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The Sulphatase of Ox Liver

7. THE INTRACELLULAR DISTRIBUTION OF SULPHATASES A AND B*

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Previous papers in this series (Roy, 1953a, b, 1954a, 1956) have shown that ox liver contains three arylsulphatases, sulphatases A, B and C, which differ both in their general properties and in their intracellular localizations. Sulphatase C apparently occurs only in the microsomes (Roy, 1956, 1958), whereas sulphatases A and B appear in several cell fractions. It was originally suggested that they occurred predominantly in the mitochondria and microsomes of rat liver (Roy, 1954b), but Viala & Gianetto (1955) showed that their distribution was consistent with their arising from the lysosomes, a conclusion that was supported by subsequent work in other species (Roy, 1958). However, these investigations all suffered from the disadvantage that sulphatases A and B were determined as one unit, the two individual enzymes not being separately determined. It is obvious that such studies could give misleading results and that important differences in the localizations of the two enzymes might not be detected. Unfortunately, the separate determination of sulphatases A and B in a mixture is a matter of some difficulty, especially when the situation is further complicated by the simultaneous presence of sulphatase C.

* Part 6: Roy (1957).

This paper describes the results of an investigation into the intracellular distribution of sulphatases A and B in ox liver. This species was chosen for study as in it the differences between these two enzymes are greater than those between the corresponding enzymes of the rat (Dodgson, Spencer & Thomas, 1955) and, further, they can readily be separated by their different solubilities in water, sulphatase A being insoluble (Roy, 1953b), whereas sulphatase B is soluble (Roy, 1954a).

A preliminary account of this work was presented at the Canberra meeting of the Australian Biochemical Society in January 1960.

EXPERIMENTAL

Cell-fractionation and general techniques. The samples of ox liver were obtained at the slaughter house within a few minutes of the death of the animal and were immediately placed in 0.25 M-sucrose at 0° for transport to the laboratory. Further treatment was commenced within 30 min. of death.

The mitochondria and microsomes were isolated from a 10% (w/v) dispersion of ox liver in 0.25 M-sucrose by the standard technique of Hogeboom (1955), as described by Roy (1958) except that the mitochondria were washed only once. The particles were extracted by suspending them in the appropriate solvent at 0° (1 ml./g. of liver) and then

centrifuging for 1 hr. at 50 000 g. Dialyses were carried out in Visking cellophan tubing against repeated changes of 100 vol. of water at 0° for the times specified below.

Enzyme assays. The general technique was a simplification of that originally described (Roy, 1953a) with dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) as the substrate. The reaction mixture had a volume of 0.8 ml. and contained 0.2 ml. of enzyme preparation. The exact composition of the various mixtures is described below. After incubation for 1 hr. at 37° proteins were precipitated by the addition of 3 ml. of 0.1M-trichloroacetic acid and removed by centrifuging. A portion (3 ml.) of the supernatant solution was pipetted into 5 ml. of 2.5N-NaOH and the extinction of the resulting solution was determined in the Unicam SP. 600 spectrophotometer at 515 m μ . The amounts of 4-nitrocatechol present were determined from suitable calibration curves.

Trichloroacetic acid was used as protein precipitant instead of the phosphotungstic acid of the original method in order to avoid the formation of the troublesome precipitate which appeared with the latter in high concentrations of electrolytes (Roy, 1954a). Similarly, 2.5N-NaOH was used in place of alkaline quinol (Roy, 1953a) as the colour of the 4-nitrocatechol ion was stable at the protein concentrations used in the present series of determinations.

The general conditions of the assays were as follows. In preliminary studies of the cell fractionation and the subsequent treatment of the fractions: 15 mM-NCS in 0.125M-acetic acid-sodium acetate buffer (acetate buffer), pH 6.0; for the assay of sulphatase A: 3 mM-NCS in 0.125M-acetate buffer, pH 5.0 (Roy, 1953b); for the assay of sulphatase B: 0.06M-NCS in 0.125M-acetate buffer, pH 5.9 (Roy, 1954a, 1955), or 15 mM-NCS in 0.5M-acetate buffer, pH 5.6. Any adjustments of pH which were required were made with 2N-acetic acid or 2N-NaOH.

