Studies on the Oestrogen Sulphatase and Arylsulphatase C Activities of Rat Liver

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Detailed studies on the hydrolysis of *p*-acetylphenyl sulphate and oestrone sulphate by rat liver preparations strongly indicate that arylsulphatase C and oestrogen sulphatase are the same enzyme. Liver is the richest source of both enzymes, which have identical intracellular distributions, being localized mainly in the microsomal fraction. Low oestrogen sulphatase and arylsulphatase C activities were present in foetal liver and these increased at a similar rate after birth. The activities of the enzymes in an ethionine-induced hepatoma were similarly low. Results of heat inactivation, mixed-substrate and competitive-inhibition experiments employing liver microsomal fractions were also consistent with one enzyme being involved. Oestradiol-17 β 3-sulphate was also hydrolysed by microsomal preparations and activity towards both this substrate and oestrone sulphate was inhibited by oestrone and oestradiol-17 β . The physiological significance of this inhibition is discussed.

Three arylsulphatase enzymes (aryl sulphate sulphohydrolase, EC 3.1.6.1) distinguishable by their relative substrate specificities, their tissue distribution and intracellular localization have been shown to exist in most mammalian species examined (Dodgson *et al.*, 1955, 1956; Roy, 1958; Dodgson & Rose, 1970). In rat liver two of the arylsulphatases (A and B) are localized mainly in the lysosome fraction of the cell (Viala & Gianetto, 1955), whereas arylsulphatase C is present in the microsomal fraction in a highly insoluble form (Dodgson *et al.*, 1954*a*, 1957*a*), and is a useful marker for the membrane component of the endoplasmic reticulum of the rat liver cell (Milsom *et al.*, 1968; Milsom & Rose, 1970).

No physiological function has yet been assigned to arylsulphatase C, and non-physiological substrates (e.g. potassium *p*-acetylphenyl sulphate and potassium *p*-nitrophenyl sulphate) are employed for its assay. However, it is noteworthy that Hanahan & Everett (1950) have shown that injected sodium oestrone [35 S]sulphate undergoes appreciable desulphation in the rat with the rapid excretion of inorganic [35 S]sulphate in the urine. Moreover, the liver was found to be the principal organ of the rat where the desulphation of injected oestrone [35 S]sulphate occurs (Dolly *et al.*, 1971). Several investigators have reported that rat and human tissues have the capacity to desulphate oestrone sulphate (see Pulkkinen, 1961; Notation & Ungar, 1969; Pack & Brooks, 1970; Schwerts & Rodesch, 1963; Sandberg & Jenkins, 1966), the responsible enzyme being localized in the microsomal fraction of the cell (Pulkkinen & Paunio, 1963; Zuckerman & Hagerman, 1969; French & Warren, 1966). Nevertheless, it has not been established that the arylsulphatase C

and oestrogen sulphatase activities of rat liver are due to two distinct enzymes. There is some evidence which suggests that two separate enzymes are responsible for the hydrolysis of *p*-nitrophenyl sulphate and oestrone sulphate by human placental and rat kidney preparations (French & Warren, 1967; Zuckerman & Hagerman, 1969).

In the present paper we describe the properties of rat liver oestrogen sulphatase and arylsulphatase C activities and present evidence that one enzyme is responsible for both of these activities.

Materials and Methods

Materials

Chemicals. The sodium salts of oestrone [35 S]sulphate and oestradiol-17 β 3- 35 S]sulphate were prepared (sp. radioactivity approx. 6mCi/mmol) as described by Dolly *et al.* (1971). The potassium salts of *p*-acetylphenyl sulphate and *p*-nitrophenyl sulphate were synthesized by the method of Burkhardt & Lapworth (1926) as modified by Milsom *et al.* (1972). Dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate), prepared by the method of Dodgson & Spencer (1956), was kindly supplied by Dr. M. Worwood of this department. Oestrone, oestradiol-17 β , oestriol, adenosine 3':5'-cyclic monophosphate (cyclic AMP), glucagon, actinomycin D and bovine serum albumin were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Glucose 6-phosphate was purchased from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Stilboestrol was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. DNA (calf thymus) and

Triton X-100 were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. and 2,5-diphenyloxazole by Beckman Instruments Inc., Palo Alto, Calif., U.S.A.

