The Steroid Sulphatase of *Patella vulgata*

By A. B. ROY
Department of Biochemistry, University of Edinburgh

(*Received* 23 *May* 1955)

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.

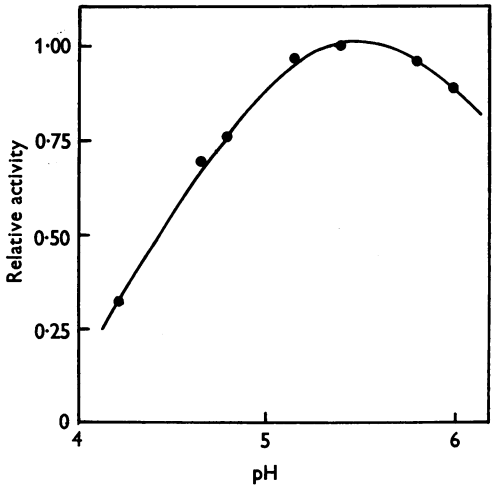

Fig. 1. Effect of pH on aryl sulphatase activity. Volume of reaction mixture 1·0 ml., containing 0·5 ml. 0·01 M nitrocatechol sulphate, 0·25 ml. enzyme and 0·25 ml. 0·5 M acetate buffers of varying pH. Incubated for 1 hr. at 37°.

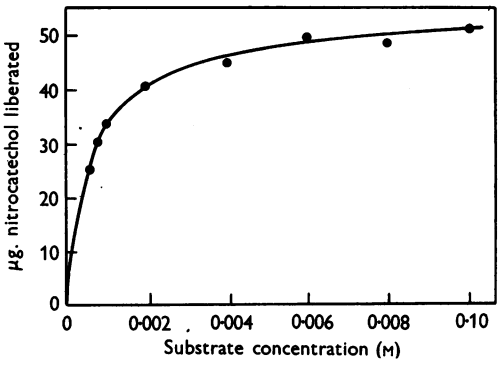

Fig. 2. Effect of varying substrate concentrations on aryl sulphatase activity. Volume of reaction mixture 1·0 ml., containing 0·25 ml. 0·5 M acetate buffer, pH 5·6, 0·25 ml. enzyme solution and 0·5 ml. nitrocatechol sulphate solutions of varying concentrations. Incubated for 1 hr. at 37°.

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.

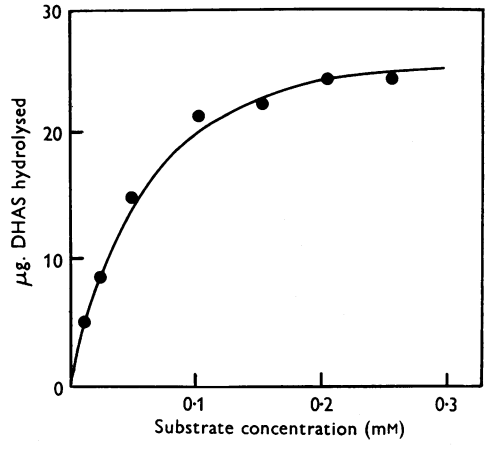

Fig. 3. Effect of varying substrate concentrations on steroid sulphatase activity. Volume of reaction mixture 1·0 ml., containing 0·25 ml. 0·5 M acetate buffer, pH 4·5, 0·25 ml. enzyme, and 0·5 ml. DHAS solutions of varying concentrations. Incubated for 1 hr. at 37°.

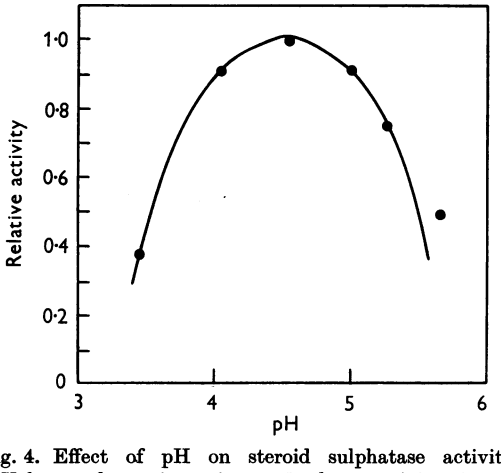

Fig. 4. Effect of pH on steroid sulphatase activity. Volume of reaction mixture 1 ml., containing 0·5 ml. 0·4 mM DHAS, 0·25 ml. enzyme solution and 0·25 ml. 0·5 M acetate buffers of varying pH values. Incubated for 1 hr. at 37°.