A few determinations were also carried out with potassium *p*-nitrophenyl sulphate (NPS) as substrate. The general procedure was similar to that described above except that 3 ml. of the deproteinized reaction mixture was pipetted into 5 ml. of M-Na₂CO₃ for colour development and the extinction of the resulting solution was determined at 400 m μ . The detailed conditions used in these assays are described in the following section.

Protein determinations. These were carried out by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine-serum albumin as standard.

RESULTS

Preliminary separation of sulphatases A and B. The mitochondria or microsomes were suspended in water to give a concentration corresponding approximately to 1 g. of liver/ml. and kept at 0° for from 4 to 18 hr. Such variations in time were without effect on the subsequent behaviour of the enzymes. The response of the arylsulphatase activity in such suspensions to changes in substrate concentration is shown in Fig. 1. The suspensions were then centrifuged for 1 hr. at 50 000 g: the supernatants were decanted and retained (the aqueous extracts) and the residues were resuspended in water and made up to the original volume (the insoluble residues). The influence of changes in substrate concentration on the arylsulphatase activities of these fractions is also shown in Fig. 1. It is obvious that the aqueous extracts and the insoluble residues, both from the mitochondria and the microsomes, differ

Fig. 1. Effect of changes in substrate concentration on the arylsulphatase activity of ox-liver mitochondria, microsomes and fractions thereof. Volume of reaction mixture, 0.8 ml., containing enzyme corresponding to approx. 150 mg. of liver; pH 6.0 in 0.125M-acetate buffer. Incubated for 1 hr. at 37°. ●, Mitochondria or microsomes; ○, aqueous extracts; +, insoluble residues from the mitochondria or microsomes prepared as described in the text.

considerably in their properties and that whereas the former behave as preparations of sulphatase A containing small amounts of sulphatase B the latter behave as typical preparations of sulphatase B.

The efficiency of the extraction of the sulphatase activity was examined only with the mitochondria, as not only did the presence of sulphatase C in the microsomes complicate the situation therein but these particles did not sediment readily after extraction with water so that it was impossible to separate quantitatively the soluble material from the sediment. The results of a typical experiment are shown in Fig. 2. The major part of the soluble-sulphatase activity is removed by one extraction with water and the remainder almost all appears in the second extract as the third contains only traces of sulphatase activity.

Sulphatases of the aqueous extract. When the aqueous extracts from either the mitochondria or the microsomes were dialysed for 2-3 days a copious flocculent precipitate appeared. This contained about 90 % of the sulphatase activity of the

extract. The remaining 10 % of the sulphatase was not precipitated even on prolonged dialysis. The precipitate was centrifuged off, washed with water and suspended in half the original volume of 0.5M-sodium acetate, pH 6. After standing overnight the remaining insoluble material was centrifuged off and the supernatant dialysed for 4 hr. to decrease the concentration of acetate. The resulting solution contained between 60 and 80 % of the original soluble-sulphatase activity: most of the remainder was present in the residue from the sodium acetate extraction, and although it could only be extracted therefrom with difficulty its properties agreed in every other respect with those of the enzyme soluble in sodium acetate.

The effect of changes in substrate concentration on the arylsulphatase activities of such soluble-enzyme preparations from the mitochondria and the microsomes is shown in Fig. 3. It is obvious that the preparations from these two sources are identical with one another and with a preparation of sulphatase A obtained by the methods described by Roy (1953*b*). In all other respects studied such enzyme preparations from mitochondria or microsomes behaved as typical preparations of sulphatase A: the values of K_m , the shapes of the pH-activity curves and the anomalous effects of changes in enzyme concentration were all indistinguishable from those of purified preparations of sulphatase A (Roy, 1953*b*). Determinations of the specific activities of the preparations showed them to be of a purity comparable with that of sulphatase A-1 (Roy, 1953*b*).

