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THE TYPE II ARYLSULPHATASES OF THE RED KANGAROO

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SUMMARY

The separation of two type II arylsulphatases (aryl-sulphate sulphohydrolases, EC 3.1.6.1) from the liver of the red kangaroo, *Megaleia rufa*, is described. The two enzymes have many properties in common with sulphatases A and B from ox liver and have been similarly named. Neither of the enzymes has been obtained in a homogeneous form but the sulphatase A cannot be grossly impure. It has a molecular weight of 100 000, an isoelectric point of 5.1–5.4, and does not polymerise as does ox sulphatase A. Its kinetic properties are analogous to those of the ox enzyme.

The sulphatase B of kangaroo liver has a molecular weight of 45 000 and an isoelectric point of 7. Kinetically it also resembles the corresponding ox enzyme.

No evidence has been obtained for the existence of a sulphatase C in kangaroo liver and a steroid sulphatase, if it be present, occurs in only very small amounts.

The results suggest that sulphatases A and B had developed quite early in the evolution of the mammals.

INTRODUCTION

The livers of most eutherian mammals contain several arylsulphatases (aryl-sulphate sulphohydrolases, EC 3.1.6.1) (refs. 1, 2). Type I arylsulphatases, sulphatase C, occur in the microsomes and are characterised by their insolubility and their insensitivity to inhibition by sulphate or phosphate. The soluble type II arylsulphatases occur in the lysosomes and, in contrast, are powerfully inhibited by sulphate and phosphate. Two enzymes of the latter type are found in the higher mammals: one, sulphatase A, is characterised by its large negative charge at pH 7.4 (ref. 3) and by its complex kinetic behaviour[4–6]; the other, sulphatase B, is characterised by being slightly positively charged at pH 7.4 (ref. 7) and by hydrolysing 4-nitrophenyl sulphate at a significant rate only in the presence of Cl^- (ref. 8). Although the arylsulphatases of mammalian tissues have been much studied only the sulphatase A of ox liver has been obtained as a homogeneous protein and characterised physically[3,9,10]. The sulphatase B isoenzymes of ox liver have been highly purified but the homogeneity of the final preparations has not been demonstrated[7]. The arylsulphatases of ox brain have also been highly purified and shown to have properties quite similar to those of the liver enzymes[11–13].

It has been suggested[14] that the livers of marsupials and of lower vertebrates contain only a single type II arylsulphatase with properties intermediate between those of sulphatases A and B of the ox. This view was based on the method of preparation of the enzyme, its behaviour on paper electrophoresis and its kinetic properties: if it were correct then further studies of the enzyme might throw light on the evolution of the arylsulphatases in the higher mammals. For this reason an investigation of the type II arylsulphatase activity of the liver of the red kangaroo, *Megaleia rufa*, was undertaken. This species was chosen because reasonable amounts of tissue were available.

EXPERIMENTAL

Kangaroo liver

This was obtained from animals (of both sexes and various ages) in the colony of red kangaroos maintained by the Commonwealth Scientific and Industrial Research Organization Division of Wildlife Research, Canberra. The livers were stored at −30° until the required amount of tissue had been accumulated (up to one year). Unpublished experiments have shown that ox and human liver can be stored under such conditions for at least this time with no change in the activity, or properties, of the arylsulphatases.

Protein determination

This was routinely carried out by the method of LOWRY *et al.*[15] using bovine serum albumin as standard. The concentration of the most purified preparation of sulphatase A was also determined refractometrically[16], assuming a value of 0.180 $ml \cdot g^{-1}$ for the specific refractive increment: the two figures agreed within 10%.

Analytical ultracentrifugation

This was carried out as previously described[9,10] in a Spinco Model E ultracentrifuge using 30-mm cells in an An-E rotor. The values of s and $\bar{s}$ were calculated as before[10] and the boundaries were analysed by the method of BALDWIN[17].

Isoelectric focussing

This was carried out as previously described[7].