Experimental animals. Male and female Medical Research Council hooded rats (200–250 g body wt.) were used, except where otherwise stated. Littermates were employed for tissue-distribution and development experiments. Male Norwegian hooded rats bearing ethionine-induced transplanted hepatomas and appropriate control rats were kindly supplied by Dr. E. Reid, Wolfson Bioanalytical Centre, University of Surrey, Guildford, U.K.

Methods

Paper electrophoresis. Samples (5 μ l) were subjected to electrophoresis on Whatman no. 1 paper with 0.1 M-sodium acetate–acetic acid buffer, pH 4.5, for 2 h at a potential gradient of 9 V/cm.

Detection and measurement of radioactivity. Radioactive spots were located and measured on electrophoretograms by scanning as described by Dolly *et al.* (1971). The radioactivity of liquid samples (50 or 700 μ l) was counted with the Packard Tri-Carb (model 3375) liquid-scintillation spectrometer in 10 ml of toluene-based scintillation fluid (1 litre of toluene, 5 g of 2,5-diphenyloxazole and 500 ml of Triton X-100).

Tissue homogenates. Crude whole homogenates were employed for the measurement of enzymes in various rat tissues and hepatomas. The homogenates were prepared by the procedure described by Milsom *et al.* (1972).

For most of the other experiments reported in the present paper rat liver microsomal fractions were employed and were prepared as described by de Duve *et al.* (1955) except that the rats were not starved and the mitochondrial fraction was not isolated. The microsomal pellet was suspended in water (adjusted to pH 7.0 with NaOH) at a concentration so that 1.0 ml of suspension was equivalent to 1 g of liver, and was stored at -20°C ; no losses of sulphatase activities were detected within 4 months.

Assay of oestrogen sulphatase and arylsulphatases A, B and C. The oestrogen sulphatase assay was based on the methods employed by Wengle (1964) and Mattock & Jones (1970) for the measurement of sulphotransferase activity. The standard incubation mixture for crude homogenates contained 100 μ l of 0.14 M-tris–HCl buffer, pH 7.7, 50 μ l of an aqueous solution of sodium oestrone [^{35}S]sulphate or sodium oestradiol-17 β 3-[^{35}S]sulphate (0.4 mM) and 50 μ l of enzyme preparation. When microsomal preparations were being studied, phosphate buffer (0.1 M- Na_2HPO_4 – NaH_2PO_4 final concn.), pH 8.0, was substituted for the tris–HCl buffer. Milsom *et al.* (1972) have shown that rat liver arylsulphatases A and B are completely inhibited by phosphate buffer

under these conditions, whereas the activity of arylsulphatase C is not significantly affected. Assay mixtures were incubated for 2–15 min at 37°C in a water bath that permitted continual shaking (75 cycles/min) of the incubation mixture. The reaction was stopped by the addition of 0.4 ml of 0.05 M- H_2SO_4 and the liberated inorganic [^{35}S]sulphate was precipitated by adding 0.4 ml of 0.1 M- $\text{Ba}(\text{OH})_2$ (each litre containing 5 ml of NH_3 , sp.gr. 0.88, and 10 ml of 0.1% phenolphthalein in ethanol). The suspension was mixed, then the excess of Ba^{2+} ions was precipitated by bubbling CO_2 through the solution until the phenolphthalein was decolorized. The mixture was centrifuged at about 500 g for 10 min and the unhydrolysed ^{35}S -labelled ester in the supernatant was measured by counting radioactivity of samples as described in the preceding section. Control determinations were made in which enzyme was added immediately before the reaction was stopped. By expressing the amount of radioactivity in the test sample as a percentage of that in the control the amount of ester hydrolysed could be calculated. Preliminary experiments with crude liver homogenates and microsomal preparations showed that there was complete precipitation of inorganic [^{35}S]sulphate and negligible co-precipitation of oestrone [^{35}S]sulphate and oestradiol-17 β 3-[^{35}S]sulphate. The esters were not hydrolysed during the precipitation of inorganic [^{35}S]sulphate. Unless otherwise stated, oestrone [^{35}S]sulphate was always used for the assay of oestrogen sulphatase. As a further check of the assay procedure, incubations were done as described above except that the reaction was terminated by immersing the tubes in a boiling-water bath for 30 s. The samples were centrifuged, the inorganic [^{35}S]sulphate was separated from the unhydrolysed ^{35}S -labelled ester by paper electrophoresis and the radioactivity of each component was measured by scanning the electrophoretograms as described previously. Both methods of assay agreed to within $\pm 5\%$.

