of the steroids tested above only oestrone satisfies these conditions, all the other steroids being typical alcohols. It might be argued that the claims of Anderton *et al.* (1948) were based on *in vivo* studies, but there is no reason to suppose that the *in vitro* reactions do not also occur *in vivo*, especially as many of the steroid sulphates have been isolated from urine. It is of interest, however, that none of the simpler alcohols studied showed the ability to conjugate with sulphate, although simple phenols did. It would therefore seem that the claim of Anderton *et al.* (1948) was an oversimplification of the situation, and that the ability of any particular hydroxyl group to conjugate with sulphate is not governed only by its pK value but also by the structure of the molecule as a whole.

SUMMARY

1. A method is described for the assay of steroid sulphate synthesis by soluble enzyme preparations from rat liver. The substrate used is dehydro*epi*androsterone, but the method may be used with any suitable steroid as substrate.

2. The properties of the enzyme system synthesizing dehydro*epi*androsterone sulphate are identical with those of the system which synthesizes aryl sulphates, and it is probable that the same enzyme system is involved in the synthesis of both types of compound.

3. The synthesis of dehydro*epi*androsterone sulphate is inhibited by *m*-aminophenol, cortisone, cortisone acetate and hydrocortisone, but is uninfluenced by salicylates.

4. The substrate specificity of the enzyme system has been investigated and has been shown to be relatively low.

5. An interpretation of the kinetic data is presented.

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A. B. ROY

LE MÉTABOLISME DES SULFATES DE STÉROÏDES

L'existence d'arylsulfates dans des substances naturelles est connue depuis de nombreuses années, et le métabolisme de ces composés a fait l'objet d'études considérables. Par contre, les alkylsulfates sont apparemment assez rares, et leur métabolisme n'a été examiné que récemment. A part le véritable alkylsulfate, le sulfate de choline qui a été isolé d'*Aspergillus sydowi* par WOLLEY et PETERSEN (1), les seuls alkylsulfates existant naturellement sont les sulfates de stéroïdes.

Le premier sulfate de stéroïde isolé fut un arylsulfate, le sulfate d'oestrone (2), mais, depuis, un grand nombre de sulfates de stéroïdes non phénoliques a été isolé (tab. I) et la présence d'un certain nombre d'autres a été

Tableau I

Stéroïdes isolés comme esters sulfuriques de sources naturelles.

Stéroïde	Source	Référence
Oestrone	Urine de jument	(2)
« Tétrahydroxycholane »	Bile de crapaud	(15)
« Pentahydroxybufostane »	Bile de crapaud	(16)
Androstérone	Urine humaine	(17)
Déhydroépiandrostérone	Urine humaine	(18)
Alloprégnan-3β-ol-20-one	Urine de jument	(19)
Alloprégn-16-ene-3β-ol-20-one	Urine de jument	(20)
Uranediol	Urine de jument	(21)
Scymol	Bile de roussette	(22)
Ranol	Bile de grenouille	(23)

envisagée d'après les résultats analytiques. Dans la suite de ce rapport, lorsque le terme « sulfate de stéroïde » sera employé, le composé dont il sera question sera le sulfate d'un stéroïde non phénolique : de telles substances ne sont pas de vrais alkylsulfates et il n'y a aucun nom correct pour les décrire. Le métabolisme de ces sulfates de stéroïdes est d'un intérêt considérable, indépendamment de toute activité physiologique du stéroïde mis en jeu, parce que, jusqu'à une date récente, on ne connaissait aucun enzyme capable d'hydrolyser ces composés (3) et l'on avait signalé que de tels composés ne

pouvaient pas être synthétisés directement par les animaux supérieurs (4).

Je m'occupe depuis assez peu de temps du métabolisme des sulfates de stéroïdes, et je voudrais ici discuter deux aspects de ce sujet, d'abord la synthèse de sulfates de stéroïdes par les enzymes du foie des mammifères et ensuite l'hydrolyse de ces sulfates de stéroïdes par la stéroïde sulfatase.

Synthèse des sulfates de stéroïdes

On sait depuis fort longtemps qu'il est possible d'obtenir à partir du foie des mammifères des préparations enzymatiques capables de synthétiser des arylsulfates tels que le méta-aminophénylsulfate (5) ou le phénylsulfate (6).

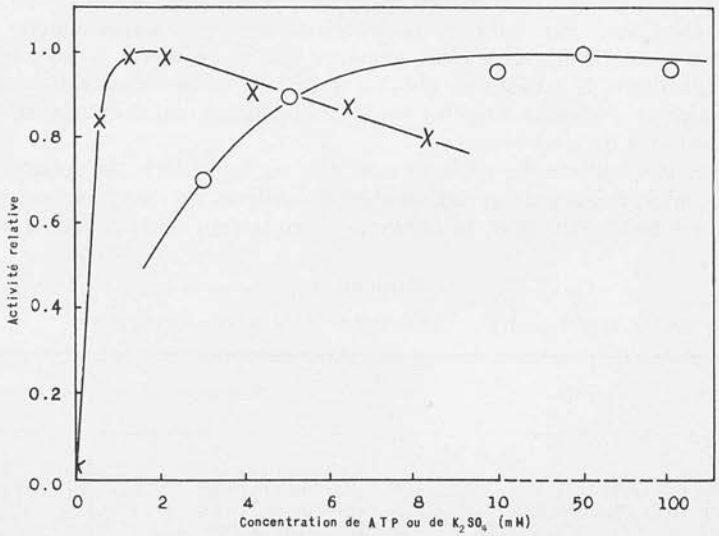

FIG. 1

Influence de ATP et K_2SO_4 sur la synthèse du sulfate de déhydroépiandrostérone par les enzymes du foie de rat
X = Effet de concentrations variables de ATP, la concentration de K_2SO_4 étant 0,01 M
O = Effet de concentrations variables de K_2SO_4, la concentration de ATP étant 0,0002 M

On n'a pas examiné la synthèse des sulfates de stéroïdes par de tels enzymes : une simple note (7) a signalé la synthèse du sulfate de déhydroépiandrostérone.

