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Properties of Steroid Sulphatase and Arylsulphatase Activities of Human Placenta

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1. The properties of enzyme activities hydrolysing the sulphate esters of dehydroepiandrosterone, oestrone and *p*-nitrophenol are reported. The preparation studied was obtained from the microsomal fraction of human placenta by ultrasonic treatment and addition of Triton X-100. 2. The behaviour of the preparation during sedimentation at 105000*g* and attempts at purification indicated that the activities were particulate. Electron microscopy demonstrated the rupture of vesicular structures approx. 0·5μ in diameter concurrent with the release of activity. 3. The three activities were always associated throughout repeated attempts at separation by sucrose-density-gradient centrifugation and Sephadex-gel filtration. On the basis of kinetic studies, stability studies and treatment with butanol and ribonuclease it was concluded that a separate enzyme is responsible for each of the three activities. Widely varying plots of activity as a function of pH were consistent with this conclusion. 4. On the basis of sensitivity of the enzymes hydrolysing dehydroepiandrosterone sulphate and oestrone sulphate to ribonuclease and sensitivity of all three enzymes to lipase, it was concluded that the three enzymes are bound to a particle containing lipid and RNA. Enzymic activity is dependent on structural integrity of the particle. 5. A spectrophotometric method for the assay of oestrone sulphate hydrolysis is described. 6. Hydrolysis of nitrocatechol sulphate by human placenta under conditions described for arylsulphatases A and B is reported.

The capacity of human placenta to hydrolyse steroid 3β-sulphates was shown by Pulkkinen (1961) and Warren & Timberlake (1962). The subsequent demonstration in several Laboratories that steroid 3β-sulphate esters play a major precursor role in oestrogen biosynthesis in the pregnant human female (Baulieu & Dray, 1963; Siiteri & MacDonald, 1963; Warren & Timberlake, 1964; Bolte, Mancuso, Eriksson, Wiquist & Diczfalusy, 1964) has focused attention on placental sulphatase activity. Further studies have demonstrated that, though certain adult tissues contain this activity (Warren & French, 1965), it is essentially absent from those of the foetus (Pulkkinen, 1961; French & Warren, 1965), and that placenta is the richest human source. Sulphatase(s) capable of hydrolysing the 3-sulphates of dehydroepiandrosterone, oestrone, cholesterol and the sulphate ester of *p*-nitrophenol have been localized primarily in the microsomal fraction of placenta (French & Warren, 1966).

The present investigation was undertaken to evaluate (*a*) the number and specificity of placental enzymes responsible for hydrolysis of steroid and non-steroid sulphate esters and (*b*) the physical characteristics of these enzymes with particular reference to solubility.

EXPERIMENTAL

The following trivial names are used in this paper: oestrone for 3-hydroxy-1,3,5(10)-oestratrien-17-one; dehydroepiandrosterone for 3β-hydroxy-5-androsten-17-one; nitrocatechol for 2-hydroxy-5-nitrophenol.

Materials. Tris, *p*-nitrophenyl sulphate, oestrone sulphate, bovine pancreatic ribonuclease A, bovine pancreatic trypsin, soya-bean trypsin inhibitor, wheat-germ lipase, lecithinase C (type I) from *Clostridium welchii* and all non-radioactive steroids were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Labelled materials ([4-^{14}C]dehydroepiandrosterone and [4-^{14}C]oestrone) were purchased from New England Nuclear Corp., Chicago, Ill., U.S.A. Triton X-100 was purchased from Ruger Chemical Co., Irvington-on-Hudson, N.Y., U.S.A. Freeze-dried venom of the eastern diamond-back rattlesnake, *Crotalus adamanteus*, was purchased from the Ross Allen Reptile Institute, Silver Springs, Fla., U.S.A. All reagents were prepared with distilled deionized water.

Synthesis of [^{14}C]oestrone sulphate was according to British patent no. 829618, with sulphamic acid as the sulphating agent. The [^{14}C]dehydroepiandrosterone sulphate was synthesized with pyridine–sulphur trioxide

adduct (Roy, 1956). The resulting potassium salts were characterized by their ability to solubilize methylene blue in chloroform (Roy, 1957). Free steroid was removed from steroid sulphates by extraction with ether. Radioactive substrates were stored in water at −20°. Non-labelled oestrone sulphate and *p*-nitrophenyl sulphate were stored in crystalline form *in vacuo* at 4°. Urea and guanidine were recrystallized from 80% (v/v) ethanol before use. Snake-venom phospholipase was prepared according to the method of Bachmann, Allman & Green (1966) except that the pH was held at pH 7·0 throughout the procedure.

Methods. Protein determinations were performed by a biuret method (Rodwell, Towne & Grisolia, 1957), with human serum albumin as the standard. All pH values were determined with a Beckman expanded-scale pH-meter at 25°; values reported are corrected to 37° according to data supplied by the manufacturer. Sephadex was prepared as recommended by the manufacturer. Extinctions were determined with a Beckman model DU spectrophotometer in 3·0 ml. cells with a 1 cm. light-path. Quantitative determination of radioactivity was carried out as previously described (French & Warren, 1965). Centrifugations were done in Lourdes model LRA and Spinco model L centrifuges. Pellets for electron microscopy were fixed in 1·67% osmium tetroxide, dehydrated in *sym.*-collidine (2,4,6-trimethylpyridine) buffer, embedded in Epon, sectioned with a diamond knife, and examined with an RCA EMU 3F electron microscope.