Arylsulphatase C activity was assayed by the method of Dodgson & Spencer (1953) as modified by Milsom *et al.* (1972) but employing one-fifth of the quantities stated. Similar assay conditions were employed with *p*-nitrophenyl sulphate as substrate except that a final concentration of 12 mM-potassium *p*-nitrophenyl sulphate was used. Unless otherwise stated, *p*-acetylphenyl sulphate was always used as substrate.

The combined activities of arylsulphatases A and B (EC 3.1.6.1) were estimated by using nitrocatechol sulphate as described by Milsom *et al.* (1972) except that in the tissue-fractionation studies assays were performed in the presence of 0.1% (v/v) Triton X-100.

One unit of sulphatase activity is defined as the number of nmol of ester hydrolysed/h under the conditions used.

Assay of other enzymes. Rhodanese (thiosulphate-cyanide sulphurtransferase, EC 2.8.1.1) was assayed as described by Rosenthal *et al.* (1950) and glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) as outlined by de Duve *et al.* (1955) except that inorganic phosphate was determined by the method of Chen *et al.* (1956).

One unit of rhodanese or glucose 6-phosphatase activity is defined as the number of μmol of substrate hydrolysed/h.

Determination of protein and DNA. Protein was measured by the method of Gornall *et al.* (1949) with dry bovine serum albumin as standard. DNA was isolated by the procedure of Munro & Fleck (1966) and measured by the method of Giles & Myers (1965).

Subcellular fractionation. This was performed essentially as described by de Duve *et al.* (1955) on 10g of liver obtained from two male rats that had been starved for 16h. All pellets were resuspended in 0.25M-sucrose (containing 1mM-EDTA and adjusted to pH 7.0 with NaOH). Oestrogen sulphatase, arylsulphatase C and the marker enzymes were assayed on the freshly prepared fractions. All other measurements were made with samples that had been stored at -20°C .

Experimental and Results

Optimum conditions for oestrogen sulphatase activity of crude liver homogenates

These were determined and subsequently employed in a survey of the general tissue distribution of the enzyme. Maximum activity in 0.07M-tris-HCl buffer was obtained at pH 7.6–7.7 and at a substrate concentration of approx. $100\mu\text{M}$ (Fig. 1). It is important to note that the inhibition by excess of substrate becomes very pronounced beyond the range shown in Fig. 1 and this, together with the very low apparent optimum substrate concentration, places a severe limitation on the type of assay method that can be used to measure enzyme activity. At the optimum conditions the rate of hydrolysis of oestrone [^{35}S]sulphate was linear for a 15min incubation period and directly proportional to the amount of enzyme added, provided that less than 20% of the substrate was hydrolysed.

Distribution of the enzymes

Tissue distributions of oestrogen sulphatase and arylsulphatase C. Table 1 shows that all the rat tissues examined were able to hydrolyse oestrone [^{35}S]sulphate and *p*-acetylphenyl sulphate, the greatest activities being found in the liver. Enzyme activities in male rat liver were significantly higher than in the female, as found for arylsulphatase C by Dodgson *et al.* (1953). These differences were also reflected in

some of the other tissues examined although, in some cases, little difference was apparent (cf. Pulkkinen, 1961; Dodgson *et al.*, 1953).

Intracellular distribution of oestrogen sulphatase. The results of a typical rat liver distribution pattern are presented in Table 2. Rhodanese, arylsulphatases A+B and arylsulphatase C were selected as marker enzymes for the mitochondrial, lysosomal and microsomal fractions respectively. DNA served as a chemical marker for the nuclear fraction. Oestrogen sulphatase was predominantly localized in the microsomal fraction and it was particularly significant that the relative specific activity (% of enzyme recovered in each fraction divided by the % of protein recovered in that fraction; see de Duve *et al.*, 1955) of this enzyme in the different fractions was always virtually identical with that of arylsulphatase C. In this type of experiment, the relative specific activity of these two enzymes was always rather high in the lysosomal fraction. However, this could be traced to contamination, and when a purer lysosomal fraction was isolated (see Milsom, 1970) the relative specific activities were much decreased although still identical.