L'enzyme a été préparé par la méthode de BERNSTEIN et MCGILVERY (5) : on fait un homogénat de foie de rat dans KCl 0,15 M et on élimine le matériel insoluble par centrifugation à 20.000 *g*. Par précipitation fractionnée avec le sulfate d'ammonium, on obtient un enzyme soluble qui est tout à fait stable quand on le maintient au froid. La dialyse de cet enzyme contre de l'eau,

distillée dans du verre, pendant 20 heures à 0° entraîne son inactivation totale, et même la dialyse contre un tampon phosphate 0,2 M, pH 6,8, détruit environ 40 % de l'activité. Comme l'enzyme dialysé est très instable et devient souvent complètement inactif si on le laisse quelques heures à 0°, la dialyse n'a généralement pas été employée. Avec des préparations enzymatiques obtenues comme il vient d'être indiqué, il est facile de démontrer la synthèse de sulfate de stéroïdes, le substrat généralement employé étant la déhydroépiandrostérone. Cette synthèse dépend de la présence dans le milieu de réaction d'ions Mg^{++}, d'ATP et d'ions SO_4^{--}; le pH optimum en tampon phosphate se situe à environ 6,8 et la concentration optima en substrat est 0,05 mM de déhydroépiandrostérone.

Il y a une différence frappante entre les quantités d'enzyme présentes dans le foie des rats mâles et dans celui des rats femelles : des préparations enzymatiques, faites dans des conditions standard à partir de foie de rat femelle, peuvent conjuguer la déhydroépiandrostérone avec le sulfate à la vitesse de 150 mμM par gramme et par heure, alors que des préparations identiques obtenues à partir de foie de rat mâle présentent la vitesse très inférieure de 80 mμM par gramme et par heure. Une telle différence est tout à fait en dehors des variations normales observées d'un animal à l'autre, à l'intérieur d'un même sexe.

La spécificité du système enzymatique a été étudiée et les résultats sont groupés dans le tableau II.

Tableau II

Vitesses relatives de synthèse des sulfates de stéroïdes par des préparations enzymatiques de foie de rat. La vitesse de 1,0 *correspond à la synthèse d'environ* 20 *mμM de sulfate de stéroïde par heure dans un volume de* 1 *ml.*

Stéroïde	Vitesse
3 α-hydroxy-5 α-androstan-17-one	0,5
3 β-hydroxy-5 α-androstan-17-one	0,9
3 α-hydroxy-5 β-androstan-17-one	0,1
3 β-hydroxy-5 β-androstan-17-one	0,25
3 β-hydroxyandrost-5-ene-17-one	1,0
17 β-hydroxyandrost-4-ene-3-one	0,4
17 α-hydroxyandrost-4-ene-3-one	0,25
3 β-hydroxy-5 α-pregnan-20-one	1,0
3 β-hydroxy-5 β-pregnan-20-one	0,6
3 β-hydroxypregn-5-ene-20-one	1,0
Cortisone	0,1
Cortisol	0,05

Pour étudier le rôle éventuel, comme substrats, de substances non stéroïdes, nous avons examiné un certain nombre d'alcools et de phénols. Parmi ces substances, seuls les phénols se conjuguent avec le sulfate. La capacité de synthèse de cet enzyme est ainsi limitée à la formation d'arylsulfates et de sulfates de stéroïdes. Cet enzyme est incapable de synthétiser les alkylsulfates

en général. Comme on le voit dans le tableau II, la spécificité de cet enzyme vis-à-vis du substrat stéroïde est assez faible puisque tous les stéroïdes étudiés, sauf les corticostéroïdes, peuvent être conjugués avec le sulfate, bien qu'il existe des variations considérables dans la vitesse de synthèse de ces différents sulfates. Ces variations sont celles que l'on peut attendre si l'on considère les facteurs stériques qui interviennent; il y a cependant une exception : la 3-β -5-β -androstanolone réagit plus vite que la 3-α -5-β -androstanolone, en dépit du fait que, dans cette dernière, le groupement hydroxyl est dans une position équatoriale plus réactive. Parmi les 5-α -androstanolones, le composé équatorial : l'isomètre 3-β, réagit plus vite.

Un certain nombre d'inhibiteurs ont été essayés sur ce système, parmi ceux-ci les salicylates et les corticostéroïdes qui ont été signalés comme activant ou inhibant la formation d'esters sulfuriques (8). Lorsque le substrat est la déhydroépiandrostérone, ni l'acide salicylique ni l'acide acétylsalicylique aux concentrations 1,0 mM n'exercent la moindre action inhibitrice. La cortisone, l'acétate de cortisone et le cortisol, aux concentrations 1,0 mM, inhibent tous la réaction d'environ 20 %.

Parmi les points les plus intéressants qui ressortent de ces recherches, se trouve la question de savoir si le système enzymatique synthétisant les sulfates de stéroïdes est identique à celui qui synthétise les arylsulfates. Une preuve directe de ce point n'a pas encore pu être obtenue, mais il existe un grand nombre de preuves indirectes qui suggèrent qu'un seul enzyme est responsable de la synthèse de ces deux types de composés. Les propriétés générales des enzymes qui interviennent dans les deux réactions sont identiques et les cinétiques des réactions sont semblables dans les deux cas. La courbe de la réaction présente une phase de latence durant laquelle la vitesse augmente jusqu'à une valeur maxima qui se maintient pendant une période plus ou moins longue, ce qui donne la phase linéaire. La formule mathématique proposée par SEGAL (9) pour expliquer les cinétiques de la formation du méta-aminophénylsulfate, basée sur la formation intermédiaire d'un « sulfate actif », peut être étendue (10) pour expliquer de la même manière les cinétiques de la formation des sulfates de stéroïdes. Une preuve un peu plus directe de l'intervention d'un même enzyme dans la synthèse des deux types de sulfates est donnée par le fait que la synthèse du sulfate de déhydroépiandrostérone est inhibée de 40 % par m-aminophénol 1,0 mM. On peut s'attendre à ceci si l'enzyme synthétisant les sulfates de stéroïdes joue aussi dans la formation des arylsulfates. Cette preuve n'est ainsi, en aucune façon, contraire à l'hypothèse selon laquelle un seul système enzymatique est mis en jeu pour la synthèse des arylsulfates et des sulfates de stéroïdes. Jusqu'ici, la synthèse des sulfates d'hydrates de carbone n'a pas été étudiée avec cet enzyme, et ceci est dû aux difficultés expérimentales rencontrées pour la détermination de ces composés. Il ne semble pas improbable cependant que le même système enzymatique intervienne aussi pour la synthèse de ce type d'ester sulfurique. Des preuves en faveur de cette manière de voir sont fournies par les récentes observations de LOEWI et KENT (8*bis*) : ces auteurs ont observé que le tissu granuleux qui synthétise rapidement des mucopolysaccharides sulfatés (8) peut aussi former des phénylsulfates à partir de phénols et d'ions SO_4^{--}. On peut donc dire que le foie du rat contient un système en-

zymatique capable de synthétiser les aryl- et les stéroïdes sulfates, et que ce système est, selon toute probabilité, identique à celui qui intervient dans la synthèse des mucopolysaccharides.