Enzyme assays. Several methods, falling into two general categories, were used to determine activities. All assays were carried out at 37°. One unit of activity in each case is defined as that hydrolysing 1 μg. of substrate/hr.

Methods of type I utilized incubation in a water bath with continual oscillation at approx. 45 cyc./min. in conical glass tubes with ground-glass stoppers. The solution contained quantities of substrate stated below and 75 μmoles of tris hydrochloride buffer, pH 7·8. Total volume (including enzyme) was always 2·1 ml.

Assay I–1. A 0·25 μmole sample of [^{14}C]dehydroepiandrosterone sulphate or 0·26 μmole of [^{14}C]oestrone sulphate was used. The reaction was stopped by addition of 10 ml. of cold ether and the free steroid was extracted. A sample of this ether was plated, and the radioactive product rendered ether-soluble by hydrolysis was quantitatively determined. Free steroid, present in the substrate or solubilized owing to the effect of tissue (Burstein & Dorfman, 1963), was taken into account with zero-time controls in which substrate was added and immediately extracted. These values were always subtracted from those of the samples.

Assay I–2. For kinetic studies, assay I–1 was modified as follows. The reaction mixture, lacking only substrate, was warmed to 37°. The reaction was begun by addition of substrate and ended by addition of 0·1 ml. of 8 N-NaOH. The reaction time was 4·0 min. ± 10 sec. Subsequent treatment was identical with that of assay I–1.

Methods of type II utilized continuous recording of a change in extinction due to hydrolysis of a sulphate ester. Assays were carried out in a total volume of 3·0 ml. in cuvettes with a 1 cm. light-path. Optical and recording equipment used was a Beckman model DU monochromator fitted with a Gilford power supply and optical-density converter, a Honeywell recorder, and thermospacers maintaining a constant temperature of 37°. The cuvette contained 133 μmoles of tris hydrochloride buffer. Assays were begun by addition of substrate in 100 μl. of water.

Assay II–1. A 0·1 μmole sample of oestrone sulphate was used; the pH was 7·1. Increase in extinction due to hydrolysis of oestrone sulphate was measured at 290 mμ. Extinction was a linear function of oestrone concentration.

Assay II–2. Unless otherwise noted, 4 μmoles of *p*-nitrophenyl sulphate served as substrate; the pH was 7·1. Hydrolysis was followed spectrophotometrically at 400 mμ (Warren & Cheatum, 1966). Extinction was a linear function of *p*-nitrophenol concentration. When this assay was carried out in the presence of salts at high ionic strength, the pH of each salt–buffer solution was individually adjusted.

Preparation of microsomes. Routine preparation of the microsomal fraction was carried out by the following modification of the method of Schneider & Hogeboom (1950). Placentas were placed on ice immediately after delivery and all procedures were at 0–4°. Trophoblastic tissue was homogenized in a Waring Blendor: 3 g. of tissue/ml. of buffer in 0·05 M-tris hydrochloride buffer, pH 7·8, with 0·25 M-sucrose, and the homogenate was centrifuged 45 min. at 10000 *g*. The supernatant was recentrifuged for 1 hr. at 105000 *g*. The resultant microsomal pellet was suspended in water at a concentration such that 1·0 ml. of suspension was equivalent to 5·0 g. of fresh placenta and stored at −20°; activity was completely retained for at least 4 months.

RESULTS

Previous work in this Laboratory (French & Warren, 1966) has demonstrated that dehydroepiandrosterone sulphatase, oestrone sulphatase and *p*-nitrophenyl sulphatase activities are localized predominantly in the microsomal fraction.

In preliminary experiments carried out to evaluate release from the microsomal fraction, activity was followed with dehydroepiandrosterone sulphate as substrate. Portions of the frozen microsomes were thawed and diluted fivefold with water so that 1·0 ml. was equivalent to 1·0 g. of fresh placenta. Activity of this preparation was 300 units/ml. All subsequent procedures were carried out at 0–4° unless otherwise specified. After treatment, the preparation was centrifuged for 30 min. at 105000 *g* and presence of activity in the supernatant was the criterion for enzyme release. The ultrasonic treatment was carried out with an MSE ultrasonic oscillator at full amperage, the preparation being cooled in an ice–acetone mixture. Results of the various agents and conditions are shown in Table 1.

Because ultrasonic treatment released activity, subsequent procedures used microsomes so treated for five 50 sec. intervals as the starting material. Agents used to release activity are shown in Table 2. Sodium hydroxide extraction was carried out by the method of Person, Felton & Zipper (1965). All agents were added as solutions to the final concentrations shown. With urea and guanidine,

Table 1. *Agents used to release dehydroepiandrosterone sulphatase from microsomes*

Assay I–1 was used: 1ml. was equivalent to 1g. of fresh placenta. After the treatments indicated, the preparations were centrifuged at 105000*g* for 30min. and the supernatants assayed. Total activity was 300 units/ml. and equals 100%.

