Studies on Sulphatases

6. THE LOCALIZATION OF ARYLSULPHATASE IN THE RAT-LIVER CELL

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Arylsulphatase in the rat occurs in greatest concentration in the liver (Dodgson, Spencer & Thomas, 1953*a*). Purification of rat-liver arylsulphatase has been hindered by the insolubility of the enzyme, as has already been noted for rabbit liver (Hommerberg, 1931; Morimoto, 1937). Previous work (Dodgson *et al.* 1953*a*) suggested that, in a water suspension of rat liver, part of the enzyme was insoluble, and was associated with the formed components of the cells. Roy (1953) arrived at a similar conclusion for ox liver and, furthermore, demonstrated that a high proportion of the total arylsulphatase of mouse liver was present in the mitochondria.

A study of the localization of arylsulphatase in the rat-liver cell was therefore undertaken as a preliminary to the purification of the enzyme. The locations of the enzymes responsible for the hydrolysis and synthesis of arylsulphates have also been compared. Both sexes were studied, since there is a difference between the sexes in the arylsulphatase content of rat liver (Dodgson *et al.* 1953*a*).

A preliminary account of this work has already been given (Dodgson, Spencer & Thomas, 1953*b*).

METHODS

Fractionation of rat liver in sucrose media. The cellular components of the livers of M.R.C. hooded rats were fractionated by differential centrifugation of suspensions in sucrose. The instructions of Hogeboom, Schneider & Pallade (1948) for hypertonic sucrose solution (0·88 M) were followed as far as the isolation of mitochondria. Separation of the submicroscopic material from the soluble components of the cells was not possible with the apparatus at our disposal. Fractionation in isotonic sucrose (0·25 M) was by the method of Schneider (1948). Before arylsulphatase assay, all fractions were suspended in the original volume of the appropriate sucrose solution, adjusted to pH 7·2, and an equal volume of 0·5 M acetate (pH 7·2) was added. The final solution corresponded to a 5% (wet wt./v) suspension.

Fractionation in water and acetate buffer. Suspensions in water or acetate buffer, prepared using a glass homogenizer (Dodgson *et al.* 1953*a*), were centrifuged at 2200 *g* for 15 min. at 0° in a refrigerated centrifuge. The residue was suspended in the original medium, adjusted to pH 7·2 and assayed for arylsulphatase activity. The supernatant solution was similarly adjusted to pH 7·2 before assay. All pH adjustments were made with acetic acid or NaOH. Hydrochloric and sulphuric acids were not used, since both chloride and sulphate ions activate rat-liver arylsulphatase (unpublished observations).

Fractionation in isotonic KCl. The procedure used was identical with that of Bernstein & McGilvery (1952*a*) for the study of the enzyme system synthesizing arylsulphates. All fractions were dissolved in isotonic KCl solution and adjusted to pH 6·65 for estimation of the activity of the synthesizing system, and to pH 7·2 for arylsulphatase assay. In the latter case a suitable dilution with KCl solution was made before assay.

Isolated nuclei. Nuclei prepared by the method of Wilbur & Anderson (1951) were washed with isotonic sucrose solution to remove phosphate ions which inhibit arylsulphatase, centrifuged, and finally suspended in isotonic sucrose solution at pH 7·2.

Arylsulphatase assay. The spectrophotometric method of Dodgson *et al.* (1953*a*) was used. Sucrose (0·44 M) did not affect the activity of rat-liver arylsulphatase, but KCl increased the activity of the enzyme by 20% in the concentration used in the assay (0·077 M).

Estimation of arylsulphate synthesis. Since potassium *p*-acetylphenylsulphate was the substrate for arylsulphatase assay, it was necessary, for strict comparison, to use *p*-hydroxyacetophenone in the estimation of synthesizing activity. The enzyme solution was incubated anaerobically with a suitable medium containing *p*-hydroxyacetophenone, and enzyme action was stopped and protein precipitated with trichloroacetic acid (TCA). After being centrifuged, the precipitate was washed with TCA. The combined supernatants contained all the synthesized arylsulphate, but not necessarily all the unconjugated phenol, since some was adsorbed on to the protein precipitate. The free *p*-hydroxyacetophenone in the supernatant was measured in alkaline solution at 323 mμ. (Dodgson & Spencer, 1953*a*). The *p*-hydroxyacetophenone conjugated as ethereal sulphate was liberated by mild acid hydrolysis, and that as glucuronide by stronger acid. After being made alkaline, the total *p*-hydroxyacetophenone in these solutions was measured and the extent of each conjugation calculated.