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.	Percentage hydrolysis after 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,

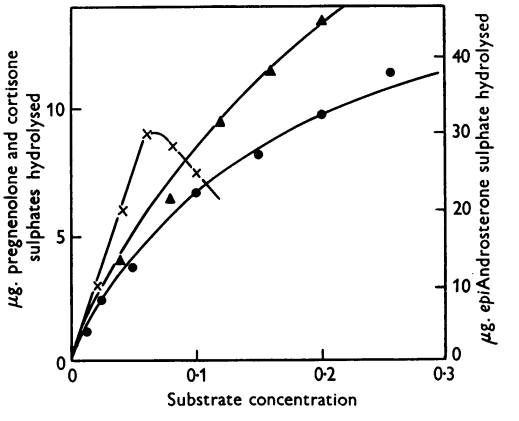

Fig. 5. Effect of varying substrate concentrations on the hydrolysis of *epi*androsterone sulphate, pregnenolone sulphate and cortisone sulphate by extracts of *Patella*. Conditions as in Fig. 3, except in the case of cortisone sulphate which was incubated for 17 hr. *epi*Androsterone sulphate, ●; pregnenolone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

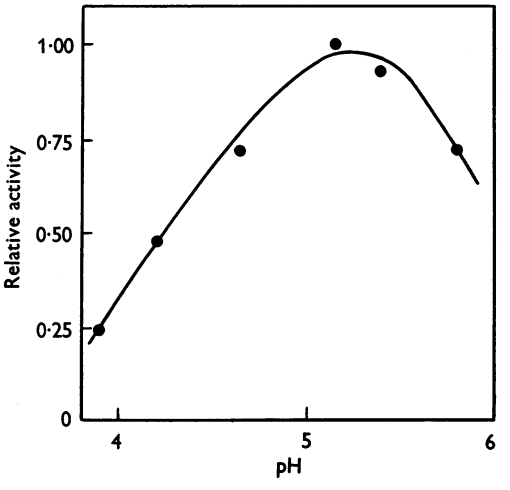

Fig. 6. Effect of pH on the hydrolysis of cortisone sulphate by extracts of *Patella*. Conditions as in Fig. 4 except that incubation time was 17 hr.

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°.

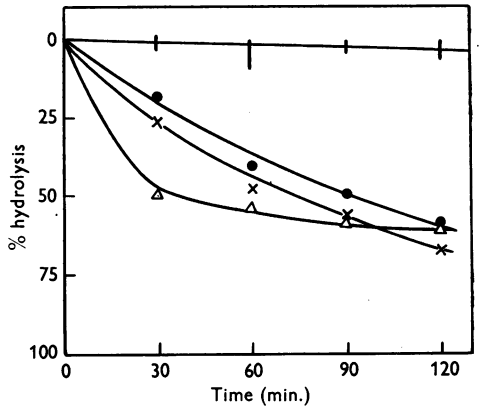

Fig. 7. Hydrolysis of steroid sulphates at pH 7 and 100°. Reaction mixture: 4 ml. 0·4 mM steroid sulphate and 4 ml. 0·02 M phosphate buffer, pH 7. DHAS, ●; pregnenolone sulphate, ×; cholesteryl sulphate, △. The corresponding points for androsterone, *epi*androsterone, 3β-hydroxy-5α-pregnan-20-one and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