Stéroïde sulfatase

La stéroïde sulfatase est la sulfatase la plus récemment découverte. Elle a été décrite pour la première fois par HENRY, THEVENET et JARRIGE (11) qui l'ont trouvée dans les jus intestinaux d'*Helix pomatia* qui sont capables d'hydrolyser le sulfate de déhydroépiandrostérone. Ces chercheurs cependant n'ont apparemment pas distingué la stéroïde sulfatase de l'arylsulfatase typique qui existe en grande quantité dans les tissus des mollusques.

La seule stéroïde sulfatase qui ait été étudiée de manière intensive est celle de *Patella vulgata* (12). La meilleure préparation de cet enzyme est obtenue à partir d'une poudre acétonique de la bosse viscérale de *Patella* par extraction avec KCl 0,1 M; on effectue ensuite une précipitation fractionnée par l'acétone à — 9° et par le sulfate d'ammonium. Les solutions de stéroïde sulfatase sont très stables quand elles sont conservées au froid. Les propriétés de cet enzyme diffèrent très peu de celles des sulfatases les mieux étudiées, les différences étant de nature quantitative. Le substrat habituel pour l'essai de la stéroïde sulfatase est le sulfate de déhydroépiandrostérone, la concentration optima en substrat est 0,2 mM en tampon acétate *p*H 4,5. La stéroïde sulfatase est fortement inhibée par les ions sulfate et phosphate.

Tableau III

Hydrolyse des sulfates de stéroïde par la stéroïde sulfatase de Patella vulgata. Les substrats sont les sulfates des stéroïdes suivants aux concentrations de 0,2 mM.

Stéroïde	Pourcentage d'hydrolyse en	
	1 h.	17 h.
3 α-hydroxy-5 α-androstan-17-one	0	1
3 β-hydroxy-5 α-androstan-17-one	26	96
3 α-hydroxy-5 β-androstan-17-one	2	0
3 β-hydroxy-5 β-androstan-17-one	0	1
3 β-hydroxyandrost-5-ene-17-one	59	96
17 α-hydroxyandrost-4-ene-3-one	1	1
17 β-hydroxyandrost-4-ene-3-one	0	0
3 α-hydroxy-5 α-pregnan-20-one	0	1
3 β-hydroxy-5 α-pregnan-20-one	5	78
3 α-hydroxy-5 β-pregnan-20-one	2	0
3 β-hydroxy-5 β-pregnan-20-one	0	1
3 β-hydroxypregn-5-ene-20-one	56	94
20 α-hydroxy-5 α-pregnane	0	0
20 β-hydroxy-5 α-pregnane	0	0
3 β-hydroxycholest-5-ene	—	50

La propriété la plus intéressante de cet enzyme est sa spécificité ainsi qu'on le voit dans le tableau III. Tous les sulfates de stéroïdes hydrolysés sont des sulfates 3 β des stéroïdes 5 α ou Δ^5, aucun autre isomère n'est hydrolysé. Cette spécificité est plus grande que celle montrée par les autres sulfatases, mais elle n'est pas inattendue si l'on considère la stéréochimie des subtrats. Les sulfates hydrolysés sont ceux dans lesquels la molécule de stéroïde atteint sa forme la plus plane. D'autres facteurs cependant doivent intervenir puisque le 17-β-hydroxyandrost-4-ène-3-one sulfate n'est pas hydrolysé par cet enzyme, et pourtant il possède une forme plane. La stéroïde sulfatase n'est pas une alkylsulfatase générale puisqu'elle ne peut pas hydrolyser certains alkylsulfates vrais tels que l'éthyl ou le benzylsulfate : à cause de cette spécificité, le nom « alkylsulfate » ne peut être employé pour décrire l'enzyme qui hydrolyse le sulfate de déhydroépiandrostérone.

Le seul sulfate de stéroïde hydrolysé par l'enzyme et qui n'a pas la configuration 3-β-5-α ou 3-β-Δ^5 est le cortisone-21-sulfate. L'hydrolyse de ce composé est très surprenante si l'on tient compte de la forte spécificité manifestée dans d'autres cas par l'enzyme, mais il se peut que l'hydrolyse du cortisone-21-sulfate soit due à une autre sulfatase existant dans les préparations enzymatiques employées. Des preuves dans ce sens sont données par le fait que le rapport des vitesses d'hydrolyse du sulfate de déhydroépiandrostérone et du sulfate de cortisone est 35/1 dans le cas de l'enzyme préparé à partir de *Patella vulgata*, mais seulement 7/1 dans le cas de l'enzyme préparé à partir de *Littorina littorea*. La nature de l'enzyme qui effectue cette hydrolyse ne peut pas être précisée, mais il se pourrait que ce soit la glucosulfatase, que l'on sait être présente dans *Littorina* en plus grande quantité que dans *Patella* (13), d'autant qu'il existe une certaine ressemblance de structure entre le cortisone-21-sulfate et le glucose-6-sulfate que l'on sait être un substrat pour la glucosulfatase.

D'après ce que l'on sait jusqu'ici, il apparait que la stéroïde sulfatase n'existe que dans les mollusques gastéropodes : on l'a trouvée dans toutes les espèces de gastéropodes examinées, mais jamais ailleurs. Buelher, Katzman et Doisy (14) ont recherché sa présence dans un grand nombre d'espèces de bactéries, mais n'ont pas trouvé cet enzyme. Comme la grenouille *Rana temporaria* sécrète dans sa bile des quantités relativement grandes de ranol sulfate, on a cherché la stéroïde sulfatase dans le foie de la grenouille mais on n'a observé aucune activité. De même, aucune stéroïde sulfatase n'a été trouvée dans le foie des mammifères. La raison de cette distribution limitée de la stéroïde sulfatase est difficile à comprendre, d'autant plus que, au moins dans certaines espèces de mollusques, cet enzyme est apparemment un enzyme digestif sécrété dans le jus intestinal. Cependant il est difficile de croire que cet enzyme joue un rôle dans la digestion, puisqu'on ne lui connait aucun substrat naturel existant normalement dans la nourriture de ces animaux.