Agent	Conditions	Enzyme released (units/ml.)	Enzyme released (% of total)
Freezing and thawing	Once	0	0
	Five times	0	0
	Ten times	0	0
Butanol	5%	48	16
Ultrasonic treatment	30sec.	0	0
	60sec.	0	0
	120sec.	5	1·6
	180sec.	50	16
	240sec.	60	20
	300sec.	40	13·3

the pH was adjusted to the values indicated with hydrochloric acid. Triton X-100 added to a final volume of 0·1% (v/v) was most effective in releasing activity; other agents are ineffective and in no case did the released material tolerate 12hr. of centrifugation at 105000*g*, suggesting that little or no activity was actually solubilized.

Properties of the microsomal enzyme preparation

All further studies described below utilized an enzyme prepared with ultrasonic treatment and Triton X-100 as follows. Stored microsomes were thawed, diluted fivefold with water and treated ultrasonically for five 50sec. intervals at full amperage. Triton X-100 was added to a final concentration of 0·1% and the solution centrifuged for 30min. at 105000*g*. The supernatant was used as the standard preparation. It contained 3·5mg. of protein/ml. and hydrolysed dehydroepiandrosterone sulphate (150 units/ml. with assay I–1), oestrone sulphate (450 units/ml. with assay II–1) and *p*-nitrophenyl sulphate (200 units/ml. with assay II–2). In addition, hydrolysis of nitrocatechol sulphate was observed. Under the conditions described by Roy (1953) for assay of arylsulphatases A (pH4·7, 3mM-nitrocatechol sulphate) and B (pH5·7, 33mM-nitrocatechol sulphate) respectively, 95 and 100μg. of nitrocatechol sulphate were hydrolysed/hr./ml. of enzyme.

After release of activity with ultrasonic treatment and Triton X-100, attempts at purification of the standard preparation were carried out by chromatography on DEAE-cellulose, protamine sulphate precipitation, ammonium sulphate precipitation, calcium phosphate-gel adsorption and exposure to heat. None was successful. In every case where a pellet resulted, the activity could not be resuspended even with a second ultrasonic treatment and addition of Triton X-100.

On the basis of observations described above it was concluded that the procedure described, i.e. ultrasonic treatment, addition of Triton X-100 and centrifugation did not solubilize the enzyme(s), but rather suspended (released) particulate fragments with which the activities were closely associated. Various procedures, listed in Table 3, were unsuccessfully used in an attempt to effect true solubilization of these activities. In each case, resulting activity is represented as a percentage of a control in which conditions were identical except for the absence of the specified agent.

Sodium dodecyl sulphate and butanol were added to final concentrations of 1% (w/v) and 5% (v/v) respectively, and the mixture was centrifuged for 30min. at 105000*g*. Incubations of wheat-germ lipase and trypsin were carried out at pH7·0 at 37° and 25° respectively, with constant agitation. Lipase was removed from solution by centrifugation and trypsin was inhibited with an excess of soya-bean inhibitor.

Protamine sulphate was added as a 10% (w/v) solution and caused a heavy precipitate; the mixtures were centrifuged 30min. at 105000*g*, pellets were resuspended, and both pellet and supernatant fractions were assayed for dehydroepiandrosterone sulphatase and oestrone sulphatase by assay I–1. The activities of the pellets increased whereas those of the supernatants decreased. Thus the protamine sulphate caused redistribution, but not destruction, of the activities. Prior digestion with ribonuclease (30min. at room temperature) did not facilitate purification with protamine sulphate.

Electron micrographs. Plate 1 is an electron micrograph of the microsomal pellet before ultrasonic treatment; Plate 2 is an electron micrograph of the pellet obtained by centrifugation of the standard enzyme, prepared as described in the Experimental section, for 12hr. at 105000*g*.

Table 2. *Agents used to release dehydroepiandrosterone sulphatase from ultrasonically treated microsomes*

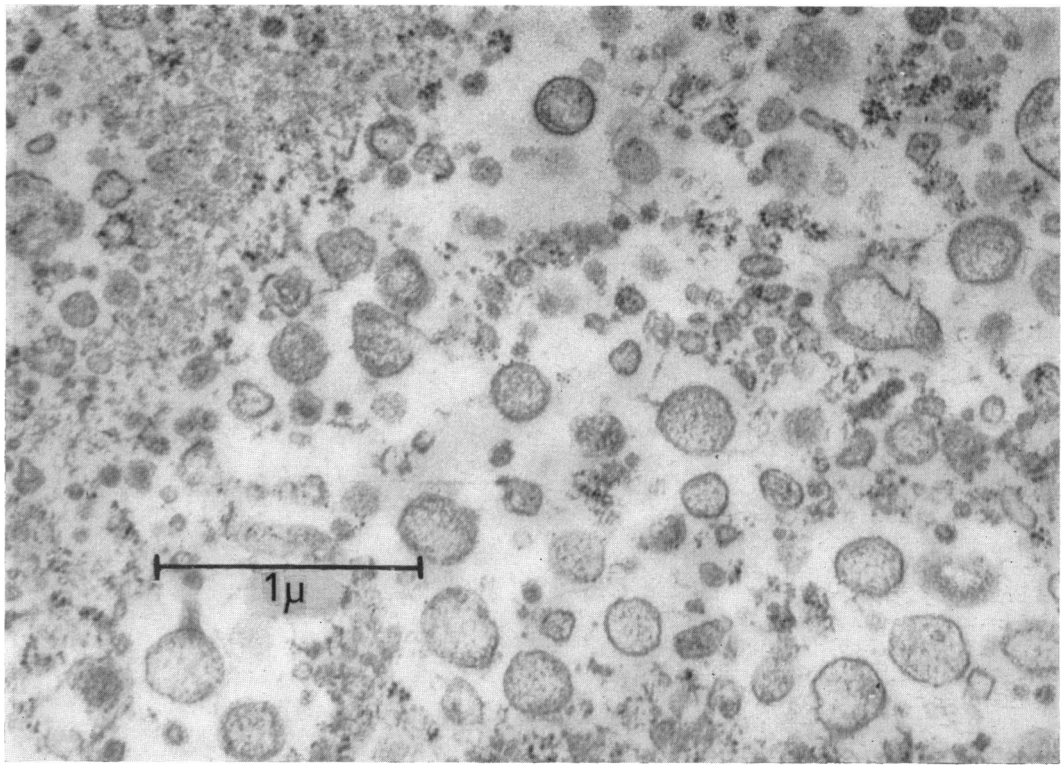
Microsomes were ultrasonically treated for five 50 sec. intervals before treatment in every case. The presence of dehydroepiandrosterone sulphatase in the supernatant after 30 min. of centrifugation at 105000*g* was the criterion of release used. In no case were any of the supernatant preparations active after 12 hr. of centrifugation at 105000*g*. Assay I–1 was used; 1 ml. of supernatant was equivalent to 1 g. of fresh placenta.