Preparation and assay of the microsomal fraction

A crude preparation of the microsomes from kangaroo liver was obtained exactly as described for ox liver[18]. Attempts to detect sulphatase C and steroid sulphatase in this preparation were made by previously described methods using 4-nitrophenyl sulphate[18] and androstenolone sulphate[19], respectively, as substrates.

Assay of the type II arylsulphatases

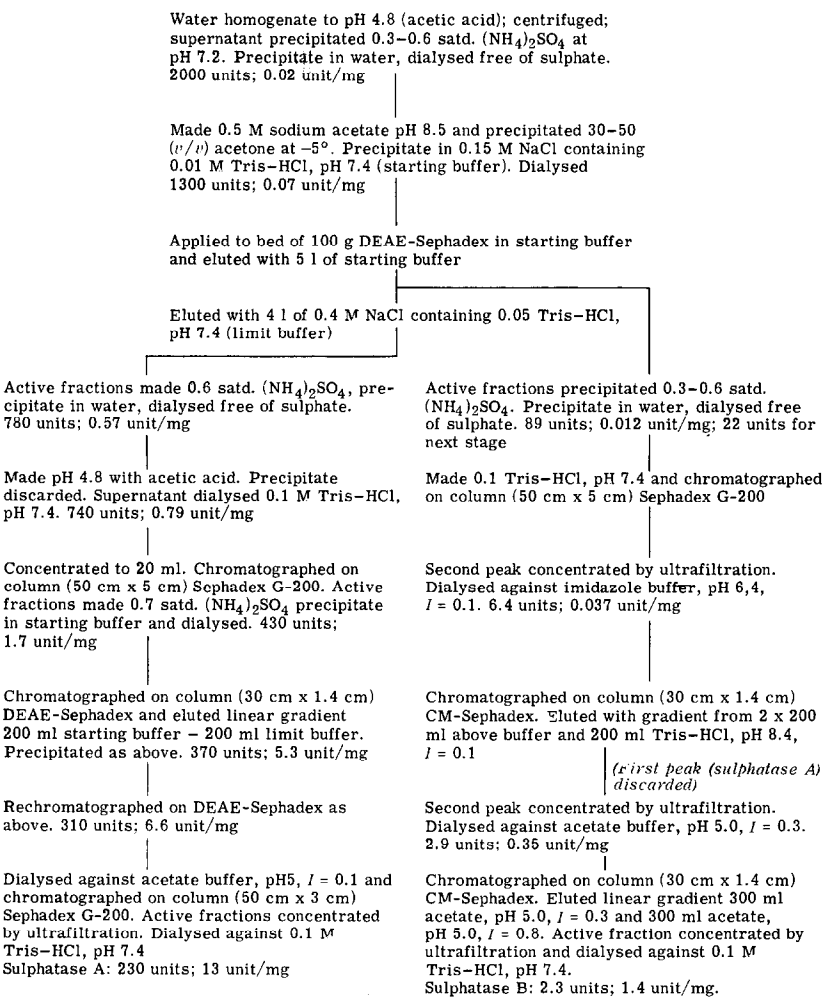
To 0.8 ml of 0.01 M nitrocatechol sulphate (2-hydroxy-5-nitrophenyl sulphate) in 0.5 M sodium acetate–acetic acid buffer (pH 5.0) were added 20 μl of enzyme solution. After incubating at 37° for 5 min the reaction was stopped by the addition of 5 ml of 0.2 M NaOH and the amount of liberated 4-nitrocatechol ($\varepsilon_{510\ nm}$, 12 600) determined spectrophotometrically. These conditions were not optimum for either of

the sulphatases of kangaroo liver but were adopted as a compromise to follow their purification.

For the assay of the purified enzymes and for kinetic studies the same general technique was adopted. In some cases the reaction mixture was buffered with 0.5 M imidazole–acetic acid buffer. When 4-nitrophenyl sulphate was used as substrate a value of 17 000 was taken for $\varepsilon_{420\ nm}$ of 4-nitrophenol in alkali.