Tout récemment, Gibian et Bratfisch (25) ont décrit dans le foie du bœuf et du rat un enzyme qui hydrolyse le sulfate de déhydroépiandrostérone. La spécificité de cet enzyme semble être tout à fait comparable à celle de la stéroïde sulfatase de *Patella* ; néanmoins, par son pH optimum voisin de 8 et par son insolubilité, elle se rapproche de l'arylsulfatase C.

Signification biologique des sulfates de stéroïdes

A la lumière de la discussion précédente, il est intéressant d'examiner quelle est la signification des sulfates de stéroïdes dans le métabolisme des mammifères. Il est bien établi maintenant que le foie des mammifères contient un système enzymatique capable de synthétiser les sulfates de stéroïdes, mais ne contient pas d'enzyme effectuant l'hydrolyse de ces substances. Le seul sulfate de stéroïde qui peut être hydrolysé dans les tissus des mammifères est le sulfate d'oestrone, or celui-ci est un arylsulfate et il peut donc être hydrolysé par les arylsulfatases des mammifères. Cette hydrolyse n'a jamais été montrée comme étant effectuée par les sulfatases des mammifères bien qu'elle soit apparemment catalysée par la sulfatase de la taka-diastase. Il est intéressant cependant, de signaler que la sulfatase A du foie a une grande affinité pour les arylsulfates polycycliques. On a dit que le sulfate d'oestrone est rapidement hydrolysé *in vivo,* mais ce fait semble encore discutable. La préparation du sulfate d'oestrone pur est extrêmement difficile, de plus, plusieurs des expériences indiquées comme prouvant l'hydrolyse *in vivo* du sulfate d'oestrone sont délicates à interpréter, puisque Young et Hawkins (26) ont montré que le SO_4^{--} précipité sous forme de sulfate de benzidine, en présence d'un ester sulfurique ^{35}S, peut être fortement contaminé par ^{35}S si l'on ne purifie pas de façon extrêmement soigneuse le sulfate de benzidine. Il est donc impossible actuellement de dire si le sulfate d'oestrone est ou non hydrolysé *in vivo.* Une telle hydrolyse n'est pas impossible théoriquement bien qu'il soit manifeste que les arylsulfates simples ne sont pas hydrolysés *in vivo,* mais ceci peut être dû à la spécificité des arylsulfatases. On peut faire aussi des critiques à la détermination de l'activité biologique du sulfate d'oestrone, étant donné que, dans beaucoup de cas, les préparations employées n'étaient pas parfaitement pures; mais si l'on considère que le sulfate d'oestrone a une activité d'oestrogène, il n'est pas possible pour autant de dire qu'il est hydrolysé *in vivo,* puisqu'on ne sait pas si le sulfate d'oestrone lui-même est dépourvu d'activité oestrogénique.

En ce qui concerne les autres sulfates de stéroïdes, du type alkylsulfate, on ne peut pas dire s'ils sont hydrolysés *in vivo* ou s'ils sont biologiquement actifs; il est tentant de penser que les sulfates de stéroïdes sont de vrais produits de détoxication c'est-à-dire qu'ils sont dépourvus d'activité biologique ; d'autre part, les glucuronides de stéroïdes seraient des formes de transport des stéroïdes puisqu'on ne peut pas douter de l'activité biologique du glucuronide d'oestriol.

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DISCUSSION

Cl. Fromageot. — L'étude des esters sulfuriques pose la question de la nature du sulfate actif ayant donné naissance à ces esters.

Dans une note qui vient de paraître dans *J. Am. Chem. Soc.* (78 (1956) 2652), Robbins et Lipmann viennent de démontrer qu'une forme au moins de sulfate enzymatiquement actif est constituée par l'adénosine-3′-phosphate-5′-phosphosulfate.

P. Jarrige. — Avez-vous réussi à séparer la stéroidesulfatase de l'arylsulfatase de *Patella* ? Nous avons nous-mêmes essayé de réaliser la séparation de ces enzymes dans le suc d'*Helix pomatia*, nous n'y sommes pas parvenus. Les fractions les plus actives en arylsulfatase l'étaient aussi en stéroidesulfatase. Ces sulfatases sont précipitées par l'acétone à 60 %, par le sulfate d'ammonium à *p*H 5,7 entre 65 et 80 %. Une préparation obtenue par le sulfate d'ammonium et dialysée précipite par l'éthanol à *p*H 4,05 et *r*/2 0,02 entre 60 et 69 %. L'activité stéroïdesulfatasique est optimum à *p*H 4,5 en tampon acétate 0,05 M. La concentration optimum en sulfate de déhydroépiandrostérone était 6.10^{-4} M. On obtenait 91 % d'hydrolyse en 4 heures à 37°. L'activité arylsulfatasique est optimum à *p*H 5,5 en tampon acétate 0,05 M et 0,005 M en phénolphtaléine sulfate.

L'action des inhibiteurs aux mêmes concentrations sur la même préparation est la suivante :

	Stéroïdesulfatase	Arylsulfatase
SO_4^{--} 0,1 M	95 % d'inhibition	30 % d'inhibition
PO_4^{---} 0,1 M	95 % »	70 % »
Borate 0,05 M	95 % »	100 % »

A. B. Roy. — Je n'ai pas pu séparer la stéroidesulfatase et l'arylsulfatase au moyen de fractionnement par le sulfate d'ammonium ou par les solvants organiques. Les deux sulfatases sont apparemment étroitement associées, mais elles peuvent être partiellement séparées par électrophorèse sur papier. Le traitement à 50° donne une très faible séparation des deux enzymes.

R. Henry. — Je désire évoquer deux points de la communication du Dr. Roy :

a) *Spécificité de conjugaison.* — Expérimentalement nous constatons que, dans les urines et dans les sérums, ce sont les 3-β-hydroxystéroïdes qui sont sulfoconjugués. Les 3-cétostéroïdes ne sont sulfoconjugués que par la fonction alcool en C_{21}. Après action des enzymes du suc digestif d'*Helix Pomatia* qui libère les 3-β-hydroxylsulfoconjugués ainsi que les glycuroconjugués, l'hydrolyse par le dioxane trichloroacétique libère des 3-α-hydroxystéroïdes dont l'androstérone.

Les essais de synthèses et d'hydrolyses des stéroïdes sulfates effectués par le Dr. Roy nous apportent quelques lumières sur ce point. En effet, *in vitro*, la vitesse de synthèse de l'androstérone sulfate par rapport à celle de l'isoandrostérone sulfate est de 1 à 2 alors que leur rapport d'hydrolyse est de 1 à 100. Il ne semble donc pas que la sulfoconjugaison soit spécifique des 3-β-hydroxystéroïdes mais que, pour mettre en évidence ces 3-α-hydroxystéroïdes, ce soit l'hydrolyse qui nécessite des moyens différents de ceux utilisés couramment.