Liver oestrogen sulphatase and arylsulphatase C activities during development

The development pattern of liver oestrogen sulphatase activity of male and female rats was virtually identical with that of arylsulphatase C (Fig. 2; see Pulkkinen, 1961, and Milsom, 1970).

Fig. 1. Effect of substrate concentration (a) and pH (b) on oestrogen sulphatase activity of rat liver homogenates

Experimental conditions were as described in the text except that 0.08M-sodium acetate-acetic acid was the buffer when enzyme activity was measured at pH 5.0 and 6.0.

Table 1. *Distribution of oestrogen sulphatase and arylsulphatase C activities in various rat tissues*

Enzyme activities are expressed as units/mg of protein. Experimental details are given in the text. Typical results for one animal of each sex are given.

Tissue	Oestrogen sulphatase		Arylsulphatase C	
	Male	Female	Male	Female
Liver	102.5	65.9	176.6	111.8
Adrenals	43.7	53.3	29.4	51.7
Kidney	26.6	27.7	39.9	35.6
Lung	25.5	12.6	41.5	19.8
Pancreas	22.1	17.4	27.1	17.2
Brain	20.8	11.1	28.8	18.0
Spleen	16.2	11.7	39.7	30.5
Heart	16.1	16.2	23.4	21.5
Small intestine	2.7	3.5	9.1	11.9
Large intestine	5.7	3.0	12.4	7.0
Testes	24.6	—	54.2	—
Ovaries	—	23.2	—	40.4
Uterus	—	14.0	—	10.2

Table 2. *Intracellular distribution of oestrogen sulphatase activity and marker enzymes in the rat liver cell*

Absolute values for the various enzymes are in units/mg of tissue. Protein and DNA concentrations are expressed as mg and μ g respectively/g of tissue. Fractionation procedures and enzyme assays are described in the text.

Fraction ...		Absolute values	Percentage recovered in					Recovery (%)
		Cytoplasmic+ nuclear	Nuclear	Mito- chondrial	Lysosomal (light mitochondrial)	Micro- somal	Cell sap	
Protein		0.19	28.1	18.1	3.7	9.5	40.6	90.0
DNA		0.26	98.6	1.4	0	0	0	100.0
Rhodanese		1.61	32.0	58.5	1.4	4.6	3.5	105.3
Arylsulphatases A and B		35.3	14.1	15.9	20.4	10.0	39.6	114.5
Arylsulphatase C		23.0	29.2	9.5	14.7	44.8	1.8	91.3
Oestrogen sulphatase		12.5	29.9	10.1	15.4	43.4	1.2	90.8

Enzyme activities of male rat hepatomas

Oestrogen sulphatase, arylsulphatase C and glucose 6-phosphatase activities were lower in hepatomas than in normal male rat liver (Table 3). The activities of the first two enzymes were decreased to the same extent. Low activities of arylsulphatase C and glucose 6-phosphatase have been observed in similar hepatomas in these laboratories (Fish, 1970).

Effects of administration of cyclic AMP, glucagon and actinomycin D on enzyme activities

Male rats were fed with a basic daily ration (15g/rat) of normal protein test diet (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) to

ensure that they were in nitrogen balance before the experiment. Compounds (dissolved in 1ml of 0.95% NaCl) were injected intraperitoneally at zero time and again at 6h. The animals were killed at 12h and the livers assayed for enzyme activities. Control rats were injected with 0.95% NaCl only. In all cases the activities of oestrogen sulphatase and arylsulphatase C were similarly affected, irrespective of the compound administered. However, glucose 6-phosphatase activities in the cases of cyclic AMP and actinomycin D appeared to be independent of those of the sulphatase (Table 4). Cyclic AMP decreased the activities of the two sulphatases in liver even though experiments *in vitro* showed that both enzymes were slightly activated (5–10%) by the

Fig. 2. *Oestrogen sulphatase and arylsulphatase C activities of liver homogenates from male and female rats during development*

Experimental details are described in the text. ○ and ●, Male and female oestrogen sulphatase activities; □ and ■, male and female arylsulphatase C activities.

Table 3. *Oestrogen sulphatase, arylsulphatase C and glucose 6-phosphatase activities of male rat liver and hepatoma*

Sulphatase and glucose 6-phosphatase activities are expressed as units/mg of protein. The values in parentheses represent the enzyme activities expressed as a percentage of the activities in control rat liver. Experimental details are described in the text.