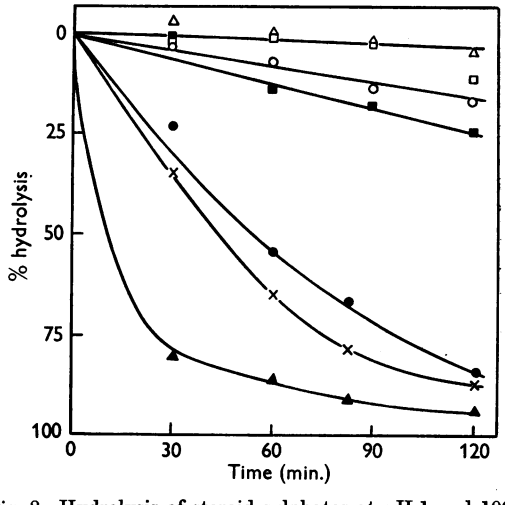

Fig. 8. Hydrolysis of steroid sulphates at pH 1 and 100°. Reaction mixture: 4 ml. 0·4 mM steroid sulphate and 4 ml. 0·2 N-H_2SO_4. DHAS, ●; pregnenolone sulphate, ×; cholesteryl sulphate, ▲; androsterone sulphate, ○; *epi*androsterone sulphate, △; 5α-pregnanolone sulphate, □; 5β-pregnanolone sulphate,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

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

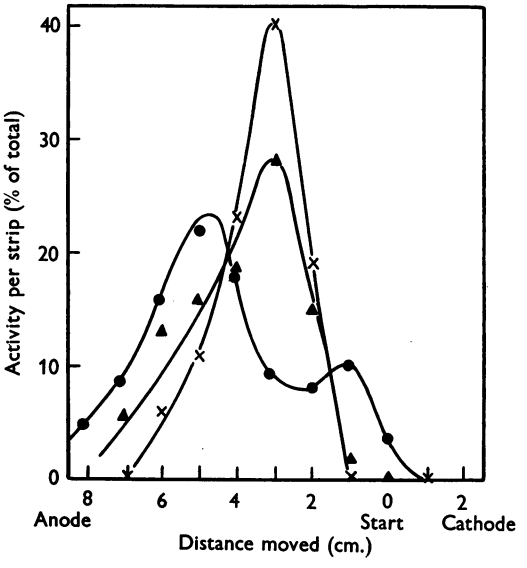

Fig. 9. Paper electrophoresis of an extract of *Patella*. 2 mg. stage 3, run 6 hr. at 500 v on Whatman no. 100 paper in 0·03 M veronal buffer, pH 7·4. Paper cut into 1 cm. strips and portions assayed with the following substrates. Nitrocatechol sulphate, ●; DHAS, ×; cortisone sulphate, ▲.

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.

The author is also very grateful to Miss Isla Sharp for her skilled technical assistance throughout this work.

REFERENCES

Butenandt, A. & Hofstetter, H. (1939). *Hoppe-Seyl. Z.* **259**, 222.

Dodgson, K. S., Lewis, J. I. M. & Spencer, B. (1953). *Biochem. J.* **55**, 253.

Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 315.

Dodgson, K. S. & Spencer, B. (1954). *Biochem. J.* **57**, 310.

Duff, R. (1949). *J. chem. Soc.* p. 1597.

Einbinder, J. & Schubert, M. (1950). *J. biol. Chem.* **185**, 725.

Fish, G. R. (1955). *Nature, Lond.*, **175**, 735.

Grant, G. A. & Glen, W. L. (1949). *J. Amer. chem. Soc.* **71**, 2255.

Haslam, R. M. & Klyne, W. (1953). *Biochem. J.* **55**, 340.

Haslewood, G. A. D. (1952). *Biochem. J.* **51**, 139.

Henry, R. & Thevenet, M. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 886.

Henry, R., Thevenet, M. & Jarrige, P. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 897.

Holden, G. W., Levi, I. & Bromley, R. (1949). *J. Amer. chem. Soc.* **71**, 3844.

Holden, R. & Bromley, R. (1950). *J. Amer. chem. Soc.* **72**, 3807.