Je signale que l'emploi de l'arylsulfatase d'*Aspergillus oryzea* nous a permis d'hydrolyser des phénolstéroïdes sulfates dans les urines de femmes enceintes.

b) *Forme de transport des stéroïdes.* — Je suis d'accord avec les précédents rapporteurs pour penser que, dans le domaine des stéroïdes hormonaux, la sulfoconjugaison est surtout une forme de transport. L'insolubilité des 17-cétostéroïdes implique que la conjugaison se fasse dans la cellule même où ils sont produits. Il serait donc intéresasnt de refaire les essais de synthèse des stéroïdes sulfoconjugués avec des broyats et des coupes de surrénales.

Enfin, à l'occasion d'expériences toutes récentes, j'ai pu constater que ces formes de transport sont encore beaucoup plus compliquées dans le sérum. A côté des fractions glucuro et sulfoconjuguées, il y a une fraction importante des stéroïdes qui est liée aux lipides.

GENERAL DISCUSSION.

It is unfortunate that one of the principal aims of this investigation has not been achieved in that no clue has yet been obtained to the physiological function of the sulphatases. A considerable amount of information on the general properties of these enzymes has, however, been made available through the work and this knowledge will be of considerable value in the further study of the role of the sulphatases in metabolic processes. The difficulty of defining their physiological role is by no means a peculiarity of the sulphatases as it is surprising how few of the apparently simple hydrolases have had any specific physiological function definitely assigned to them.

The decision to use nitrocatechol sulphate as the substrate was a fortunate one. Without the availability of this substrate progress in the study of the mammalian arylsulphatases would have undoubtedly been slow and the complexity of these enzymes would not readily have been apparent, as is shown by the early work of Dodgson, Spencer & Thomas (1953) who, using p-acetylphenyl sulphate as substrate, detected only one sulphatase in rat liver. Use of the simpler substrates such as

phenyl sulphate or p-acetylphenyl sulphate would have allowed the detection of only the sulphatase C type of enzyme which has a high affinity for these simple substrates and which rapidly hydrolyses them. The contrary is the case with sulphatases of the A or B type. It is probable that sulphatase B would never have been detected, at least in ox liver, without the use of nitrocatechol sulphate as this type of arylsulphatase hydrolyses substrates such as phenyl sulphate only very slowly, if at all. Sulphatase A can be detected by its ability to hydrolyse p-nitrophenyl sulphate (Maengwyn-Davies & Friedenwald, 1954), but only if extremely high concentrations of enzyme are used, under which conditions problems caused by the presence of endogenous inhibitors, such as phosphates, become important. Nitrocatechol sulphate is undoubtedly the substrate of choice in any investigation of sulphatases A or B, although the anomalous kinetics shown by the former may make the interpretation of the results difficult, at least from a quantitative point of view. In this connexion it is perhaps worthy of note that although the interpretation of the kinetics of sulphatase A proposed in the second paper of the series has since been shown

to be wrong, the empirical relationship between the enzyme concentration and the reaction velocity which is described in that paper is still of value in comparing the concentrations of different preparations of sulphatase A, provided that the conditions specified are strictly adhered to.

The choice of ox liver as the enzyme source was also fortunate. In that tissue all three arylsulphatases are present in readily detectable amounts and the soluble sulphatases, sulphatases A and B, are easily separated by the usual techniques of enzyme chemistry. In other species the situation may not be so favourable. In the rat, for instance, enzymes corresponding to the sulphatases A and B of the ox can be detected after separation by paper electrophoresis but they cannot readily be separated by the techniques successfully used in the fractionation of the ox enzyme. Guinea-pig liver seems to be completely lacking in sulphatase C, while in mouse liver the amount of the latter enzyme which is present is extremely small. This fact presumably explains the difficulties encountered in the early stages of the work when phenyl sulphate was used for the assay of mouse liver sulphatases. The choice of ox liver as the

enzyme source and of nitrocatechol sulphate as the substrate therefore contributed in no small way to the relatively rapid progress of the initial stages of this investigation.

As described in the experimental section the occurrence of three arylsulphatases in the livers of the ox and of the rat has definitely been established. Unpublished observations have shown similar enzymes to exist in the mouse and the rabbit while in the guinea-pig at least two of them are present. The enzymes may readily be distinguished by their specificity, by their response to inhibitors and by their intracellular distribution. Sulphatase C, a group 1 arylsulphatase, has a high affinity for simple substrates such as p-nitrophenyl sulphate or p-acetylphenyl sulphate and rapidly hydrolyses them. It is not inhibited to any significant extent by phosphate ions nor by sulphate ions. Sulphatases A and B, or group 2 arylsulphatases, have, on the contrary, little affinity for the simple aryl sulphates and hydrolyse these compounds only very slowly. They have, however, a high affinity for nitrocatechol sulphate which they rapidly hydrolyse. The group 2 arylsulphatases are also characterised by being

strongly inhibited by phosphate and sulphate ions. The distinction between the two groups of arylsulphatases is therefore relatively clear-cut, at least in the case of the mammalian enzymes, although when the arylsulphatases in general are considered it seems likely that these two groups represent only the extreme types of a series of related enzymes.

The two groups of arylsulphatases also differ in their intracellular localisations. In rat liver sulphatase C is apparently localised entirely in the microsomes while sulphatases A and B occur in both the mitochondrial and microsomal fractions, the major part of the activity being associated with the mitochondria. Viala & Gianetto (1955) have suggested that the group 2 arylsulphatases occur not in the mitochondria nor in the microsomes but in the lysosomes, a type of cell particle which is differentiated from the typical mitochondrion by its smaller size and by its very characteristic complement of enzymes. According to Applemans, Wattiaux & Duve (1955) the lysosomes occur in both the mitochondrial and microsomal fractions when these are prepared in isotonic sucrose solution by the now standard technique of

Hogeboom (1955), the method used in the present investigation. The lysosomes differ very strikingly from the mitochondria in that they lack cytochrome oxidase, which is a typical enzyme of the true mitochondria, but contain many hydrolases with pH optima on the acid side of neutrality - arylsulphatase, β-glucuronidase, acid phosphatase, cathepsin, ribonuclease and deoxyribonuclease. This view of Viala & Gianetto (1955) that the group 2 aryl-sulphatases occur in the lysosomes is supported by the finding that the sulphatases A and B of the microsomal fraction are readily extracted by water, as are the corresponding enzymes of the mitochondrial fraction, but in striking contrast to the purely microsomal sulphatase C which is insoluble in water under all normal conditions. The possibility that some of the group 2 arylsulphatases do in fact occur in the microsomes cannot be completely discounted, however, as the routine preparations of ox liver sulphatase C described in the experimental section always contain small amounts of sulphatases A and B which are not readily soluble in water, suggesting that these latter enzymes might have a truely microsomal origin.