Tissue	Oestrogen sulphatase	Arylsulphatase C	Glucose 6-phosphatase
Control liver*	102.0 (100)	167.2 (100.0)	6.6 (100.0)
Hepatoma WDA/P4†	77.4 (75.9)	123.1 (73.6)	6.3 (95.5)
Hepatoma WDB/P3†	33.8 (33.1)	53.8 (32.2)	3.5 (53.0)
Hepatoma VAP/11†	6.0 (5.9)	11.7 (7.0)	0.7 (10.6)
Hepatoma VAP/11†	7.5 (7.4)	12.3 (7.4)	0.7 (10.6)

* Average results for three animals.

† Results for single samples.

compound over a wide concentration range (0.07–66.0 μ M). Glucagon decreased the activities of all three enzymes tested, a noteworthy finding in view of the autophagy induced in rat liver by injection of this compound (see Deter & de Duve, 1967; Deter *et al.*, 1967).

Properties of oestrogen sulphatase and arylsulphatase C activities of rat liver microsomal fractions

The experiments described above indicated that a single enzyme was possibly responsible for the

activities shown by tissue preparations towards oestrone sulphate and *p*-acetylphenyl sulphate. Further experiments with microsomal preparations were therefore performed.

Hydrolysis of different sulphate esters by rat liver microsomal fractions. Table 5 shows the relative K_m values obtained with different sulphate esters under optimum conditions for each substrate. The observed activities towards the compounds are also presented. The affinity of enzyme for substrate was high in the cases of the oestrogen sulphates and certainly much more appropriate to physiological concentration than in the case of the simple (non-physiological)

phenolic esters. However, the latter compounds were hydrolysed at an apparently greater rate. It is noteworthy that, under conditions where the lysosomal arylsulphatases A and B are known to be completely inhibited (i.e. in phosphate buffer at high pH values), the microsomal preparations showed some activity towards nitrocatechol sulphate. Zuckerman & Hagerman (1969) have claimed that rat kidney microsomal preparations cannot hydrolyse this ester.

Effects of incubation and preincubation temperature. The optimum temperature of incubation was identical for the hydrolysis of oestrone [^{35}S]sulphate and *p*-acetylphenyl sulphate (Fig. 3*a*). The temperature-inactivation curve in the absence of substrate was virtually the same for the two activities (Fig. 3*b*). In the latter experiment microsomal suspensions (2.1 mg of protein/ml of water at pH 7.0) were incubated in the absence of substrate for 15 min at different temperatures. After rapid cooling in an ice-bath they were assayed for both enzyme activities in the normal way.

Mixed-substrate experiments. The rates of hydrolysis of *p*-acetylphenyl sulphate and oestrone

[^{35}S]sulphate by rat liver microsomal fractions were measured (0.1M- Na_2HPO_4 - NaH_2PO_4 buffer, pH 8.0) for each substrate alone and when both substrates were present together. The results were then compared with the theoretical value calculated on the assumption that one enzyme catalyses the desulphation of both substrates. The K_m values for oestrone [^{35}S]sulphate and *p*-acetylphenyl sulphate were first determined for the particular microsomal preparation to be employed. Values of 10 μM and 6.7 mM respectively were obtained. The relative concentration (defined as the ratio of the substrate concentration to the K_m) of each substrate subsequently selected for the assays was 3.0. Assuming that both substrates were hydrolysed by a single enzyme, the observed velocities (v_a and v_b) for each substrate when assayed alone were then employed to calculate the theoretical velocity (v_t) which should be observed when the substrates are mixed together, by using the expression (Dixon & Webb, 1964):

$$v_t = (v_a + v_b) \cdot \left(\frac{1 + \sigma}{1 + 2\sigma} \right)$$

Table 4. *Effects of cyclic AMP, glucagon and actinomycin D on activities of hepatic enzymes in male rats*

Results are quoted as average values for three animals. See the text for experimental details. Sulphatase and glucose 6-phosphatase activities are expressed as units/mg of protein. Values in parentheses represent the enzyme activities expressed as a percentage of the activities in control rat liver.