		Enzyme released	
Agent	Conditions	(units/ml.)	(% of total)
None	—	50	17
Digitonin	1%	0	0
Sodium dodecyl sulphate	1%	4	1
Sodium deoxycholate	0·1%	10	3
	0·3%	15	5
Guanidine	4·2 M, pH 8	21	7
	3·0 M, pH 5	0	0
Urea	5·5 M, pH 5	0	0
	5·5 M, pH 8	0	0
	2·8 M, pH 8	0	0
Butanol	5%	79	26
Ribonuclease	80 μg./ml.	0	0
	320 μg./ml.	0	0
Snake-venom phospholipase	0·5 mg./ml.	18	6
Lecithinase	1·25 mg./ml.	0	0
Lipase	1·4 mg./ml.	0	0
	2·7 mg./ml.	0	0
	12·2 mg./ml.	0	0
	24·2 mg./ml.	0	0
NaOH	pH 12·5	60	20
Tween 20	0·3%	30	10
	0·65%	11	4
	1·0%	13	4
Triton X-100	0·1%	150	50
	0·3%	96	32
	0·5%	72	24

Table 3. *Agents used to release dehydroepiandrosterone sulphatase from particles suspended by ultrasonic treatment and Triton X-100*

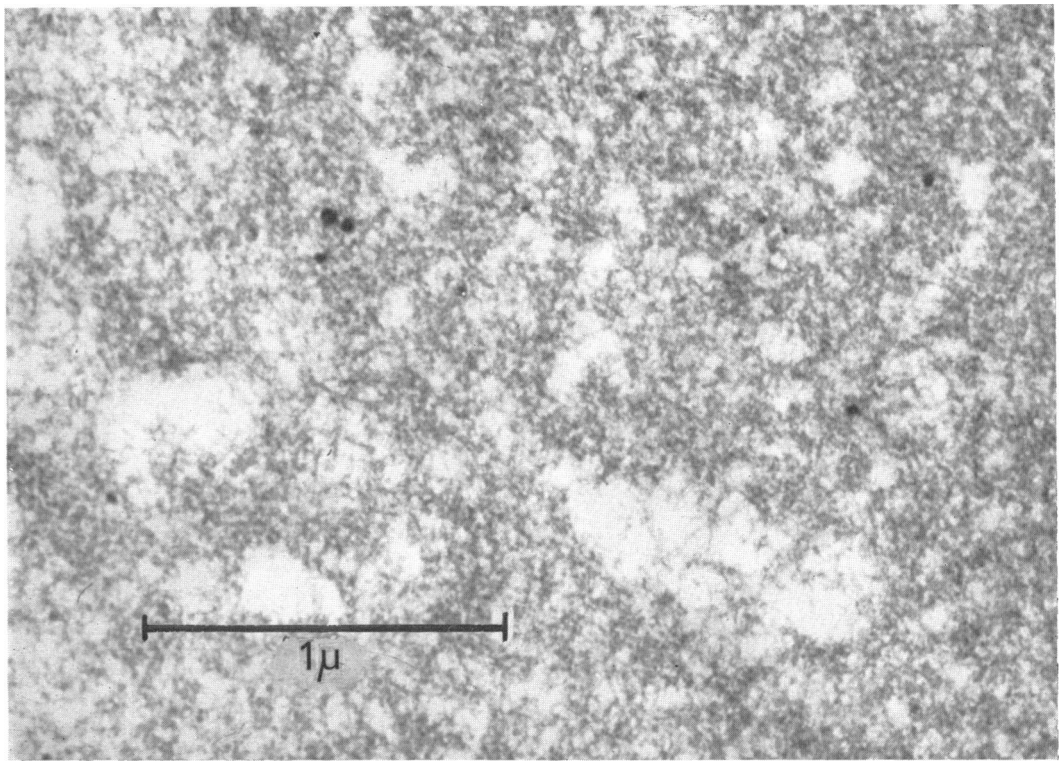
The starting material for these experiments was enzyme obtained by ultrasonic treatment, addition of Triton X-100 and centrifugation as described. Treatment with the agents was performed according to procedures described in the text. Assay I–1 was used for both dehydroepiandrosterone sulphatase (DSase) and oestrone sulphatase (OSase) activities. In no case were supernatant preparations active after prolonged centrifugation at 105000*g*.