Be this as it may, the liver arylsulphatases

of the rat are localised predominantly in the particulate fractions of the cell with only small, perhaps negligible, amounts in the soluble fraction. The small amounts of soluble enzyme normally found may well be derived from lysosomes ruptured during the isolation procedure. Unpublished observations have shown similar, although not identical, distributions of the arylsulphatases in the livers of the mouse, rabbit and guinea-pig.

One of the most important questions arising from the study of the arylsulphatases is that of the nature of their natural substrate, or substrates. This problem is made even more interesting in the case of the group 2 enzymes which have such a very high affinity for the highly 'non-physiological' compound, nitrocatechol sulphate, but very little affinity for the simple aryl sulphates, some of which do occur in natural products. It is commonly argued that if an enzyme has a high affinity for a substrate and is an efficient catalyst for the appropriate reaction, then it is probable that the substrate under investigation is closely related to the 'physiological' substrate of the enzyme. It is difficult to see how this can be the case with the group 2 arylsulphatases as there

is no known compound of natural occurrence which has a structure comparable to that of nitrocatechol sulphate. The only physiological substance at all related to the latter would seem to be adrenaline sulphate (Richter, 1940), although to judge by the results of Dodgson, Rose & Spencer (1955) it is probable that in this compound the sulphate group will be esterified with the hydroxyl group in the para position to the side chain, while in nitrocatechol sulphate the sulphate grouping is meta to the nitro group. Whether or not such a compound would be hydrolysed by the group 2 aryl-sulphatases cannot be stated at present. Although aryl sulphates are important constituents of urine the nature of the phenols involved seems to be unknown, at least in the case of normal animals. The only adequately characterised aryl sulphates of normal urine, apart from indoxyl sulphate, are oestrone sulphate and p-ethylphenyl sulphate which were isolated from the urine of pregnant mares (Schachter & Marrian, 1938) and from normal goat urine (Grant, 1948) respectively. Naturally occurring aryl sulphates other than those of urine are rare. The only known compounds of this type seem to be bufothionine, found in toad venom

(Wieland & Wieland, 1937), and tyrosine-O-sulphate which has recently been claimed as a constituent of fibrinogen (Bettleheim, 1954). It seems highly unlikely that any one of these compounds could be the natural substrate for the arylsulphatases. At the most they are present in only small amounts and their turnover is presumably negligible so that the large amounts of arylsulphatase present in most mammalian tissues would seem to be greatly in excess of any requirement for the metabolism of these compounds. The conclusion would therefore seem to be inescapable that the physiological substrate, or substrates, of the arylsulphatases have yet to be discovered. The widespread occurrence of these enzymes would suggest that their substrates must have a similar distribution, even if they occur in only small amounts.

The possibility must be borne in mind that the arylsulphatases may not be functioning as such in vivo. For instance, the work of Viala & Gianetto (1955) has shown that the lysosomal hydrolases become active only after the particles have been lysed, so that presumably in the intact cell these enzymes cannot be functioning in the way they do

in vitro. Again, the group 2 arylsulphatases are strongly inhibited by phosphate ions and it is therefore difficult to see how these enzymes can act *in vivo* unless their immediate environment has a composition very different from that of the general intracellular fluid which is so very rich in phosphates. Another possible explanation is, of course, that *in vivo* the arylsulphatases, whatever their function, are not sensitive to these ions.

The available evidence for any arylsulphatase activity *in vivo* is not satisfactory. Garton & Williams (1949) fed phenyl sulphate (100-500 mg./kg.) to rabbits and showed that some 90% of the dose was excreted unchanged in the urine. Hawkins & Young (1954) administered sulphate esters labelled with ^{35}S to rats at a dosage of approximately 60 mg./kg. and found that only about 5% of phenyl sulphate and about 10% of 1- or 2-naphthyl sulphates were hydrolysed *in vivo*. These results are unfortunately not as conclusive as might have been hoped because of the specificity of the arylsulphatases. The substances administered are substrates for the group 1 arylsulphatases but not for the group 2 enzymes which are present in much larger amounts.

Therefore, although the results seem to demonstrate that the group 1 arylsulphatases have little activity *in vivo* they give no information about the quantitatively more important group 2 arylsulphatases. Another complicating factor in all experiments of this type is that of the permeability of the cells to sulphate esters: this problem does not appear to have been investigated and therefore the apparent inactivity of the arylsulphatases *in vivo* might be due simply to the fact that the substrates are not obtaining access to the intracellular enzymes.

In contrast to the above results are those of Hanahan & Everett (1950) who claimed that in rats oestrone sulphate, labelled with ^{35}S, was very rapidly hydrolysed *in vivo*. In view of the detailed studies of Hawkins & Young (1954) the methods used by Hanahan & Eeverett (1950) must be viewed with suspicion as it seems not unlikely that the samples of benzidine sulphate isolated from the urine by the latter workers would be grossly contaminated with unhydrolysed oestrone sulphate.

The only other evidence pertinent to the activity of the arylsulphatases *in vivo* is indirect and therefore capable of many different interpretations.

For instance, stilboestrol sulphate has oestrogenic activity (Bishop, Richards & Perry, 1951) and this has been given as evidence for the hydrolysis of the sulphate ester *in vivo* but it is obvious that several other interpretations are possible. Again, there is the observation that insulin sulphate ester retains its hormonal activity (Glendenning, Greenberg & Fraenkel-Conrat, 1947). This has also been quoted in favour of an arylsulphatase activity *in vivo* but other interpretations are possible as insulin acetate, which would almost certainly be hydrolysed in the body, has no hormonal activity. Not without interest in this connexion is the observation of Richter & MacIntosh (1941) that the conjugated from of adrenaline which is present in urine, almost certainly adrenaline sulphate (Richter, 1940), has no biological activity suggesting that hydrolysis of this substance cannot occur *in vivo*.

