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 dehydro*epi*androsterone sulphate (DHAS). This report is of considerable interest as it had previously been believed (Roy, 1956*a*) that such an enzyme was lacking from mammalian tissues. Work from this Laboratory (Roy, 1953, 1954*a*, 1956*c*) 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 optimum activity in the region of pH 8. Their preparations of steroid sulphatase hydrolysed oestrone sulphate, which would suggest that an arylsulphatase 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of the arylsulphatase, as it has been noted that this substrate is not readily hydrolysed by mammalian arylsulphatases (unpublished observations).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 synthesized as previously described (Roy, 1956*a*), and the enzyme was obtained from ox liver exactly as described for the preparation of sulphatase C (Roy, 1956*c*). 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, 1956*a*, *b*). To 0·2 ml. of 0·5M-2-amino-2-hydroxymethylpropane-1:3-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°, and the reaction was then stopped by the addition of 5 ml. of ethanol. The precipitated proteins were 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, 1956*b*) and the methylene blue salt of the DHAS extracted into 5 ml. of $CHCl_3$. The amount of DHAS present was then determined colorimetrically, exactly as previously described (Roy, 1956*a*).

Control determinations, in which the enzyme was incubated separately from the remainder of the reaction mixture and was added only immediately before the ethanol, were always carried out. These control values were identical with those obtained when the buffered enzyme was incubated separately from the unbuffered substrate. 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, 1956*a*), this method cannot be used for accurate kinetic studies as the difference usually found between these two values is 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

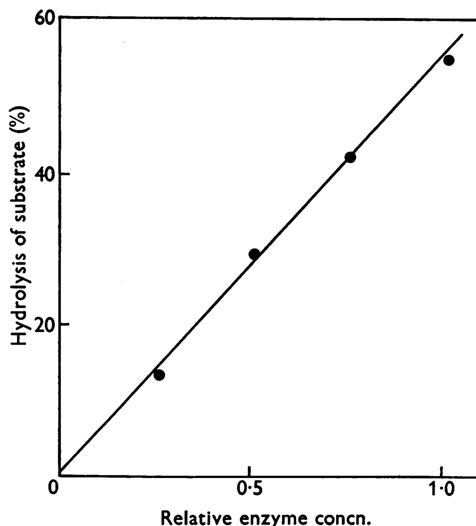

Fig. 1. Effect of enzyme concentration on reaction velocity. Volume of reaction mixture: 0·8 ml., containing 3 mg. of enzyme preparation. Substrate concentration: 0·2 mM-DHAS at pH 7·8 in 0·13M-tris buffer. Incubated for 1 hr. at 37°.

* Part 5: Roy (1956*c*).

below. The degree of hydrolysis of the substrate on incubation with the enzyme was directly related to the enzyme concentration (Fig. 1).

Cell fractionation and assay of arylsulphatases

Cell fractionation was carried out in 0·25M-sucrose by the standard method of Hogeboom (1955), a glass homogenizer fitted with a nylon pestle being used. The arylsulphatase activities of the fractions were determined as previously described, 0·03M-nitrocatechol sulphate at pH 6·0 being used for the assay of sulphatases A + B (Roy, 1954*b*) and 0·004M-*p*-nitrophenyl sulphate at pH 7·8 for sulphatase C (Roy, 1956*c*). With both the time of incubation was 15 min. Steroid sulphatase was assayed as described 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 activity 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. The optimum pH in 0·13 M-tris buffers was in the region of 7·7–7·9 (Fig. 3).

The high specificity of the steroid sulphatase of *Patella vulgata* (Roy, 1956*a*) 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 with the mammalian enzyme.

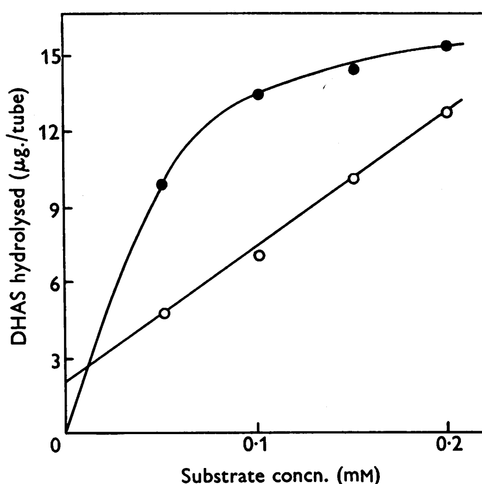

Fig. 2. Effect of substrate concentration (*s*) on reaction velocity (*v*). Volume of reaction mixture: 0·8 ml., containing various concentrations of DHAS at pH 7·8 in 0·13M-tris buffers. Incubated for 1 hr. at 37°. ●, Plot of *v* against *s*; ○, plot of 10^6 (*s*/*v*) against *s*.