		Enzyme released (% of control)	
Agent	Conditions	DSase	OSase
Sodium dodecyl sulphate	1%	2	—
Butanol	5%	20	—
Lipase	0·5 mg./ml., 45 min.	41	—
Trypsin	10 μg./ml., 10 min.	110	—
	10 μg./ml., 2·5 hr.	95	—
	10 μg./ml., 3·0 hr.	100	—
Protamine sulphate			
Pellets	0·0%	—	—
	0·5%	52	81
	1·0%	80	93
	2·0%	75	91
Supernatants	0·0%	100	100
	0·5%	21	35
	1·0%	21	31
	2·0%	21	37



EXPLANATION OF PLATE 1

Electron micrograph of the microsomal pellet. Precipitation of the microsomal pellet between 10000*g* and 105000*g* and fixation for electron microscopy are described in the Experimental section.



EXPLANATION OF PLATE 2

Electron micrograph of a pellet centrifuged from the standard enzyme preparation. A sample of enzyme was prepared by ultrasonic treatment of microsomes and addition of Triton X-100 in the usual manner. After centrifugation for 1 hr. at 105000*g*, the supernatant was centrifuged for 12 hr. at 105000*g* and the resultant pellet fixed for electron microscopy as described in the Experimental section. The final supernatant was devoid of activity.

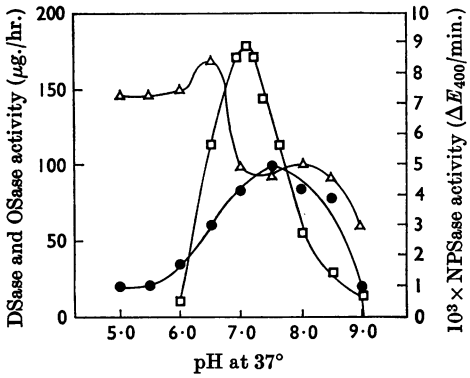


Fig. 1. Dehydroepiandrosterone sulphatase, oestrone sulphatase and *p*-nitrophenyl sulphatase activities as a function of pH. All assays were carried out in acetate buffer from pH 5 to 6 and in tris buffer from pH 6 to 9. Dehydroepiandrosterone sulphatase (DSase) (●) and oestrone sulphatase (OSase) (△) were determined by assay I–1 and are expressed in μg. hydrolysed/hr. *p*-Nitrophenyl sulphatase (NPSase) (□) was determined by assay II–2 and is expressed as ΔE_{400}/min. Each point represents two to four determinations; pH values were checked immediately after the assays.

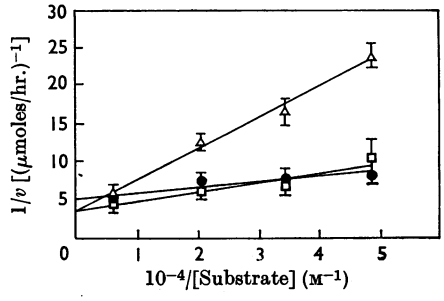


Fig. 2. Inhibition of dehydroepiandrosterone sulphate hydrolysis by oestrone sulphate and *p*-nitrophenyl sulphate. Assay I–2 was used. The graph illustrates the inverse of velocity with dehydroepiandrosterone sulphate alone (●) and in the presence of 100 μg. of *p*-nitrophenyl sulphate (□) and 25 μg. of oestrone sulphate (△). The lines were calculated by the method of least mean squares. Each point represents the mean and standard deviation of four determinations.

Vesicles present in the microsomal fraction are clearly absent from the preparation that had been treated ultrasonically and with Triton X-100. These electron micrographs, particularly in view of the sudden release of dehydroepiandrosterone sulphatase during ultrasonic treatment demonstrated in Table 1, strongly suggest the rupture of vesicular subcellular structures concomitant with release of sulphatase activity.

Effect of pH. Fig. 1 demonstrates the pH optima of the three activities. *p*-Nitrophenyl sulphatase was assayed by method II–2 with 100 μg. of substrate. Oestrone sulphatase and dehydroepiandrosterone sulphatase were assayed by method I–1 with acetate buffer from pH 5 to 6 and tris buffer from pH 6 to 9. Dehydroepiandrosterone sulphatase has an optimum at pH 7·5; *p*-nitrophenyl sulphatase has a very sharp optimum at pH 7·1. The curve for oestrone sulphatase is of particular interest. Two optima are present, one at pH 6·5 and another at pH 8·0. Since the substrate p*K* values are distant from the pH values used, the variations in activity with pH most likely reflect ionization changes at the active site or sites.

Site specificity

Because of failure to effect solubilization and further purification, the number and specificity of sites involved in the hydrolysis of dehydroepiandrosterone sulphate, oestrone sulphate and *p*-nitrophenyl sulphate were evaluated by kinetic analysis, stability, sucrose-density-gradient centrifugation and gel filtration.