Estimates of K_m and V were obtained by the method of WILKINSON[20] and are given $\pm$ their standard errors. Initial rates, based on the amount of hydrolysis occuring in 5 min, were used in these calculations. The nomenclature of CLELAND[21] is used in the discussion of the reaction kinetics.

One unit of arylsulphatase is defined as the amount of enzyme which will, under optimum conditions (see Table III), hydrolyse 1 μmole of nitrocatechol sulphate per min.



Scheme 1. Preparation of sulphatases from 5 kg kangaroo liver.

TABLE I

THE YIELDS AND SPECIFIC ACTIVITIES OF THE TYPE II ARYLSULPHATASES FROM OX AND KANGAROO LIVER

	Sulphatase A		*Sulphatase B*	
	Ox	*Kangaroo*	*Ox*	*Kangaroo*
Yield (units/kg liver)	140	97	25	0.4
Specific activity (units/mg protein)	140	27	130	1.4

Preparation of the type II arylsulphatases

The techniques used in the preparation of the enzymes have been described in detail in previous publications dealing with sulphatases A (ref. 9) and B (ref. 7) of ox liver and only an outline of the purification procedure is given in the following scheme. Although the purification of sulphatase A can be regarded as satisfactory that of sulphatase B could certainly be improved but this has not been possible in the present instance because of the scarcity of material.

RESULTS

The application of the above purification procedures clearly showed that the liver of the red kangaroo contained two soluble type II arylsulphatases which had properties comparable to those of the sulphatases A and B of ox liver and which were therefore named similarly. No isoenzymes of sulphatase B were detected. The yields and specific activities (determined under the optimum conditions described below) of these enzymes are compared with those of the corresponding enzymes from ox liver in Table I.

Sulphatase A

Physical properties

The final preparation of sulphatase A was eluted from Sephadex G-200 as a single symmetrical peak of enzyme activity which did not coincide exactly with the protein peak. The preparation also gave a single boundary during sedimentation in the ultracentrifuge: values for the sedimentation coefficients are given in Table II. At pH 7.5 the sedimenting boundary was apparently symmetrical but analysis[19] showed that the preparation was not homogeneous with respect to sedimentation coefficient.

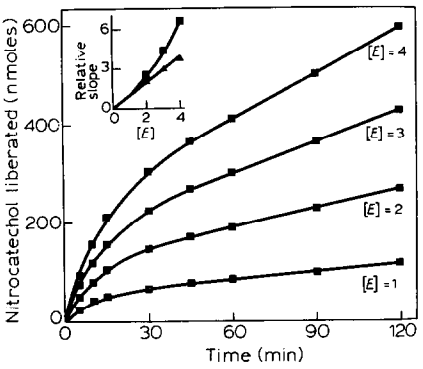
The molecular weight of the enzyme was shown to be 100 000 at both pH 5.0 and 7.5 (ionic strength 0.1, temp. 25°) by zone chromatography[22] on Sephadex G-200.

TABLE II

THE SEDIMENTATION COEFFICIENTS OF A PREPARATION OF KANGAROO SULPHATASE A

Determined at 20° in buffers of ionic strength 0.1. $\bar{v}$ assumed to be 0.715.

pH	*Concn. (mg/ml)*	$s_{20,w}(S)$	$\bar{s}_{20,w}(S)$
7.5	0.13	6.2	5.99
5.0	0.10	6.1	5.58



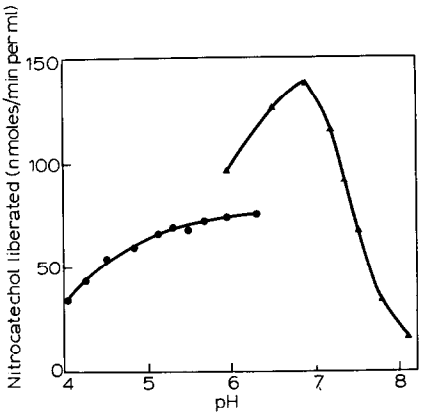


Fig. 1. Progress curves for the hydrolysis of 10 mM nitrocatechol sulphate by different concentrations of sulphatase A at pH 6.9 in 0.5 M imidazole–acetic acid buffers. The relative enzyme concentrations are indicated on the figure. The inset shows a plot of the initial slope (▲, calculated from the hydrolysis during the first 5 min) and the final slope (■, between 60 and 120 min) against the relative enzyme concentration.