Potassium *p*-acetylphenylsulphate was completely hydrolysed by 0·2 N-HCl in 7 min. at 100°. No *p*-acetylphenylglucuronide was available, but it is unlikely that any appreciable hydrolysis of this compound would occur under the conditions used for the hydrolysis of the arylsulphate (cf. Garton, Robinson & Williams, 1949). Most glucuronides can be completely hydrolysed by 5 N-HCl in 2 hr. at 100° (Hanson, Mills & Williams, 1944) and these conditions have been adopted here. As a means of estimating glucuronide synthesis, the method could not be considered satisfactory, since slight charring occurred. However, with the medium and enzyme extracts used in this study, no glucuronide synthesis was observed.

The enzyme solution (3 ml.) and a similar volume of anaerobic medium (Bernstein & McGilvery, 1952*a*) containing 180 μg. *p*-hydroxyacetophenone were incubated at 37·5° for 2 hr. in a 15 ml., stoppered centrifuge tube. At the end of the incubation period, 4 ml. of 5% (w/v) TCA were added and the precipitate which formed was separated by centrifugation. The supernatant was decanted into a measuring cylinder through a cotton-wool plug held in a small funnel. The residue was shaken with a further 5 ml. of 5% TCA, centrifuged, and the supernatant decanted through the funnel. The funnel was washed with water until the volume of the collected fluid was 16 ml. Three 5 ml. portions were taken. One portion was diluted to 10 ml. with N-NaOH and the extinction at 323 mμ. was measured (E_{t1}); another portion was made acid with 1 ml. of N-HCl, heated in a boiling-water bath for 10 min., cooled, diluted to 10 ml. with N-NaOH and E_{323} measured (E_{t2}); the remaining portion was made acid with 5 ml. 10N-HCl, heated in a boiling-water bath for 2 hr., cooled, diluted to 15 ml. with 10N-NaOH, and E_{323} measured (E_{t3}). Control determinations in which *p*-hydroxyacetophenone was absent were carried out in an identical manner, and the extinctions at 323 mμ. before and after acid hydrolysis were E_{c1}, E_{c2} and E_{c3}. Then

μg. *p*-hydroxyacetophenone conjugated with sulphuric acid

$$= (E_{t2} - E_{c2}) - (E_{t1} - E_{c1}) \times 217{\cdot}8,$$

and μg. *p*-hydroxyacetophenone conjugated with glucuronic acid

$$= (E_{t3} - E_{c3}) \tfrac{15}{10} - (E_{t2} - E_{c2}) \times 217{\cdot}8,$$

where 217·8 is a factor derived from the molecular extinction coefficient of *p*-hydroxyacetophenone at 323 mμ. (Dodgson & Spencer, 1953*a*) and the dilutions made. Experiments showed that potassium *p*-acetylphenylsulphate added to rat-liver preparations could be recovered quantitatively (96–100%).

The identity of the compound hydrolysed under the mild acid conditions was established as an arylsulphate acted on by a glucuronidase-free arylsulphatase concentrate (Dodgson & Spencer, 1953*b*). Hydrolysis by acid or by arylsulphatase of the protein-free, anaerobic incubation mixture from an actively-synthesizing preparation resulted in the liberation of identical amounts of *p*-hydroxyacetophenone. Before hydrolysis with arylsulphatase, it was necessary to remove phosphate and sulphate ions with barium hydroxide at pH 11·5, and then to adjust to pH 5·5 (Dodgson & Spencer, 1953*b*). It was not possible to detect spectrophotometrically the presence of *p*-acetylphenylsulphuric acid at its position of maximum absorption (252·5 mμ.) owing to the presence of adenosine triphosphate and its hydrolysis products, which absorb strongly at this wavelength. However, the low absorption maximum peak of unchanged *p*-hydroxyacetophenone (323 mμ.) which exists in the unhydrolysed incubation mixtures increased in intensity after mild acid hydrolysis without any change in wavelength. This suggested that further *p*-hydroxyacetophenone had been liberated, and it was therefore assumed that *p*-acetylphenylsulphate had been synthesized.

Using a more tedious method, similar to that already described, but precipitating protein with ethanol, it was possible to recover all the added phenol from whole suspensions of rat liver before incubation. After incubation under anaerobic conditions, recovery of *p*-hydroxyacetophenone as a free phenol and a conjugated phenol was quantitative, but under aerobic conditions (Bernstein & McGilvery, 1952*a*) recovery was low (50–70%). This suggested that *p*-hydroxyacetophenone is metabolized to a product which, after treatment with acid, is spectroscopically different from the original phenol.