Kinetics. Kinetic analysis was used to investigate the number of active sites. Fig. 2 shows inhibition of dehydroepiandrosterone sulphate hydrolysis by oestrone sulphate and *p*-nitrophenyl sulphate. Assays were carried out by method I–2, with 0·30 ml. of enzyme and 4·0 min. incubations. Each point represents the mean and standard deviation of at least four determinations. Intercepts were calculated by the method of least mean squares. The inhibition of dehydroepiandrosterone sulphate hydrolysis by oestrone sulphate appears to be competitive, suggesting that the site hydrolysing dehydroepiandrosterone sulphate also binds oestrone sulphate. At the concentration used (0·4 μmole) *p*-nitrophenyl sulphate had no observable effect. At the same concentration, however, *p*-nitrophenyl sulphate exerted a marked inhibitory effect on hydrolysis of oestrone sulphate. Fig. 3 illustrates the inhibition of *p*-nitrophenyl sulphate hydrolysis by dehydroepiandrosterone sulphate and oestrone sulphate. The inhibition is clearly not of the competitive type in either case.

Fig. 4 demonstrates inhibition of oestrone sulphate hydrolysis by dehydroepiandrosterone sulphate and *p*-nitrophenyl sulphate with assay II–1. Owing to consistently observed substrate inhibition at high concentrations of oestrone sulphate and sensitivity limitations at low concentrations, only a narrow range of substrate could be used. However, over this limited range, it is clear that the inhibitions are not of the competitive type.

Despite the inherent ambiguity of kinetic

analysis, particularly with an impure enzyme preparation, it would be difficult to reconcile these data with a single binding site (Dixon & Webb, 1964), since competitive inhibition was demonstrated in only one case.

Stability. Variation in the three activities with time at 56° (pH7·3) is shown in Table 4. Each value represents the mean of at least four assays. Enzyme was kept at 56 ± 0·5°. At the times shown, samples were taken out, cooled to 0° and assayed. Assays I–2, II–1 and II–2 were used. At 4·5hr. no significant differences were observed. At 9·0hr. and 14·5hr. values were significantly different from each other; in every case $P < 0·05$. If a single site is responsible for the hydrolysis of all three substrates, configurational changes must decrease the catalytic activity for the three substrates at different rates. Alternatively, more than one catalytic site is present.

Sucrose-density-gradient centrifugation. Gradients were constructed by carefully pipetting three 2·5ml. layers of decreasing sucrose concentration into Spinco no. 40 centrifuge tubes. The microsomal enzyme preparation (2·5ml.) was carefully layered on the top. Gradients were centrifuged for 3hr. at 105000*g* and slowed without use of the brake. After centrifugation the contents of the tube were divided into four fractions (from top to bottom) and samples were assayed. Repeated runs were

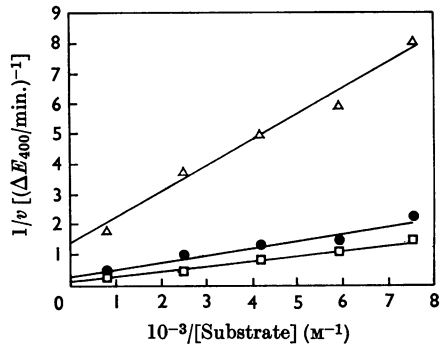


Fig. 3. Inhibition of *p*-nitrophenyl sulphate hydrolysis by dehydroepiandrosterone sulphate and oestrone sulphate. Assay II–2 was used. The Figure illustrates the inverse of velocity with *p*-nitrophenyl sulphate alone (□), in the presence of 10 μg. of dehydroepiandrosterone sulphate (●) and in the presence of 37·5 μg. of oestrone sulphate (△). Each point represents the mean of at least four determinations. The method of least mean squares was used to calculate intercepts and slopes.

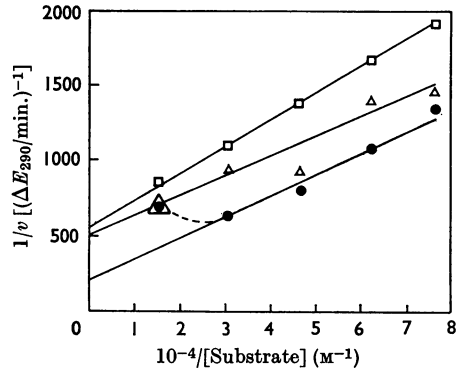


Fig. 4. Inhibition of oestrone sulphate hydrolysis by dehydroepiandrosterone sulphate and *p*-nitrophenyl sulphate. Assay II–1 was used. The Figure illustrates the inverse of velocity with oestrone sulphate alone (●), in the presence of 100 μg. of *p*-nitrophenyl sulphate (△) and in the presence of 50 μg. of dehydroepiandrosterone sulphate (□). Each point represents the mean of at least four determinations. The slopes and intercepts were calculated by the method of least mean squares. The point representing the highest concentration of oestrone sulphate alone was not used because of the obvious substrate inhibition.

Table 4. *Effects of various agents on microsomal dehydroepiandrosterone sulphatase, oestrone sulphatase and* p*-nitrophenyl sulphatase*

Experimental conditions are described in the text. Assays I–1, II–1 and II–2 were used for dehydroepiandrosterone sulphatase (DSase), oestrone sulphatase (OSase) and *p*-nitrophenyl sulphatase (NPSase) respectively. Values are expressed as percentages of a control in which a sample of the standard enzyme preparation was subjected to identical conditions except for the absence of the agent. In the case of heating the control sample was kept at 0°.