The small amounts of arylsulphatase which were not precipitated by dialysis against water were quite different from the above-described prepara-

Fig. 2. Extraction of arylsulphatases from ox-liver mitochondria by successive treatments with water (1-3) and 0.15M-KCl (4-6). General conditions of assay were as given in Fig. 1 except that the substrate concentration was fixed at 15 mM-NCS. Open areas represent the arylsulphatase activity which had been extracted by the solvent in question; filled areas indicate the arylsulphatase activity remaining in the residue.

Fig. 3. Effect of changes in substrate concentration on the activity of sulphatase A prepared from liver mitochondria (●) or microsomes (○). Conditions were as given in Fig. 1 except that the pH was 5.0 in 0.125M-acetate buffer. The line is the appropriate curve for an authentic preparation of sulphatase A (replotted from Roy, 1953*b*).

tions and corresponded to a sulphatase B, indistinguishable from the enzymes described below. The presence of a sulphatase B in the aqueous extracts was confirmed by paper electrophoresis as previously described (Roy, 1954a).

Sulphatase of the insoluble residue. The data shown in Fig. 1 indicated that the insoluble residues of both the mitochondria and the microsomes must contain a sulphatase B, presumably along with a sulphatase C in the latter. Soluble preparations of sulphatase B were obtained from the insoluble residues by a number of techniques.

When the suspension, buffered by the addition of 0.1 vol. of 0.2M-sodium citrate buffer, pH 6.2, was stirred vigorously at room temperature for 30 min. with 1 vol. of butan-1-ol and the resulting emulsion was broken by centrifuging, a considerable proportion of the sulphatase activity was found in the aqueous phase. This solution, the butanol-solubilized enzyme, contained (after dialysis to remove any butanol) about 50 and 25 % of the sulphatase activity of the insoluble residues from the mitochondria and the microsomes respectively.

When the suspensions of the insoluble residues were taken to pH 4.2-4.5 with 2N-acetic acid, incubated at 37° for 15 min. and then centrifuged, the solutions so obtained, the acetate-soluble enzymes, contained sulphatase activity in amounts similar to those in the corresponding butanol-solubilized enzymes.

Finally, when the insoluble residues were suspended in 0.15M-KCl in place of water and then centrifuged, the resulting extract, the KCl-soluble enzyme, contained considerable amounts of sulphatase activity, about 70 and 50 % from the mitochondrial and microsomal residues respectively. The efficiency of the extraction by KCl is shown in Fig. 2: again it is obvious that the major part of the sulphatase is removed in the first and second extractions, the third containing only traces of activity. Further small amounts of sulphatase could be obtained from the residue remaining after the KCl extraction by treatment with butanol as described above but it was not possible to obtain all the sulphatase activity from the insoluble residues in a soluble form.

In Fig. 4 is shown the effect of changes in substrate concentration on the arylsulphatase activity of these three types of preparation (butanol-solubilized, acetate-soluble and KCl-soluble) and on the sulphatase B activity of the aqueous extracts of the mitochondria and microsomes and of the residue from the KCl extraction of the mitochondria. These assays were carried out in 0.5M-acetate buffer, not in 0.125M-acetate buffer as previously (Roy, 1954a) because it is now known that the behaviour of impure preparations of sulphatase B is greatly influenced by the concentra-

tion of the acetate buffer used (Dodgson & Wynn, 1958): in particular many of the irregularities in the behaviour of relatively impure preparations disappear in more concentrated buffers. It is obvious from the results shown in Fig. 4 that these preparations of sulphatase B are identical with one another and with a purified preparation of sulphatase B. The pH-activity curves, determined in 0.5M-acetate buffers at a substrate concentration of 15 mM-NCS, were similarly indistinguishable from one another. The specific activities of the various

Fig. 4. Effect of changes in substrate concentration on the sulphatase B activity of various preparations obtained as described in the text. The points on the graph indicate the mean values and the vertical lines show the ranges of the activities where these are greater than the diameter of the points. The line is the appropriate curve for an authentic preparation of sulphatase B-2 (Roy, 1954a). General conditions of assay were as given in Fig. 1 except that the pH was 5.6 in 0.5M-acetate buffer.