RESULTS

Fractionation of sucrose suspensions of rat liver

The arylsulphatase activities of the various fractions are reported in Table 1. The so-called 'nuclear' fractions from both hypertonic and isotonic sucrose contained blood cells, undamaged liver cells, and granular material, in addition to nuclei. The uncontaminated nuclei isolated from 1 g. of liver by the method of Wilbur & Anderson (1951) had negligible arylsulphatase activity when assayed over 18 hr., and the activity of the 'nuclear' fractions therefore cannot be attributed to nuclei.

Table 1. *The arylsulphatase activities of fractions separated by differential centrifugation of suspensions of rat liver in sucrose solutions*

(The figures represent the arylsulphatase activity of the fraction expressed as percentage of that of the whole suspension, and are the averages of those from four rats in each case. Ranges are given in parentheses.)

Fraction	Activity	
	Males	Females
Hypertonic sucrose		
'Nuclei'	22·1 (12·1–36·0)	22·6 (14·4–31·8)
Mitochondria	22·3 (20·2–25·4)	24·2 (16·0–32·6)
Supernatant (containing the microsomes)	44·3 (32·1–51·7)	44·4 (41·8–46·8)
Recovery	88·7 (86·8–90·5)	91·2 (85·8–96·8)
Fractions combined before assay	85·0 (81·9–88·8)	93·9 (89·1–98·4)
Isotonic sucrose		
'Nuclei'	12·3 (9·6–15·1)	15·3 (14·0–16·2)
Mitochondria	8·7 (4·2–17·2)	12·3 (9·3–15·0)
Microsomes	60·5 (48·7–76·1)	63·9 (60·3–71·0)
Supernatant	2·2 (0–4·1)	2·6 (0–5·4)
Recovery	83·7 (74·2–93·9)	94·1 (91·1–99·5)

Neither can blood cells contribute significantly to the activity of these fractions, since rat blood has an arylsulphatase activity of only about 200 units/100 ml. when assayed by the method of Dodgson *et al.* (1953*a*). Furthermore, removal of blood before fractionation by perfusion of the livers with ice-cold sucrose solutions had no effect on the activity of the nuclear fractions. It must be assumed that the activity is attributable to undamaged and partially damaged cells, and contaminating granular material. The difference in activity of the nuclear fractions isolated from isotonic and hypertonic sucrose solutions is probably owing to the greater concentration of intact cells and contaminating granular material resulting from the method of centrifuging used for the latter solution. The results would suggest that the glass homogenizers (cf. Brown, Dodgson, Sherwood & Spencer, 1952) rupture only 85% of the cells under the conditions recommended by Hogeboom *et al.* (1948) and Schneider (1948). Similar activity due to intact cells was found in the 'nuclear' fractions by Hawkins (1952) for the amine oxidase of rat liver.

It appears that the arylsulphatase of the rat-liver cell is localized in the granular material and, under the conditions of fractionation in isotonic sucrose, there is virtually no enzyme in solution, i.e. in the final supernatant. On these grounds it is reasonable to assume that the activity of the final supernatant from hypertonic sucrose solution is due solely to the submicroscopic granules, or microsomes, contained in it. Fractionation of the liver cells in both sucrose media showed that the enzyme was mainly concentrated in the microsomes, but the proportion of the activity of the original suspension localized in the mitochondria and microsomes differed with the tonicity of the sucrose solution used (Table 1). The mitochondria isolated by both methods were stained characteristically by Janus B green, and the microsome fractions contained only a trace of granular material of mitochondrial size. The reason for the discrepancy between the results obtained by the two methods is not therefore immediately apparent. It is possible, however, that some disintegration of mitochondria might have occurred in isotonic sucrose, since this medium does not sustain the morphological integrity of these granules (Schneider, 1948), or that the mitochondrial fractions from the hypertonic sucrose solution may have been contaminated with microsomes. The exact derivation of microsomes is still under discussion (Schneider & Hogeboom, 1951). A redistribution of the enzyme during the fractionation procedures cannot be ruled out, since Rutenberg, Cohen & Seligman (1952) demonstrated histochemically that diffusion of arylsulphatase from rat-kidney slices occurs under certain conditions, with subsequent transference to adjacent, inactive dog-liver slices fixed by formalin.

There was no apparent difference in the localization of arylsulphatase in the liver cells of male and female rats. The sum of the activities of the various fractions gave only 84–94% recovery. Recombination of the fractions before assay did not improve recoveries (Table 1), and the loss of activity must be an actual physical loss, and not due to the removal of an activator or a coenzyme.