	Enzyme activity (% of control)		
Agent	DSase	OSase	NPSase
Ribonuclease	118	40	53
Butanol, 7·5%	90	114	42
Lipase	41	38	37
Trypsin	100	100	100
56°, 9hr.	38	18	29
56°, 14·5hr.	23	9	13

carried out with sucrose concentrations of 0·0M, 0·5M, 1·0M; 0·5M, 1·0M, 1·5M; 1·0M, 1·5M, 2·0M; 1·5M, 2·0M, 2·5M. No clear-cut separation of dehydroepiandrosterone sulphatase, oestrone sulphatase and *p*-nitrophenyl sulphatase was obtained in any case.

Gel filtration. Gel filtration was carried out four times with Sephadex G-200 on a column (2·25cm. × 30cm.) with a flow rate of 2·4ml./hr./cm.^{2} and eluents were collected in 7·0ml. fractions. The elution pattern consistently demonstrated complete exclusion without separation of the three activities by the Sephadex G-200 gel. Therefore all three either have a molecular weight in excess of 200000 or are associated with particles.

Nature of enzyme insolubility

The nature of the non-protein material associated with the activities and its relevance to enzyme activity and insolubility were investigated by the use of lipase, ribonuclease, trypsin and salt perturbation. All studies were carried out on the standard enzyme preparation described above.

Effect of trypsin. Digestion with trypsin was carried out with 0·01mg. of trypsin/ml. at pH7·1 at 25° for 3hr. without destruction of any activity (Table 4) despite extensive liberation of trichloroacetic acid-soluble material that absorbed at 280mμ.

Effect of ribonuclease. A 10ml. portion of enzyme was incubated for 2hr. at 25° at pH7·3 with 0·5mg. of bovine pancreatic ribonuclease. Oestrone sulphatase and *p*-nitrophenyl sulphatase activities were decreased whereas dehydroepiandrosterone sulphatase activity was slightly above control value. The absence of proteolytic activity from the ribonuclease was assured by control experiments in which yeast glucose 6-phosphate dehydrogenase was incubated in 0·05M-tris hydrochloride buffer at pH7·3 with and without ribonuclease in amounts and under conditions used above. No differences in activity were seen whether ribonuclease was present or not.

Ribonuclease-induced decrease in activity implies dependence on RNA. It is unlikely that RNA would be directly related to enzymic activity. A possible explanation involves a catalytic protein or proteins that are bound to an RNA-containing fragment, the structural integrity of which is required for tertiary structure of the enzyme(s) commensurate with activity.

Effect of lipase. Presence of a lipid moiety was investigated by incubating the enzyme preparation with wheat-germ lipase. A 10ml. portion of enzyme preparation was incubated with 21mg. of lipase at 37° at pH7·3 for 45min.; the lipase was removed by centrifugation at 85000*g* for 10min. This lipase incubation destroyed approx. 60% of all three activities.

The possibility of proteolytic activity in the lipase was eliminated by control experiments. These involved incubation of human serum albumin and human placental glutamate dehydrogenase in 0·05M-tris hydrochloride buffer, pH7·3, with and without lipase in amounts and under conditions equal to those used above for 45min. No differences in trichloroacetic acid-soluble extinction at 280mμ (albumin) or activity (glutamate dehydrogenase) were seen regardless of whether lipase was present or not. Therefore it may be inferred that the lipase exerts its effect by destruction of lipid, the presence of which is required for proper conformation of the enzymic protein.

Effect of butanol. Butanol was added to a concentration of 7·5%. Oestrone sulphatase, *p*-nitrophenyl sulphatase and dehydroepiandrosterone sulphatase were assayed by methods II–1, II–2 and I–1 respectively. Dehydroepiandrosterone sulphatase and oestrone sulphatase were not significantly changed by this treatment, whereas *p*-nitrophenyl sulphatase was markedly decreased. Butanol has been noted for its ability to destroy lipoprotein complexes (Morton, 1955). Sensitivity of *p*-nitrophenyl sulphatase to this agent further

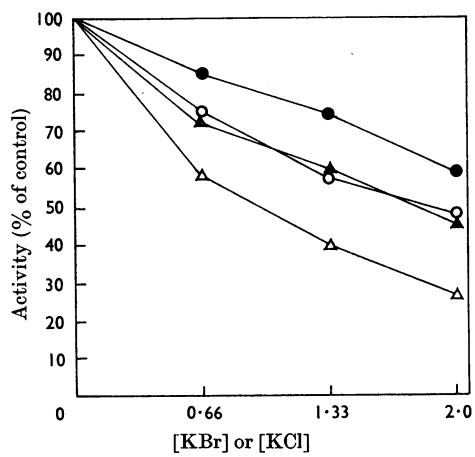


Fig. 5. Inhibition of *p*-nitrophenyl sulphatase by KCl and KBr after treatment with lipase. A sample of the standard enzyme preparation was incubated with 2·6mg. of lipase/ml. Assays II–2 of control enzyme (not incubated with lipase) were carried out with KCl (○) and KBr (△) and of lipase-treated enzyme with KCl (●) and KBr (▲) at the concentrations indicated. Values are expressed as percentages of treated or non-treated enzyme respectively, in buffer alone. The lipase-treated control had 37% of the activity of the non-treated control. Each point represents the mean of at least two determinations.

suggests the association of the enzyme with a membrane fragment.