It must be accepted, therefore, that it is at least possible that the arylsulphatases do not function as such *in vivo*. Unfortunately no alternative function can be suggested although it is tempting to postulate that they might play a part in some reaction undergone by the sulphated

derivatives of ATP recently discovered by Robbins & Lipmann (1956a, b). It must be stressed, however, that the arylsulphatases play no part in the synthesis of aryl sulphates by the mechanisms so far studied. The possibility that there are other pathways available cannot be ruled out. Such a pathway could be the transfer of a sulphate grouping from, say, an aryl sulphate to some suitable acceptor, a reaction which might well be catalysed by an aryl-sulphatase. There is no evidence for the existence of such a reaction but it is perhaps pertinent that Fishman & Green (1956) have recently shown that a similar reaction, the transfer of a β-glucuronide residue, is catalysed by β-glucuronidase. The physiological importance of this reaction is, of course, unknown.

This latter point is interesting as it is striking how many points of resemblance there are between the β-glucuronidases and the arylsulphatases. In both cases the enzymes are localised, at least in part, in the lysosomes and they do not exhibit their characteristic activity until the particles have been lysed; both enzymes are present in mammalian tissues as a complex of closely related forms, the relationships between which are obscure;

as far as can be judged from the available data neither of the enzymes has any significant activity in vivo; and finally, the natural substrates for either of the hydrolases are quite unknown. It is hard to believe that these many points of resemblance are entirely fortuitous and it might with justification be predicted that in the future some intimate connexion between the arylsulphatases and the β-glucuronidases will be discovered. An obvious link between these enzymes is provided by the mucopolysaccharides which contain both glucuronic acid and sulphuric acid. Unfortunately, although Meyer, Linker & Rapport (1951) have shown that β-glucuronidase can hydrolyse degradation products of mucopolysaccharides there is no similar role obvious for the arylsulphatases which are, as far as is known, without action on these compounds. In fact, some specimens of chondroitin sulphate are inhibitors of the group 2 arylsulphatases. The specimen used in the experiments reported in paper number two was prepared by the extraction of cartilage with alkali and, as described, it did not inhibit sulphatase A. More recent experiments have shown that chondroitin sulphate prepared by extraction with KCl (Einbinder & Schubert, 1950) did inhibit the

the group 2 arylsulphatases. This inhibition is probably of no physiological significance as the chondroitin sulphate had to be used in quite high concentrations, in the region of 0.5 mg./ml.

With regard now to the steroid sulphatase of ox liver some of the same comments apply, although in this case a physiological substrate, dehydroepiandrosterone sulphate, is known to be a normal metabolic product in many animals. The problem of a possible substrate for this enzyme does not, therefore, occur and there seems to be no reason to believe that steroid sulphatase is not functioning as such *in vivo*, although no such activity has yet been demonstrated. In view of this it might be of interest to investigate the biological activity of dehydroepiandrosterone sulphate: it seems to be generally held that this compound is without androgenic activity but the evidence for this view has not been stated. If steroid sulphatase were functioning as such *in vivo* it might be expected that dehydroepiandrosterone sulphate would be biologically active. Androsterone sulphate would, on the other hand, not be expected to have androgenic activity as this substance is not a substrate for steroid sulphatase. This latter

point would indeed seem to be the case to judge by the preliminary results of Venning, Hoffman & Browne (1942).

The situation is rather different in the case of the molluscan steroid sulphatase. At least in the snails Helix pomatia and Otala punctata (Savard, Bagnoli & Dorfman, 1954) steroid sulphatase seems to be a digestive enzyme as it occurs in the intestinal juices of these molluscs. It therefore presumably plays some role in the normal digestive processes of these animals. What this role can be is quite obscure as there seems to be no known substrate for the enzyme which is likely to be a normal constituent of the diet of either of these snails, or indeed of any mollusc. The most interesting property of steroid sulphatase, its high specificity, has been dealt with in the experimental section and as the enzyme is of little interest in connexion with the general plan of the work it need not be discussed further at this stage, except for one minor point. Since the work was published similar enzyme preparations from Littorina littorea (The common Periwinkle) have been examined. This species is particularly rich in glucosulphatase (Dodgson & Spencer, 1954) and

the enzyme preparations obtained from it hydrolysed cortisone-21-sulphate much more rapidly than did those from Patella vulgata. In the former case the ratio of the rates of hydrolysis of dehydroepiandrosterone sulphate and cortisone sulphate is 7:1, compared with a value of 35:1 for the ratio in the latter. This is some slight further evidence for the suggestion made in the original publication that the hydrolysis of cortisone sulphate is due not to steroid sulphatase but to glucosulphatase. Should this indeed be the case then an interesting possibility emerges, namely, that the steroid sulphatase activity might itself be due not to a true steroid sulphatase but to some other enzyme, perhaps a glycosulphatase of some as yet unknown type. This would help to explain the otherwise peculiar occurrence of steroid sulphatase activity in the digestive juices of molluscs. Glycosulphatases of several types might well be expected to occur therein in view of the richness of the diet of many molluscan species, especially the more primitive marine types, in carbohydrate sulphates. That numerous glycosulphatases are still to be discovered seems very probable in view of the fact that none of these enzymes which are

known at present will hydrolyse seaweed polysaccharide sulphates, yet it would seem that enzymes capable of hydrolysing these compounds must exist. If they do, then their most likely source would seem to be the digestive juices of those molluscs whose diet normally contains their substrates in such large amounts.

The question of steroid sulphate synthesis by rat liver preparations also needs little comment. The function of such a system is obvious as many of the compounds synthesised *in vitro* have been isolated from urine. There is little doubt, therefore, that the steroid sulphate synthesising system functions *in vivo* as it does *in vitro*. The amounts of the system present in liver seem to be adequate for any likely synthesis of steroid sulphates by the rat - in that species the liver could, under optimal conditions, synthesise approximately 10 mg. of steroid sulphate per day, an amount many times greater than that excreted in the urine. The mechanism of the synthesis of steroid sulphates is apparently closely allied to that of aryl sulphate synthesis as it involves the formation of an 'active sulphate' followed by transfer of the sulphate grouping to the acceptor molecule. This has been shown by