Fig. 5. Effect of varying concentrations of NaCl on the hydrolysis of NPS by sulphatase B. Volume of reaction mixture, 0.8 ml.; substrate concentration 30 mM-NPS in 0.5M-acetate buffer, pH 5.9. Incubated for 1 hr. at 37°. ●, Butanol-solubilized enzyme; ○, data of Webb & Morrow (1959) replotted to allow comparison.

preparations showed them all to be rather purer than sulphatase B-2 (Roy, 1954a).

Webb & Morrow (1959, 1960) have recently shown that, contrary to previous views, sulphatase B will hydrolyse NPS when the reaction mixture contains Cl^- ions. This is also true for preparations of the butanol-solubilized enzyme. Fig. 5 shows the dependence of the hydrolysis of NPS upon the presence of Cl^- ions and includes comparable data replotted from Webb & Morrow (1959). It is obvious that the responses of the two preparations are identical.

Quantitative distribution of sulphatases A and B. The mitochondrial and microsomal fractions were prepared as usual and each was successively extracted twice with water and twice with 0.15M-KCl. These extracts were dialysed for 3 days, the resulting precipitates were removed by centrifuging and then were resuspended in water. The suspensions so obtained and the supernatants from the centrifugings were assayed for sulphatases A and B respectively. The residues remaining after the extractions of the mitochondria and microsomes were suspended in water, dialysed and assayed for sulphatase B.

The results of a typical experiment are shown in Table 1. This again shows that most of the sulphatase A is removed by the first treatment with water, both from the mitochondria and the microsomes, although the extraction is apparently less complete from the latter, probably because they pack only extremely loosely on centrifuging. About 20% of the sulphatase B was also found in the first aqueous extract but virtually none in the second: this would suggest that this fraction of the sulphatase B originated from cell particles that had been damaged during their isolation. The major part of the remainder of the sulphatase B appeared in the first KCl extract.

With the microsomes a considerable proportion of the sulphatase activity remained in the insoluble residue. Some of this must be due not to sulphatase B but to the sulphatase C which is localized in the microsomes. It is difficult to assess accurately

the contribution due to the latter but from sulphatase determinations made in the presence of 0.01M- KH_2PO_4 , which is a powerful inhibitor of sulphatase B (Roy, 1954a) but not of sulphatase C (Roy, 1956), it seems probable that from 60 to 70% of the total insoluble sulphatase activity in microsomal residue is due to sulphatase C.

An exact value for the ratio of the activity of sulphatase A to sulphatase B in the two cell fractions is difficult to obtain because of this doubt about the true value of the sulphatase B activity in the microsomal residue. If, however, it be taken that 65% of the apparent sulphatase B activity is in fact due to sulphatase C then the ratio of A to B in the mitochondria is 0.30, whereas that in the microsomes is 0.26. The ratios are therefore very similar, if not identical, in the two fractions. These values for this ratio should also be compared with that of 0.26 obtained for the activities of the two enzymes purified to stages A-1 (Roy, 1953b) and B-1 (Roy, 1954a) respectively from a sample of acetone-dried liver prepared from the same animal as used in the above-described cell-fractionation studies. The use of the same animal for both types of experiment was essential as there are considerable variations of the ratio of sulphatase A to sulphatase B in different animals.

DISCUSSION

First, it must be pointed out that although the cytological significance of the mitochondrial and microsomal fractions isolated from ox liver was not investigated and the two fractions were defined purely on the basis of their sedimentation rates there is no reason to believe that they are of a different nature from the corresponding fractions of rat liver.

The results described above conclusively show that enzymes having the properties of sulphatases A and B can be obtained by identical procedures from both the mitochondrial and microsomal fractions prepared by standard procedures from a dispersion of ox liver in 0.25M-sucrose. Further,

Table 1. *Extraction of sulphatases A and B from ox-liver mitochondria*

Details of the procedure are given in the text. Sulphatase A activity is expressed as $\mu\text{g.}\frac{1}{2}$ of nitrocatechol liberated/hr. from 3 mM-NCS at pH 5.0 in 0.125M-acetate buffer (Roy, 1953b), and sulphatase B activity as $\mu\text{g.}$ of nitrocatechol liberated/hr. from 15 mM-NCS in 0.5M-acetate buffer, pH 5.6.