The effects of the agents discussed above on the three activities are summarized in Table 4, where they are expressed as percentages of the appropriate control.

Effect of high salt concentrations. The sensitivity of *p*-nitrophenyl sulphatase to several concentrations of potassium chloride and potassium bromide before and after incubation with lipase and ribonuclease as described above was evaluated. Treatment with ribonuclease decreased activity to 50% of control value, but sensitivity to potassium chloride and potassium bromide was unchanged. Treatment with lipase decreased activity to 37% of the original value, but decreased sensitivity of the remaining activity to both salts was noted as shown in Fig. 5.

DISCUSSION

Pulkkinen (1961) has reported the hydrolysis of *p*-nitrophenyl sulphate but not dehydroepiandrosterone sulphate by foetal tissues; the latter observation was confirmed in this Laboratory (French & Warren, 1965). This observation would require the presence of at least two sulphatases in human tissues. Roy (1957) concluded on the basis of inhibition studies that *p*-nitrophenyl sulphate and dehydroepiandrosterone sulphate were hydrolysed by separate enzymes in liver.

Unequivocal proof of the number of enzymes responsible for the various sulphatase activities in placenta would be best obtained by extensive purification and physical separation of the respective proteins. Insolubility of the steroid and arysulphatases has prevented such purification and rigorous study of these enzymes. Nevertheless, the following evidence strongly suggests the presence in placental microsomes of three enzymes hydrolysing dehydroepiandrosterone sulphate, oestrone sulphate and *p*-nitrophenyl sulphate: (1) Competitive inhibition was demonstrated in only one of the six cases studied. (2) At 56° the three activities decreased at different rates. (3) Oestrone sulphatase and *p*-nitrophenyl sulphatase are sensitive to ribonuclease whereas dehydroepiandrosterone sulphatase is not, and *p*-nitrophenyl sulphatase is sensitive to butanol whereas oestrone sulphatase and dehydroepiandrosterone sulphatase are not. (4) The widely varying pH curves are compatible with this hypothesis since the pK values of the substrates are well below the pH values studied.

No firm conclusions may be drawn on the specificity of the three enzymes. The kinetic data suggest that the site hydrolysing dehydroepiandrosterone sulphate binds oestrone sulphate but not *p*-nitrophenyl sulphate.

The investigations described above provide the following evidence that the enzymes are tightly bound to a particle or membrane: (1) Release into supernatant is obtained only by vigorous ultrasonic treatment and use of detergent. (2) In no case was a preparation obtained that was not precipitated by prolonged centrifugation at 105000g. (3) The three activities, probably due to three separate enzymes, were always associated physically during sucrose-density-gradient centrifugation and Sephadex-gel filtration. (4) Oestrone sulphatase and *p*-nitrophenyl sulphatase were sensitive to ribonuclease, whereas all three activities were sensitive to lipase.

An enzyme might be tightly associated with the microsomal fraction in two ways. First, a soluble enzyme might be trapped in a compartment. Removal of such an enzyme from this compartment would probably produce a soluble, active enzyme. Another possibility is that of an enzyme tightly bound to a membrane, possibly by hydrophobic bonds between the hydrophobic side chains of the protein and a hydrophobic area of the membrane. In the former case, the sequence of amino acids is that of a soluble protein, and the relationship of hydrophobic to hydrophilic side chains required by an aqueous medium confers the proper tertiary structure (Fisher, 1964). In the latter case, however, destruction of the membrane would necessarily be accompanied by loss of activity since rearrangement of the tertiary structure would be required to provide a suitable environment for the newly exposed hydrophobic groups.

The experiments described above strongly suggest that the placental sulphatases are insoluble enzymes of the latter type. Lipase treatment rendered the *p*-nitrophenyl sulphatase less sensitive to high concentrations of potassium chloride and potassium bromide. These data imply a heterogeneity of the enzyme bearing particles with respect to some physical characteristic (Warren, Stowring & Morales, 1966). The simplest scheme consistent with these data is as follows. Ultrasonic treatment produces particles of various sizes. The enzyme molecules associated with the smallest particles are least protected from the disrupting effects of salt by the lipid; these small particles are also the more readily destroyed by lipase. Other enzyme molecules bound to larger fragments are less sensitive to salt; lipase removes part of the lipid but enough remains to exert a protective effect. Therefore incubation with lipase results in an overall decrease in salt-sensitivity.

Sensitivity of *p*-nitrophenyl sulphatase to potassium chloride and potassium bromide was not changed by treatment with ribonuclease although both *p*-nitrophenyl sulphatase and oestrone sulphatase are sensitive to ribonuclease. These

observations are most simply explained by the hypothesis that the enzymes are dependent on the structural integrity of RNA for activity.

The concept of solubility as applied to proteins has in the past rested on an operational definition; presence in the supernatant after centrifugation at 105000*g* has been the commonly accepted criterion. The results presented above demonstrate that, in some cases, a more rigorous definition, based on the relationship of hydrophobic to hydrophilic amino acid residues (Fisher, 1964), may be more useful. Dallner, Siekevitz & Palade (1965) have tentatively concluded that some enzymes may be incorporated into membranes directly after synthesis. Our results, particularly the sensitivity of the enzymes to ribonuclease and lipase, are nicely explained by this assumption.

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