Fig. 2. The effect of pH on the hydrolysis of 10 mM nitrocatechol sulphate by sulphatase A. ●, 0.5 M sodium acetate–acetic acid buffers; ▲, 0.5 M imidazole–acetic acid buffers.

Increasing the ionic strength to 2 with NaCl, at pH 5.0, increased the apparent molecular weight by about 25% but this could have been caused by any of several factors other than an actual increase in molecular weight.

Isoelectric focussing gave two incompletely resolved peaks of enzyme activity

TABLE III

SOME KINETIC PARAMETERS FOR THE SULPHATASES A AND B OF KANGAROO LIVER

The values for sulphatase A were determined in 0.5 M imidazole–acetic acid buffers and for sulphatase B in 0.5 M acetic acid–sodium acetate buffers. With the latter enzyme and 4-nitrophenyl sulphate as substrate the reaction mixture contained 1 M NaCl unless otherwise specified. Reaction temp. 37°. The values in all cases refer to initial velocities.

	Sulphatase A	*Sulphatase B*
Substrate: Nitrocatechol sulphate		
Optimum substrate concn. (mM)	10	10
K_m (mM)	2.68 ± 0.30	1.41 ± 0.30
pH optimum	6.9	5.8
Relative activity in: 0.15 M NaCl or KCl	1.05	0.52
0.025 M K_2SO_4	0.74	0.56
0.025 M NaH_2PO_4	0.03	0.02
Substrate: 4-Nitrophenyl sulphate		
Optimum substrate concn.	>Saturated	>Saturated
K_m (mM)	106 ± 23	57.2 ± 5.5
pH optimum	6.5	6.3
Relative activity in: 0.15 M NaCl or KCl	1.01	2.9*
0.025 M K_2SO_4	0.48	0.84
0.025 M NaH_2PO_4	0.01	0.07
$V_{\text{nitrocatechol sulfate}}/V_{\text{4-nitrophenyl sulphate}}$	1.3	3.5
K_i (mM) for K_2SO_4	38**	4; 150***

* Relative to the activity in the absence of Cl^-.

** Competitive inhibition.

*** Non-competitive inhibition: first value for K_i slope, second for K_i intercept.

with isoelectric points of 5.1 and 5.4. It has not been shown that these represent two isoenzymes.

Enzymic properties

Progress curves for the hydrolysis of nitrocatechol sulphate by sulphatase A at pH 6.9 in 0.5 M imidazole buffer are shown in Fig. 1. The curves are clearly not typical of enzyme reactions. The reaction velocity falls steadily during the first 45 min of incubation and then remains constant for at least a further 1.5 h. The latter

TABLE IV

THE EFFECT OF VARYING IMIDAZOLE CONCENTRATIONS ON K_m AND V FOR THE HYDROLYSIS OF NITROCATECHOL SULPHATE BY SULPHATASE A

Ionic strength 1.0 and pH 7.2.

Imidazole concn. (M)	K_m *(mM)*	V *(μmoles/min per ml)*
0.25	8.4 ± 0.47	0.896 ± 0.034
0.50	9.23 ± 0.97	1.01 ± 0.097
0.75	12.0 ± 1.6	1.12 ± 0.10
1.00	14.0 ± 2.8	1.11 ± 0.16

rate is not directly related to the enzyme concentration but the initial rate, calculated from the extent of hydrolysis occurring in the first 5 min, is so related (inset, Fig. 1). The shapes of the progress curves in 0.5 M acetate buffer (pH 5.5) were similar to those in Fig. 1.