Jayle, M. F. & Beaulieu, E. E. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 1391.
Klyne, W. (1955). *Progress in Stereochemistry*, vol. 1. London: Butterworths.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Munson, P. I., Gallagher, T. F. & Koch, F. C. (1944). *J. biol. Chem.* **152**, 67.
Paterson, J. Y. F. & Klyne, W. (1948). *Biochem. J.* **43**, 614.
Roy, A. B. (1953*a*). *Biochem. J.* **53**, 12.
Roy, A. B. (1953*b*). *Biochem. J.* **55**, 653.
Roy, A. B. (1954*a*). *Biochem. J.* **57**, 465.
Roy, A. B. (1954*b*). *Biochim. biophys. Acta*, **15**, 300.
Roy, A. B. (1955). *Biochem. J.* **59**, 8.
Savard, H., Bagnoli, E. & Dorfman, R. I. (1954). *Fed. Proc.* **13**, 289.
Sobel, E. & Spoerri, P. E. (1941). *J. Amer. chem. Soc.* **63**, 1259.
Soda, T. (1936). *J. Fac. Sci. Tokyo Univ.* **3**, 150.
Soda, T., Katsura, T. & Yoda, O. (1940). *J. chem. Soc. Japan*, **61**, 1227.
Stitch, S. R. & Halkerston, I. D. (1953). *Nature, Lond.*, **172**, 398.
Venning, E. H., Hoffman, M. M. & Browne, J. S. L. (1942). *J. biol. Chem.* **146**, 369.
Vlitos, A. J. (1953). *Contr. Boyce Thompson Inst.* **17**, 127.

A Study of the Irradiation of Catalase by Ionizing Radiations in the Presence of Cysteine, Cystine and Glutathione

By W. M. DALE and C. RUSSELL
Department of Biochemistry, Christie Hospital and Holt Radium Institute, Manchester

(*Received* 7 *April* 1955)

Any claim that H atoms generated by ionizing radiations in aqueous solutions of biochemical substances are the active agent responsible for the radiation effects observed deserves special attention, since oxidative reactions via OH and O_2H radicals are by far more common. Such claims are of particular importance in the field of enzymes for which no example of such a mechanism was known until it was found that catalase was inactivated by H atoms produced by X-irradiation of an aqueous solution of the enzyme (Forssberg, 1947). This conclusion was based on the experimental observation that cysteine, present in solutions of catalase during irradiation, did not protect the enzyme from inactivation, but on the contrary increased the inactivation. It was suggested that cysteine was scavenging the OH radicals, thus leaving the H atoms to act upon the enzyme. On the other hand, cystine was assumed to react with H atoms, because it protected the enzyme. A similar situation obtained with reduced and oxidized glutathione.

It was also found that keto acids and unsaturated acids such as pyruvic and fumaric acid protected catalase, but that saturated acids, such as succinic acid, did not.

Evidence against Forssberg's conclusions was put forward by Sutton (1952) and Bella (1949). Sutton found that H atoms produced by means other than irradiation did not inactivate catalase, and that irradiation of the enzyme in an atmosphere of oxygen was much more damaging than irradiation in an atmosphere of hydrogen. Bella irradiated pyruvic and lactic acid, and found that no lactic acid was produced from pyruvic acid, but that pyruvic acid was formed from lactic acid. Thus, the work of Sutton and Bella seemed to indicate that H atoms played no part in the inactivation of catalase by irradiation.

It was therefore decided to re-investigate the original problem, that is, whether cysteine and glutathione decrease the radiation effect on the enzyme, i.e. protect, or whether, on the contrary, they increase this effect. Further, does cystine protect the enzyme?

MATERIALS AND METHODS

Crystalline catalase was prepared from ox liver by the method of Tauber & Petit (1952) and Tauber (1953). This was found to be the simplest and quickest method. The preparation was electrophoretically homogeneous and had Kat. F. 39000.

Cysteine hydrochloride (British Drug Houses, Ltd.) was recrystallized and freed from iron by the method of Warburg (1927).

Electrophoresis. The electrophoretic properties of the enzyme were studied by the technique of paper electrophoresis, using barbitone–sodium barbitone buffer (pH 8·6, $I=0{\cdot}1$). Paper strips (Whatman no. 1) 38×7 cm. were used in a Shandon Vertical Electrophoresis tank (Shandon Scientific Co., Cromwell Place, London, S.W. 7). Catalase solutions (12·5 μl.; catalase content 2·2–4·5 mg./ml.) were applied at a spot 5 cm. from the centre line towards the cathode. Electrophoresis (150 v, *ca.* 4 v/cm.) was allowed to proceed at room temp. for about 20 hr., during which catalase migrated towards the anode. The strips were air-dried and developed with methyl orange–eosin Y in aqueous