Fractionation of water and acetate-buffer suspensions

It was previously noted (Dodgson *et al.* 1953*a*) that centrifugation of water or acetate-buffer suspensions of rat liver resulted in a distribution of the arylsulphatase between the supernatant and the residue. Microscopic examination of such fractions showed that the residue, containing about 10–20% of the activity, was composed of nuclei, blood cells, partially damaged and undamaged liver cells, and some granular material. The supernatant solution (having 60–80% of the activity) contained only granular material. These observations are in accord with those made in sucrose media.

Agglutination of granular material occurs at certain pH values and in the presence of electrolytes (Schneider & Hogeboom, 1951). The effect on the fractionation of the arylsulphatase of varying the pH of an acetate-buffer suspension was studied and the results are presented in Fig. 1. The decrease in activity of the supernatant solution following the lowering of pH can be correlated with an increase in the agglutination of the granular material. The

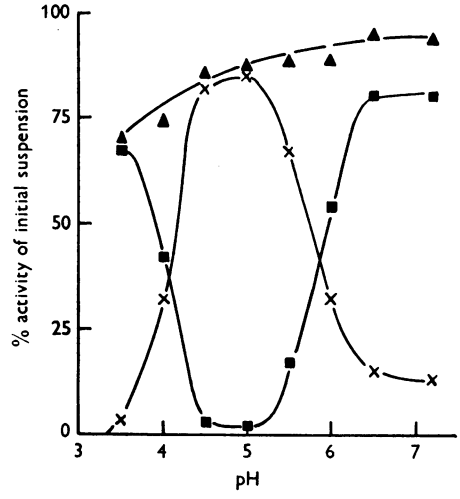

Fig. 1. The arylsulphatase activity of the supernatant (■), and residue (×) separated by centrifugation (2200 *g*), of suspensions of rat liver in acetate buffer at different pH values. The total recovery (supernatant and residue) is also shown (▲).

progressive agglutination resulted in an increase in the amount of granular material in the residue and a consequent rise in the enzymic activity of this fraction. At pH 5·0, where agglutination was maximal, the supernatant was free from granules of mitochondrial size. Since at this pH the supernatant had only about 2% of the activity of the original suspension, it can be assumed that the microsomes were also agglutinated and spun down. At more acid pH values agglutination decreased, and there was a consequent increase in the granular content and arylsulphatase activity of the supernatant. Similar results were obtained with water suspensions, when the pH was adjusted with acetic or hydrochloric acids. These results confirm that arylsulphatase is mainly localized in the granular material of the liver cell.

At all pH values there was a loss of enzymic activity during fractionation which could not be recovered by combination of the fractions before assay. Near pH 7 the loss was approximately 5–6%, but this increased at lower pH values until at 3·5 it was 30%. The loss observed in the present study was less than that reported previously (Dodgson *et al.* 1953*a*) owing to a stricter regulation of temperature during centrifugation, made possible by the use of a refrigerated centrifuge.

Fractionation of suspensions of rat liver in isotonic potassium chloride

The results are presented in Table 2. The residue spun down at 2000 *g* consisted of undamaged and partially damaged cells contaminated with granular material, whilst that removed at 18000 *g* contained only granular material. These two fractions accounted for approximately 95·7% of the recovered arylsulphatase, the remainder being in the supernatant solution, which was free from particulate matter when viewed in the light-microscope. Combination of the fractions in all possible ways before assay did not increase the activity beyond that of the sum of the separate activities.

The supernatant solution from 1 g. of liver, which contained only 4·2% of the arylsulphatase activity of the original suspension, conjugated 125·6 μg. *p*-hydroxyacetophenone/hr. (range 48·8–189·0, four rats). Other simple treatments gave liver extracts possessing synthetic activity, together with traces of arylsulphatase. Thus an acetone-dried powder of the final potassium chloride supernatant solution had an arylsulphatase activity of 226 units/g., and conjugated 714 μg. *p*-hydroxyacetophenone/g./hr. Adjustment to pH 5·4 of a 10% (wet wt./v) suspension in isotonic potassium chloride solution, and removal of the agglutinated particulate material by centrifugation at 2200 *g* (cf. Fig. 1) gave a supernatant solution with an arylsulphatase activity of 9·25 units/ml. (0·46% of the activity of the original suspension), and a conjugating activity of 4·9 μg. *p*-hydroxyacetophenone/hr./ml.