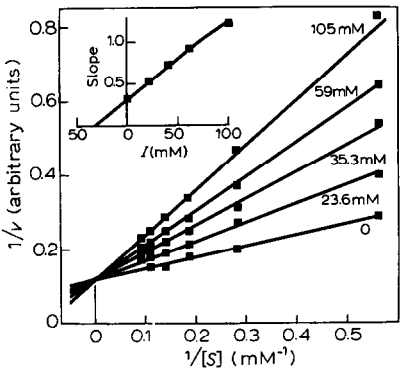
Both nitrocatechol sulphate and 4-nitrophenyl sulphate are hydrolysed by the enzyme and some kinetic parameters are summarised in Table III. Fig. 2 shows the effect of pH on the hydrolysis of nitrocatechol sulphate: the activity in imidazole buffers is considerably greater than that in acetate buffers. The hydrolysis of 4-nitrophenyl sulphate is similarly activated by imidazole. This activation is not due to a general acid-base catalysis of some stage in the enzyme reaction because changes in imidazole concentration, at constant ionic strength, have little effect on V although they do significantly alter K_m (Table IV). The initial velocity of the sulphatase A reaction is considerably depressed by SO_4^{2-} (Table III) and, as shown in Fig. 3, this inhibition is competitive. It is essentially linear competitive although at high concentrations of SO_4^{2-} a more complex type of inhibition may become important as is shown by the slight curvature of the plot in the inset of Fig. 3. The value of K_i is 38 mM SO_4^{2-}.

The behaviour of SO_4^{2-} is, however, complex and under certain conditions these ions can activate the reaction as shown in Fig. 4 which illustrates progress curves in the presence of SO_4^{2-}. An increased velocity during the final linear phase of the reaction is obvious.

Sulphatase B

Physical properties

The molecular weight of the sulphatase B from kangaroo liver is 45 000, as determined by chromatography on Sephadex G-200 (ref. 22). The isoelectric point, determined by isoelectric focussing, is about 7: there was no indication of interactions of the type found with the sulphatase B of ox liver[7].



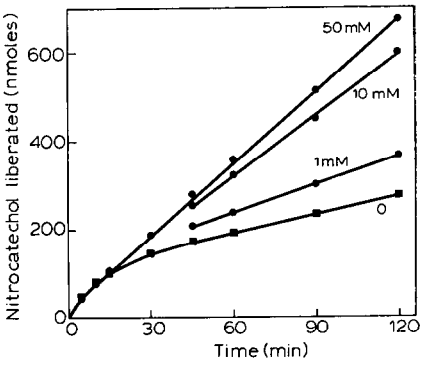


Fig. 3. The inhibition of sulphatase A by K_2SO_4. Substrate, nitrocatechol sulphate; pH 6.9 in imidazole buffer; inhibitor concentrations as shown on figure. The inset shows the replot of the slopes of the lines against inhibitor concentration.

Fig. 4. Progress curves for the hydrolysis of 10 mM nitrocatechol sulphate by sulphatase A in the presence of K_2SO_4 at the concentrations shown on the figure. General conditions as in Fig. 1.

Enzymic properties

A progress curve for the hydrolysis of nitrocatechol sulphate by sulphatase B is shown in Fig. 5: the curve is quite different from those for sulphatase A (Fig. 1) and shows only a slight instability of the enzyme under the conditions of the incubation. The extent of hydrolysis is directly related to the enzyme concentration for reaction times of at least 10 min.

Both nitrocatechol sulphate and 4-nitrophenyl sulphate are hydrolysed by the sulphatase B of kangaroo liver and some kinetic parameters have already been listed in Table III. The hydrolysis of 4-nitrophenyl sulphate by this enzyme, as by the sulphatase B of ox liver[7,8], is strongly activated by Cl^- (Fig. 6). There is no activation by imidazole, either with nitrocatechol sulphate (Fig. 7) or 4-nitrophenyl sulphate as substrate.