Compound	Amount injected (mg)	Enzyme activities		
		Oestrogen sulphatase	Arylsulphatase C	Glucose 6-phosphatase
Cyclic AMP	10.0	87.8 $\pm$ 2.0 (79.5)	135.2 $\pm$ 7.9 (77.3)	3.5 $\pm$ 0.18 (100.0)
Glucagon	2.0	79.7 $\pm$ 9.4 (72.2)	138.6 $\pm$ 20.1 (79.2)	3.8 $\pm$ 0.37 (80.0)
Actinomycin D	0.4	90.9 $\pm$ 3.6 (82.3)	136.8 $\pm$ 14.3 (78.2)	3.2 $\pm$ 0.32 (91.4)
Glucagon plus actinomycin D	2.0 0.4	90.9 $\pm$ 3.5 (82.3)	143.3 $\pm$ 7.1 (81.9)	2.9 $\pm$ 0.34 (82.9)

Table 5. *K_m values and rates of hydrolysis for rat liver microsomal fractions on various phenolic sulphate esters*

Assays were performed in the presence of phosphate buffer as described in the text (see also Milsom *et al.*, 1972) except that nitrocatechol sulphate was present at a final concentration of 40 mM in the presence of 0.1M- Na_2HPO_4 - NaH_2PO_4 buffer, pH 7.4. Desulphation of oestradiol-17 β 3-[^{35}S]sulphate was measured under the conditions described for oestrone [^{35}S]sulphate.

Substrate	K_m (μM)	Enzyme activity (units/mg of protein)
<i>p</i> -Nitrophenyl sulphate	5.5 $\times 10^3$	514.4
<i>p</i> -Acetylphenyl sulphate	6.7 $\times 10^3$	505.4
Oestrone [^{35}S]sulphate	20.8 (10.0-24.4)*	307.1
Oestradiol-17 β 3-[^{35}S]sulphate	10.3 (6.7-15.4)*	164.4
Nitrocatechol sulphate	—	96.8

* Average of several determinations on different microsomal preparations; the range is given in parentheses.

Fig. 3. Effects of temperature of incubation (a) and preincubation (b) on oestrogen sulphatase and arylsulphatase C activities of rat liver microsomal fractions

Experimental details are given in the text. In all cases enzyme and substrate were incubated for 15 min. ○, Arylsulphatase C; ●, oestrogen sulphatase.

Fig. 4. Inhibition of oestrone $[^{35}\text{S}]$ sulphate hydrolysis by p -acetylphenyl sulphate (Lineweaver-Burk plot)

For experimental details see the text. ○, No inhibitor present; △, 20 mM inhibitor; □, 40 mM inhibitor.

where σ is the relative concentration of each substrate. This calculated theoretical value (453.4 units/mg of protein) was in good agreement with the experimental value (411.9 units/mg of protein) and it thus seemed highly probable that a single enzyme was involved.

The competitive nature of the inhibition of oestrone $[^{35}\text{S}]$ sulphate hydrolysis by p -acetylphenyl sulphate is shown in Fig. 4. The K_i value obtained (5.5 mM) was very similar to the K_m value (6.7 mM) for the hydrolysis of p -acetylphenyl sulphate. Similarly, the inhibition of p -acetylphenyl sulphate hydrolysis by oestrone $[^{35}\text{S}]$ sulphate was also competitive. The K_i value (11.2 μM) was fairly similar to the K_m value (20.8 μM) for the hydrolysis of

oestrone $[^{35}\text{S}]$ sulphate. Collectively these findings provided strong evidence that a single enzyme hydrolyses both substrates.

Inhibition by oestrogens. Preliminary experiments established that oestrone, oestradiol-17 β , oestriol and stilboestrol all inhibited oestrogen sulphatase activity. Subsequently, by working at substrate concentrations of 15–50 μM and inhibitor concentrations of 2.5–12.5 μM [oestrogens were dissolved in ethanol and the final concentration of ethanol in incubation mixtures was 4.5% (v/v)], it was demonstrated that oestrone was a competitive inhibitor of oestrogen sulphatase (K_i 19.1 μM). Oestrone (25–50 μM) also competitively inhibited the hydrolysis of p -acetylphenyl sulphate but the K_i value (87.4 μM) was higher than that obtained for the hydrolysis of oestrone sulphate.

Oestradiol-17 β also competitively inhibited the hydrolysis of oestrone $[^{35}\text{S}]$ sulphate (K_i 3.2 μM) and oestradiol-17 β 3- $[^{35}\text{S}]$ sulphate (K_i 3.7 μM). This is in accord with the view that both these substrates are hydrolysed by the same enzyme (Notation & Ungar, 1969).