	Mitochondria		Microsomes	
	A	B	A	B
First aqueous extract	3980	2940	1140	1610
Second aqueous extract	135	120	427	0
First KCl extract	87	8250	57	2950
Second KCl extract	33	625	64	487
Residue from the extractions	—	2390	—	4500*

* This figure is falsely high because of the presence of sulphatase C in the microsomes.

the ratios of sulphatase A to sulphatase B are similar in the mitochondria and the microsomes. These observations confirm the view that sulphatases A and B occur in the lysosomes, or at least in some particle which distributes itself between the mitochondrial and microsomal fractions as these are normally obtained. An alternative explanation would be that the two enzymes were in fact present in the mitochondria and microsomes themselves but, although small amounts could occur therein and escape detection by the methods used above, it would seem that this possibility can be discounted as it is unlikely that the two enzymes would occur in the same proportions in the two cell fractions and that they could separately be prepared therefrom by identical and mild techniques: the very different structures of the mitochondria and microsomes would make this latter suggestion extremely unlikely. It seems justifiable to conclude therefore that sulphatases A and B are present in some cell fraction which contaminates the mitochondrial and microsomal fractions, as do the lysosomes. Although other similar particles may exist, the strong similarities between the behaviour of the sulphatases and the behaviour of the typical lysosomal enzyme acid phosphatase (Viala & Gianetto, 1955) make it very likely that the particles involved are indeed lysosomes.

There are, however, important differences in the ease of extraction of sulphatases A and B. Sulphatase A can readily be extracted from the particles by water, in contrast with sulphatase B, which requires treatment with iso-osmotic potassium chloride, with butan-1-ol or with a lowered pH to effect solution. This difference could have several possible explanations. First, both sulphatases A and B might initially be released in a soluble form by treatment with water and only the latter might subsequently be adsorbed by particulate material. Such a situation seems unlikely as it is hardly to be expected that sulphatase B would be virtually quantitatively adsorbed by both the mitochondria and the microsomes, having regard to their very different structures. One of two other possibilities seems more probable: either the two enzymes are localized differently within the lysosomes or the lysosomes are heterogeneous, some containing sulphatase A, some sulphatase B. If the first possibility be correct then it is likely that sulphatases A and B respectively occur in the sap and insoluble framework (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) of the lysosomes; if the second be correct then sulphatase A must probably occur in the sap of the lysosomes, as originally defined, and sulphatase B occur in some related but structurally different particle. It is impossible to distinguish between these possibilities at present, but evidence pointing to a

heterogeneity in the lysosomes is accumulating. Beaufay, Bendall, Baudhuin, Wattiaux & de Duve (1959) have suggested that there may be as many different types of lysosome as there are lysosomal enzymes and, further, that uricase may be associated either with another type of particle or with the insoluble framework of the lysosome. Again, Conchie & Levvy (1960) have concluded that the results of their investigation of the distribution of glycosidases in mammalian liver can best be explained on the basis of the occurrence of a number of different types of lysosome-like particle. It is obvious that the final solution to these problems will be possible only with the development of even more refined techniques of fractional centrifuging than those used by Beaufay *et al.* (1959).

It should be noted that the availability of methods for the preparation of ox-liver mitochondria on a large scale (Beinert, 1957) makes the above-described techniques suitable for the preparation of sulphatases A and B. The two enzymes are readily obtained in a fair degree of purity and the tedious and expensive preparation of large amounts of acetone-dried liver is avoided. Further, the fact that the relative amounts of sulphatases A and B obtained by the two very different types of procedure are similar gives added weight to the view (Roy, 1954*a*) that both enzymes are true components of liver tissue and not artifacts.