The enzyme is inhibited by SO_4^{2-} and, as shown in Fig. 8, the inhibition is non-competitive with the values of K_i (Table III) obtained from replots of the slopes and the intercepts of the usual reciprocal plot being different. Further, the replot of

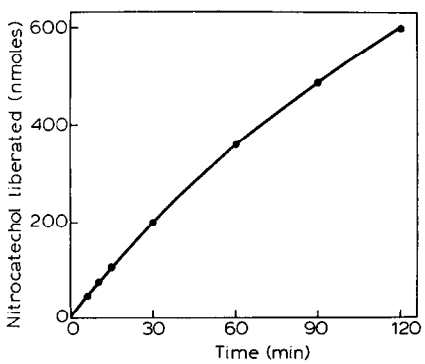
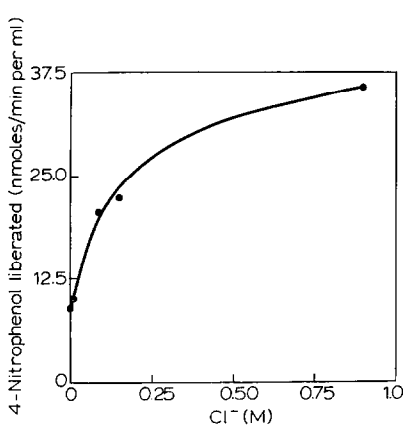


Fig. 5. Progress curve for the hydrolysis of 10 mM nitrocatechol sulphate at pH 5.8 in 0.5 M acetate buffer.



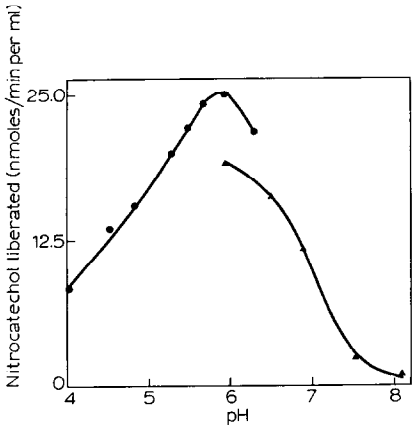


Fig. 6. The effect of Cl^- on the hydrolysis of 4-nitrophenyl sulphate by sulphatase B at pH 6.2 in 0.5 M imidazole buffer.

Fig. 7. The effect of pH on the hydrolysis of 10 mM nitrocatechol sulphate by sulphatase B in 0.5 M acetate buffers (●) and 0.5 M imidazole buffers (▲).

the slope against inhibitor concentration (inset, Fig. 8) gives a straight line only near the origin so that the value for K_i slope may only be an apparent one.

Microsomal sulphatases

No enzyme corresponding to sulphatase C could be detected, under a wide range of conditions, in a crude preparation of microsomes from kangaroo liver. Traces of steroid sulphatase activity, shown by the hydrolysis of androstenolone sulphate, were apparently present but if these were real then the concentration of the enzyme was no more than 1% of that in the microsomes of ox liver. This apparent activity has not been investigated further.

DISCUSSION

The results show that kangaroo liver contains two type II arylsulphatases

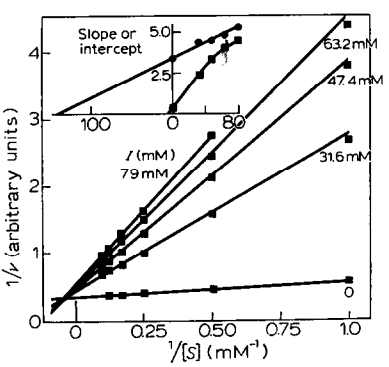


Fig. 8. The inhibition of sulphatase B by K_2SO_4. Substrate, nitrocatechol sulphate; pH 5.8 in 0.5 M acetate buffer; inhibitor concentrations as shown on figure. The inset shows the replots of the slopes (■) and intercepts on the abscissa (●) of these lines against inhibitor concentration.

which have properties resembling those of sulphatases A and B of ox liver. The two enzymes are, however, more difficult to separate (see scheme) than those of ox liver and the amount of sulphatase B which is present (or at least which can be isolated) is very much less (Table I). Neither of the enzymes has been obtained in a homogeneous form but the preparation of sulphatase A cannot be grossly impure.