Discussion

The results obtained in the present investigation strongly support the conclusion that the hydrolysis of p -acetylphenyl sulphate and oestrone sulphate by microsomal preparations can be attributed to the action of a single enzyme, the so-called arylsulphatase C first described by Dodgson *et al.* (1953, 1954a, 1955). The most convincing evidence is provided by the temperature-inactivation and (particularly) the competitive inhibition studies. The latter experiments established that each substrate competitively inhibited the hydrolysis of the other, whereas the K_i

value obtained for each ester (when used as an inhibitor) was virtually identical with the K_m value for that ester (when used as a substrate). In contrast, French & Warren, (1967), although demonstrating that oestrone sulphate and *p*-nitrophenyl sulphate were inhibitors of each other's hydrolysis by human placental 'solubilized' enzyme preparations, claimed that the inhibition was not competitive in either case. Similar findings were made by Zuckerman & Hagerman (1969) for rat kidney microsomal preparations and these authors concluded that the two substrates were competing for two different catalytic sites.

Unequivocal proof of the existence of one enzyme must await its purification and this is likely to prove difficult. Attempts to obtain soluble preparations active towards *p*-acetylphenyl sulphate have been made by Dodgson *et al.* (1957*a,b*). Although a pseudo-soluble preparation was obtained by treatment of the microsomal pellet with the non-ionic detergent Lissapol-N, and a soluble preparation of low activity was obtained by treatment of microsomal fractions with crude pancreatic enzymes in the presence of Lissapol-N, preliminary studies with similar preparations have proved unhelpful. For example, rat liver microsomal fractions treated with Lissapol-N and washed with acetone as described by Dodgson *et al.* (1959) contained 21% of the original arylsulphatase C activity but only 7.5% of the oestrone sulphatase activity. Similarly, addition of Lissapol-N to such 'washed' preparations only slightly increased the arylsulphatase C activity (1.3-fold) but increased that towards oestrone [35 S]sulphate considerably (3-fold). This may well indicate that lipid material removed from the membranous component of the microsomal preparation may be of very different significance with respect to the hydrolysis of the small and relatively non-lipophilic substrate, *p*-acetylphenyl sulphate, than to the hydrolysis of the larger, relatively lipophilic steroid sulphate. In this connexion it is noteworthy that the truly soluble arylsulphatase of *Alcaligenes metalcaligenes* (Dodgson *et al.*, 1954*b*) readily hydrolyses *p*-acetylphenyl sulphate and *p*-nitrophenyl sulphate but is without appreciable activity towards oestrone [35 S]sulphate (J. O. Dolly, unpublished work). Similarly, the soluble lysosomal arylsulphatases A and B show no significant activity towards oestrone [35 S]sulphate (J. O. Dolly, unpublished work). Therefore at present reservations must be made about the results of any experiments with oestrogen sulphate esters if the integrity of the lipid component of the endoplasmic reticulum is markedly disturbed.

The finding of Zuckerman & Hagerman (1969) that rat kidney was the most active source of oestrogen sulphatase activity is difficult to understand. This is not attributable to differences in the strain of rats used since we have found liver to be the organ richest

in activity in Wistar rats as well as in animals of the M.R.C. hooded strain. Moreover, in the latter strain we have shown the liver to be the site of the desulphation *in vivo* of injected oestrone [35 S]sulphate (Dolly *et al.*, 1971).

The common identity of oestrogen sulphatase and arylsulphatase C would certainly help to clarify further the anomalous position of the arylsulphatase enzymes since their natural substrates are largely unknown. The physiological significance of oestrogen sulphatase to the rat is not clear although the amount present in the liver is sufficient to deal with quite high concentrations of oestrone [35 S]sulphate (Dolly *et al.*, 1971). Possible clues to the significance may come from the findings that oestrone and oestradiol-17 β were good product-inhibitors of the hydrolysis of their sulphate esters. This inhibition appeared to be complex in nature and it proved difficult to determine K_i values. However, oestradiol-17 β seemed to be a much better inhibitor than oestrone. It could be argued that this type of product inhibition indicates that the enzyme is concerned in some delicate regulation of free oestrogen concentrations. It is tempting to relate this possibility to the suggestions of Sunshine *et al.* (1971) about the role of oestrogens in the binding of ribosomes to the endoplasmic reticulum of the rat liver.

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