SUMMARY

1. It has been shown that sulphatases A and B occur in the same proportions in both the mitochondrial and microsomal fractions of ox liver.

2. Sulphatase A can be obtained from both fractions by extraction with water. Such extracts also contain traces of sulphatase B, which may originate from cell particles damaged during the isolation procedure.

3. Sulphatase B cannot be extracted from the particles by water but can be obtained in solution by extraction with 0.15M-potassium chloride, by treatment with butan-1-ol or exposure to a lowered pH.

4. The properties of sulphatases A and B prepared as described above are identical with those of the corresponding enzymes obtained by fractional precipitation with acetone and ammonium sulphate.

5. It is concluded that both sulphatases A and B occur in the lysosomes but are either differently localized within these particles, sulphatase A occurring in the sap and sulphatase B in the insoluble framework, or the lysosomes themselves are heterogeneous, some containing sulphatase A, some sulphatase B.

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The Influence of the Induction of Alloxan-Diabetes on the Incorporation of Amino Acids into Protein of Rat Diaphragm

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Insulin *in vitro* stimulates the incorporation of labelled amino acids into protein of isolated diaphragm from the normal rat (Sinex, MacMullen & Hastings, 1952; Krahle, 1953; Manchester & Young, 1958; Wool & Krahle, 1959) and from the hypophysectomized or adrenalectomized rat (Kostyo, 1959; Manchester, Randle & Young, 1959). On the basis of these and other observations, it is to be expected that the rate of incorporation of labelled amino acids into protein of isolated diaphragm from the alloxan-diabetic rat would be less than that of diaphragm from the normal rat, and that treatment of the alloxan-diabetic rat with insulin would restore the rate of incorporation to normal. This possibility, in both the newly diabetic and the 'long-term' diabetic animal, has now been investigated. The ability of plasma from rats, in various stages of alloxan-diabetes, to stimulate the uptake of glucose and incorporation of amino acid into protein by diaphragm from normal rats has also been studied.

MATERIALS AND METHODS

Animals. Albino Wistar female rats of about 130 g. wt. were used throughout. They were fed *ad libitum* a standard laboratory diet (Bruce & Parkes, 1949) and had free access to water at all times.

Alloxan-diabetes. Diaphragm was taken from rats at two widely different periods after they had been treated with

alloxan. What are termed 'short-term' alloxan-diabetic rats had received alloxan 2 days (48 hr.) before removal of the diaphragm; 'long-term' alloxan-diabetic rats had received alloxan at least 2 weeks previously. In both instances the rats were starved 24 hr. before a subcutaneous injection of alloxan was given [alloxan monohydrate, British Drug Houses Ltd., 200 mg./kg. in 1% (w/v) NaCl]. Under these conditions an intense hyperglycaemia developed, and 48 hr. after the administration of alloxan the blood sugar usually exceeded 500 mg./100 ml. even though food had been withdrawn 24 hr. previously.

No deaths usually occurred within 48 hr. of the administration of alloxan, but unless the rats were given insulin daily for at least a week after the injection of alloxan, many deaths took place within 2-7 days of this treatment. The following procedure was therefore adopted for the preparation of 'long-term' diabetic animals: each animal received 2 units of protamine-zinc (PZ) insulin (Burroughs Wellcome and Co. Ltd.) subcutaneously on the evening of days 2-7 after treatment with alloxan. On days 8-10 the daily dose of insulin was decreased to 1 unit and on days 11-12 to 0.5 unit. During the period of days 2-10 inclusive, drinking water was replaced by 1% (w/v) glucose solution in order to prevent hypoglycaemia. After day 12, treatment with insulin was discontinued unless the animal showed signs of impending diabetic coma, when soluble insulin (1 unit/day) was administered for a day or two. This procedure made possible the survival, 14 days after the administration of alloxan, of about 60% of the number treated. All these animals when deprived of insulin for 2 or more days had non-fasting blood-sugar levels above 500 mg./100 ml., but after the withdrawal of food the blood sugar dropped to about 215 mg./100 ml. (range 100-500 mg./100 ml.). At