Table 2. *Fractionation of rat liver in isotonic* KCl

(The figures represent the arylsulphatase activities of the fractions expressed as a precentage of that of the initial suspension, and are the average of those for two male and two female rats. Ranges in parentheses.)

Fraction	Activity
Residue 2000 *g*	31·5 (28·0–36·4)
Residue 18 000 *g*	59·0 (54·3–63·6)
Supernatant	4·2 (1·5–6·8)
Recovery	94·7 (89·5–102·5)

DISCUSSION

Arylsulphatase is apparently concentrated in the granular material of the cytoplasm of rat-liver cells and is absent from the nuclei and from the soluble components of the cytoplasm. The distribution of the enzyme as found by mechanical cytological separation agrees with the broad histochemical picture (Rutenberg *et al.* 1952), which shows rat-liver nuclei to be devoid of arylsulphatase whilst the cytoplasm as a whole is active. Although the proportion of the activity found in the microsomes depends on the tonicity of the medium used in the fractionation procedures, the microsomes contain greater concentrations of the enzyme than do the mitochondria. This is in contrast to the finding for mouse liver, where the mitochondria account for some 70% of the total activity (Roy, 1953). However, Roy points out that his results are only semi-quantitative owing to destruction of liberated 4-nitrocatechol by whole suspensions of liver. This may be considerable (Dodgson & Spencer, 1953*a*). The proportion of the total arylsulphatase found in the granules must be greater than that actually recorded in Table 1 since the incomplete disintegration of the cells and the mechanical loss of the enzyme must be taken into account. It is interesting to note that as well as arylsulphatase, other enzymes which occur in the microsomes (Schneider & Hogeboom, 1951) are not confined to these particles, but are also distributed amongst the other granular fractions.

The work of Bernstein & McGilvery (1952*a*, *b*) suggests that the *in vitro* biosynthesis of arylsulphates is at least a two-stage process. The first reaction, which is rate-limiting, is the activation of sulphate ions by adenosine triphosphate (ATP) in the presence of an unknown cofactor. The activated sulphate then conjugates with the phenol under the control of an enzyme which may be called arylsulphate synthetase. After centrifugal fractionation of suspensions of rat liver in potassium chloride solutions the fractions (residue at 2000 *g*, residue at 18000 *g*, and supernatant) did not possess the synthesizing system when assayed separately by an

aerobic method. Combination of the high-speed residue with the supernatant solution, however, resulted in an activity comparable with that of the original suspension, but the supernatant liquid was anaerobically active alone if sufficient ATP was added. This suggested that the granular material of the cytoplasm supplied only the ATP necessary for sulphate activation, and this conclusion was also reached by DeMeio & Tkacz (1952). The distribution of the synthesizing system in suspensions of rat liver in potassium chloride solutions as found by Bernstein & McGilvery (1952*a*, *b*) was entirely different from that found for arylsulphatase in sucrose media during the present work. Whereas the synthesizing system is absent from the granular material of the cytoplasm, arylsulphatase is almost entirely concentrated in this fraction of the cell. Repetition of the potassium chloride fractionation according to Bernstein & McGilvery (Table 2) showed that the low- and high-speed residues contained the bulk of the arylsulphatase, and that the supernatant solution, which had considerable synthesizing activity, was almost devoid of the hydrolytic enzyme. Unfortunately, quantitative comparison between the two enzyme systems is not possible, owing to the different ATP requirements of the various fractions, which affects the level of arylsulphate synthesis. Thus, granular material inhibits arylsulphate formation by virtue of its ATP-ase activity (R. H. DeMeio, personal communication). With whole suspensions of rat liver under aerobic conditions there is also a competition for the *p*-hydroxyacetophenone by a process other than that of conjugation (see under Methods).

Although the difference in distribution of the hydrolytic and synthesizing systems indicates that arylsulphatase and arylsulphate synthetase are different enzymes, it is not possible to make an unequivocal differentiation on these grounds alone. Since the activation of sulphate is the rate-limiting process, only a small proportion of the total arylsulphate synthetase of the suspension may be required in the *in vitro* conditions. In this case, the traces of arylsulphatase, found in all extracts possessing synthesizing activity, might be responsible for conjugation. Final differentiation between the two enzymes must await the preparation of purer concentrates of the enzymes and cofactors concerned, and the purification of rat-liver arylsulphatase is being further studied with this in mind.

SUMMARY

1. The arylsulphatase of rat liver is localized in the granular material of the cytoplasm, mainly in the microsomes. Nuclei do not contain the enzyme.

2. The distribution of arylsulphatase amongst the cellular components of the liver cell is different from that of the system which synthesizes arylsulphates.

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