The arylsulphatases which were partly characterised[14] in crude preparations from the livers of the brush-tailed possum, *Trichosurus vulpecula*, the wombat, *Lasiorhinus latifrons*, and the echidna, *Tachyglossus aculeatus*, clearly correspond to the sulphatase A of kangaroo liver. No enzyme corresponding to sulphatase B was found in these species but the methods used in these investigations, carried out with limited quantities of material and when the physical properties of sulphatase A and B were unknown, would not have detected small amounts of sulphatase B such as occur in kangaroo liver.

To judge by its chromatographic behaviour, the sulphatase A of kangaroo liver has a molecular weight of 100 000 both at pH 7.5, ionic strength 0.1, and pH 5.0, ionic strength 0.1 or 2. Ox sulphatase A, on the other hand, forms a tetramer at pH 5.0, ionic strength 0.1, and a dimer at pH 7.5, ionic strength 2, when its concentration is greater than about 10 μg/ml. Nevertheless, the tetramer dissociates at pH 5 when the concentration is lowered to approximately 1 μg/ml (ref. 10) as was shown by the elution from Sephadex G-200 of plateaux of known protein concentration. Insufficient amounts of the kangaroo enzyme were available to allow this technique to be used and its molecular weight was estimated by zone chromatography[22]. The actual concentration of enzyme protein across the zone was unknown but if it is assumed that the specific activity of pure kangaroo sulphatase A be similar to that of the ox enzyme then the enzyme concentration at the peak of the zone was about 8 μg/ml. Therefore, if the sulphatase A of the kangaroo formed a polymer which was only slightly less stable than that of the ox enzyme it might not have been detected because dissociation could have occurred. On the other hand, making the same assumption, the concentration of the enzyme protein in the sedimentation velocity experiments was about 0.25 mg/ml and again no evidence of polymerisation was obtained. It therefore appears justifiable to state that the sulphatase A of kangaroo liver does not polymerise under the same conditions as does sulphatase A from the ox: it may, however, do so at much higher concentrations of protein.

Kangaroo sulphatase A has a higher isoelectric point than the ox enzyme, 5.1–5.4 compared with 3.6 (ref. 3), which suggests that the former might contain relatively fewer free carboxyl groups. The polymerisation of ox sulphatase A is probably due mainly to hydrophobic interactions[3] but the participation of other forces cannot be excluded and, in particular, hydrogen bonding between unionised carboxyl groups might contribute to the stability of the tetramer which is the stable species below pH 5.5. The rather high isoelectric point of kangaroo sulphatase A might therefore be related to its apparent inability to form a stable polymer.

One of the most characteristic properties of the sulphatase A of ox liver is its anomalous kinetics[4,23,24], a property shared by other sulphatases A (refs. 5, 6, 13). Although the kinetics of the reaction catalysed by the sulphatase A of kangaroo liver are not identical with those of the ox enzyme they are of a similar nature and probably have a similar origin—the activation of the enzyme, in the later stages of its reaction, by sulphate[4,6], as suggested by Fig. 4.

The sulphatase B of kangaroo liver resembles the sulphatase B of ox liver in having a molecular weight of 45 000. In other properties also the two enzymes are quite similar although there is no evidence for the existence of isoenzymes of the kangaroo enzyme.

It is interesting that the inhibitions of sulphatases A and B by sulphate are respectively competitive (Fig. 3) and non-competitive (Fig. 8). This is also the pattern of inhibition found with the corresponding ox enzymes[25] and, as previously discussed[25], it implies that sulphate is the last-released product in sulphatases A and the first-released product in sulphatases B. It is tempting to suggest that this may be a general distinction between these two groups of sulphatases.

Kangaroo liver apparently does not contain a sulphatase C, or at least not in amounts comparable to those present in most eutherian mammals[1]. There is likewise no convincing evidence that kangaroo liver contains any steroid sulphatase but it must be stressed that the method used[19] is not suited for the detection of small amounts of this enzyme.

Finally, the presence of both sulphatase A and sulphatase B in the liver of the red kangaroo, *Megaleia rufa*, clearly shows, despite previous suggestions[14], that these enzymes developed quite early in the evolution of the mammals.

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