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 optimum for all the possible substrates. The results show that mammalian steroid sulphatase has a high substrate specificity, exactly comparable with that of the steroid sulphatase of *P. vulgata*, as only the 3β-sulphates of the 5α- or Δ^5-series of steroids were hydrolysed at a significant

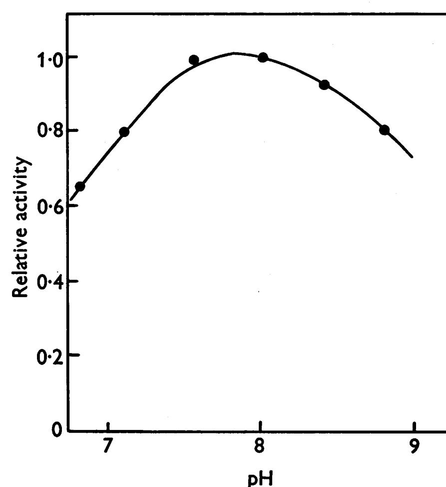

Fig. 3. Effect of varying pH on the reaction velocity. Conditions were as described for Fig. 2 but the substrate concentration was held at 0·2 mM-DHAS and the pH of the buffers varied.

Table 1. *Specifiicity 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 0·13M-tris buffers. Incubation was for the time indicated at 37°. The substrates were the potassium salts of the sulphates of the steroids listed below. With 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-en-17-one	31	79
17α-Hydroxyandrost-4-en-3-one	—	0
17β-Hydroxyandrost-4-en-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-en-20-one	28	76
20α-Hydroxypregnane	—	0
20β-Hydroxypregnane	—	0
Cortisone	—	0

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 from five to fifteen times greater than that for DHAS: this variation in the ratio of the rates of hydrolysis of the two substrates suggested that two separate enzymes 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, 1956*c*), 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.

Table 2. *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.

		Relative activity	
	Concn. (M)	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

Table 3. *Relative activities of sulphate enzymes in rat liver*

Enzymic activities are expressed as μmoles of sulphate ester hydrolysed or synthesized/hr./g. wet wt. of liver under the conditions specified in the text.

	Sulphatases			
Sex	A+B	C	Steroid sulphatase	DHAS synthesis
Female	170	16	0·17	0·17
Male	160	43	0·45	0·07

Rat-liver sulphatases

Some experiments were carried out with rat liver as the enzyme source. This tissue was chosen for cell-fractionation studies as, unlike ox liver, it 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 localized 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, and of the DHAS-synthesizing system (Roy, 1956*b*), present in rat liver are shown in Table 3. 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 methods at present available (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.

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 support for the suggestion (Roy, 1956*a*) 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 value of their optimum pH. With the molluscan enzyme the optimum is pH 4·5, whereas with the mammalian enzyme the optimum is pH 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 enzymes occur 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 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 synthesize 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 been previously noted by Dodgson, Spencer & Thomas (1953). It is of interest to compare these figures for the sulphatase activity of rat liver with the ability to produce SO_4^{2-} ion from cysteine, via cysteinesulphinic acid. According to Awapara (1955) rat liver can form approximately 3 μM-SO_4^{2-} ion/g./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-} ion by sulphatase activity could be much greater than its rate of production from cysteine. No information is available on the actual activities *in vivo*.

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 with the steroid sulphatase of *P. 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 localized 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 optimum substrate concentration is 0·2 mM-dehydro*epi*androsterone sulphate and the optimum pH is 7·8 in 2-amino-2-hydroxymethylpropane-1:3-diol buffers.

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 arylsulphatase present. The enzyme is present in amounts comparable with those of the system synthesizing steroid sulphate.

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Histones from Normal and Malignant Cells

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The functions of the histones in the cell nucleus have been the subject of considerable speculation, prompted by the reports of the tissue specificity of the histones (Stedman & Stedman, 1950*a*). Although a difference between the basic protein in the sperm and somatic tissues of the salmon and fowl has been amply demonstrated (Stedman & Stedman, 1950*a*; Daly, Mirsky & Ris, 1950), differences between various somatic tissues have not been so well defined. Cruft, Mauritzen & Stedman (1954) reported that the histones from tumours had a lower isoelectric point than those

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