kinetic studies, as described in the experimental section, and also by the separation of an 'activating enzyme' and a 'transferring enzyme' by the method of DeMeio, Wizerkaniuk & Schreibman (1955). It is virtually certain that the same active sulphate is involved in the formation of both aryl and steroid sulphates and that the two processes differ only in the nature of the 'transferring enzymes' required for the second stage of the reaction. The only point of general interest arising from the kinetic studies was the suggestion that the first reaction, the formation of the active sulphate, was reversible. Robbins & Lipmann (1956b) have recently shown that the initial reaction in the formation of active sulphate, the reaction between ATP and inorganic sulphate to give adenosine-5'-phosphosulphate and pyrophosphate, is reversible. This reaction is followed by a rapid transformation of the adenosine-5'-phospho-sulphate into adenosine-3'-phosphate-5'-phospho-sulphate through the action of a specific kinase. This latter compound is the true active sulphate (Robbins & Lipmann, 1956a). Although the action of the kinase would be expected to be irreversible this reaction would not be detected by simple

kinetic studies of the overall reaction provided that the velocity of the first step, the sulphurolysis of ATP, were rate limiting. This is likely to be the case to judge from the available data. The specificity of the steroid sulphate synthesising system is much as would be expected from a consideration of the stereochemistry of the substrates. The 3-sulphates of the relatively planar 5α and Δ^5 steroids were formed much more rapidly than those of the corresponding 5β steroids which are highly non-planar. The configuration of the hydroxyl group at the 3 position seemed to be relatively unimportant. From the study of the relatively few substrates of other types which were available it seemed that sulphation at other positions occurred very slowly, if at all. This is in agreement with the fact that all the steroid sulphates which have been isolated from urine are 3-sulphates.

The observation that simple alcohols did not give rise to sulphate esters is of interest in that it supported the thesis of Anderton, Smith & Williams (1948) that alcohols do not form sulphate esters <u>in vivo</u>, at least in mammals. In contrast

to this are the recent observations of Bridgewater & Ryan (1957). These authors have described an enzyme system present in the liver of the frog, Rana temporaria, which is capable of synthesising the sulphate ester of ranol, a true alcohol. This enzyme system must be quite distinct from that of the rat as it is present in the microsomal fraction of frog liver. The sulphate synthesising system of rat liver is present in the soluble fraction of the cell and is actually inhibited by the microsomes, presumably because of the large amounts of ATP-ase present therein (DeMeio, Wizerkaniuk & Fabiani, 1953). It is proposed to investigate the differences between these two sulphate synthesising systems whenever the opportunity arises.

Looking to the future there seem to be two main problems. Firstly, there is the obvious necessity to elucidate the nature of the complex kinetics of sulphatase A. This problem is probably purely artificial in that it is unlikely to have any direct bearing on the functioning of sulphatase A in vivo as the anomalies appear to be due, at least in part, to the use of nitrocatechol sulphate as

the substrate. For the detailed study of this problem it would be advantageous to have available large amounts of a highly purified enzyme preparation and with this end in view possible methods for the large scale preparation of sulphatase A are being investigated. The method described in the experimental section is in many ways a convenient one but it suffers from the disadvantage of using an acetone powder as a starting material and of utilising fractional precipitation with acetone at low temperatures as the initial step in the purification. This latter procedure is not suited for large scale work because of the difficulty of dealing with large volumes of liquid at low temperatures, the limiting factor being the capacity of the available refrigerated centrifuges. In view of the extremely complex nature of the kinetics of sulphatase A it would be pointless to speculate further on this topic until considerably more information is available.

The second outstanding problem is that of the physiological role of the arylsulphatases, or indeed of the sulphatases in general. A suitable method of tackling this problem is not apparent but an attempt is at present being made to synthesise

various selenium analogues of aryl sulphates and of related compounds in the hope that these might prove to be highly specific inhibitors of the arylsulphatases. Should this be the case then the administration of these compounds to experimental animals might give some clue to the physiological function of the arylsulphatases. The very widespread occurrence of the arylsulphatases throughout living things suggests that the function of these enzymes must be of considerable importance, despite the fact that no clue has been obtained in the present studies to the possible nature of this function.

A number of possible functions can be suggested but it is important to note that there is no evidence whatever in favour of any of them. It could be postulated that the aryl sulphates take part in a series of reactions which are more or less analogous to the many reactions of phosphate esters and that these reactions are controlled by the arylsulphatases. There is, for instance, the suggestion of Bean & Hassid (1955) that the L-galactose of seaweed polysaccharides is formed from D-galactose via the D-galactosyl sulphate. Another function for the arylsulphatases which seems not improbable

is that of sulpho-transferases. Such a function would be extremely difficult to investigate at present as neither the donor nor the acceptor molecules are known and it would seem that any progress in this direction must depend upon the adequate characterisation of those sulphate esters which are present in the tissues. Finally, there is the possibility that the arylsulphatases might have a part to play in the general electrolyte metabolism of the cell by virtue of the fact that hydrolysis of a sulphate ester liberates a hydrogen ion. Such a fundamental function would be in keeping with the widespread distribution of the enzymes and with their localisation in the cell particles which are well known to be involved in the maintainance of concentration gradients of electrolytes. The restriction of the sulphatases to the particulate fractions of the cell and of the sulphate synthesising system to the soluble fraction would seem to be a necessary prerequisite for any function involving ion transport. In this connexion it is perhaps of interest to recall the several previous suggestions that the mucopolysaccharides and the sulphated polysaccharides of marine molluscs may be

involved in the control of electrolyte movements (Belanger, 1954; Soda, 1936).

Although the function of the arylsulphatases is still as obscure as it was when this work was commenced seven years ago it seems that if the present interest in the metabolism of sulphate esters is maintained then the next decade should witness significant advances in the knowledge of the role of these compounds, and of their respective hydrolases, in biochemistry.

SUMMARY.

1. Methods are described for the preparation of three arylsulphatases from ox liver. These enzymes have been named sulphatases A, B and C.
2. The properties of these enzymes are discussed. One of them, sulphatase A, shows highly anomalous kinetics during the hydrolysis of nitrocatechol sulphate and an interpretation of these kinetics is presented.
3. The intracellular localisation of the enzymes have been determined - sulphatases A and B occur in both the mitochondrial and microsomal fractions while sulphatase C is found only in the microsomes.
4. The properties of a steroid sulphatase present in ox liver are described. The enzyme is closely associated with, but is distinct from, sulphatase C. The specificity of the enzyme is high as it will hydrolyse only the 3β-sulphates of the 5α or Δ^5 steroids.
5. A similar steroid sulphatase occurring in the mollusc Patella vulgata has been studied.
6. An enzyme system synthesising steroid sulphates has been obtained from rat liver and its properties examined.
7. The significance of the results is discussed in general terms.

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