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Report

Oestrone sulphate, adipose tissue, and breast cancer

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Summary

Oestrone sulphate, the oestrogen in highest concentration in the plasma, may play a role in the induction and growth of breast cancers. By enzymolysis and radioimmunoassay, oestrone sulphate concentrations were measured in 3 biological fluids. High concentrations of the conjugate (up to 775 nmol/l) were detected in breast cyst fluids from some premenopausal women, the concentrations in blood plasma (0.91–4.45 nmol/l) being much lower. Concentrations in the plasmas from postmenopausal women with (0.23–4.63 nmol/l) or without (0.18–1.27 nmol/l) breast cancer were still lower. Oestrone sulphate concentration in cow's milk or cream (0.49–0.67 nmol/l) was also low: dietary intake in these fluids is probably of little consequence. The capacity of breast tissues for hydrolysis of oestrone sulphate was examined in two ways:
1. In tissue slices incubated with 85 pM (^{3}H) oestrone sulphate solution at 37° C, cancers (131–412 fmol/g tissue/hr) and adipose tissues (23–132 fmol/g tissue/hr) hydrolysed significantly more sulphate than did benign tissues (1–36 fmol/g tissue/hr).
2. In tissue homogenates incubated with 5–25 μM [^{3}H] oestrone sulphate at 37° much higher capacities for hydrolysis (nmol/g tissue/hr) were demonstrated with a Km of 2–16.5 μM: cancers (34–394) and benign tissues (9–485) had significantly higher sulphatase activities than adipose tissues (9–39). On a protein basis, however, the sulphatase activities in the 3 tissues were comparable. It is concluded that oestrone sulphate is present in breast cysts and blood plasma and that *in vitro*, the conjugated hormone can be hydrolysed by breast tissues. The biological significance of these findings *in vivo* remains to be established.

Introduction

It is currently believed that for a cancer to arise in the breast, 1. a promoting oestrogenic environment and 2. a carcinogenic factor are needed (1). Thus the high incidence of breast cancers amongst western women may be related to unmodulated oestrogen action and to factors, as yet unknown, associated with the western diet (2). These beliefs are supported by the finding of a lower incidence of breast cancer in subjects with low oestrogen levels, eg men and oophorectomised women (3), and of a higher incidence in populations with a high dietary intake of fat (4), especially animal fat, or of milk (5).

The major circulating oestrogen in men and non-pregnant women is oestrone sulphate (6, 7). This hormone is also found in milk (8) and in high concentrations in the fluid of breast cysts (9). It is a major constituent of some preparations used to treat menopausal symptoms, a treatment which has been reported to be associated with a slight but

significant increase in risk of breast cancer (10). Although the conjugated hormone is, itself, biologically inactive, failing to bind to the oestrogen receptor (11, 12), it is readily cleaved by ubiquitous sulphatases in tissues (13), including established breast cancers (14, 15, 16), to yield the active hormone oestrone. A recent report (17) has suggested that this metabolism may represent the major pathway by which oestrogen is produced in the breast cancers of postmenopausal women. Equally, the hydrolysis of oestrone sulphate in the pre-malignant breast might play a significant role in providing the promoting, oestrogenic environment necessary for carcinogenesis. In this paper, therefore, we report on studies of the levels of oestrone sulphate in three biological fluids (milk, plasma, and cystic fluid) and of the capacity of tissues of the breast to cleave the conjugated hormone by hydrolysis.

Materials

Milk and cream: 4 samples of domestic milk and 2 samples of cream were purchased at random in Edinburgh and used on the day of purchase.

Plasmas: blood (10–80 ml) was collected from each of 10 normal males, 10 normal premenopausal women (5 of whom were taking oral contraceptives), 27 normal postmenopausal women who were volunteers attending the Edinburgh Breast Screening Clinic, 20 women with early breast cancer presenting at the Department of Clinical Surgery's Breast Clinic, and 10 women hospitalised for conditions not involving breast disease (including gastric ulcer, rectal prolapse, femoral hernias, and rectal carcinomas). The heparinised blood was placed on ice and centrifuged within 1 hour of collection. Plasmas were stored at −20° C until analysis.

Cyst fluids: fluid was collected by aspiration from each of 26 breast cysts in 18 patients with benign disease attending either the Departmental Clinic or the Edinburgh Breast Screening Clinic.

Mammary tissues: Breast tissues were obtained from patients with either breast cancer or benign disease, at the time of mastectomy or of diagnostic biopsy. For determination of oestrone sulphate hydrolysis by tissue slices, twenty-one samples of normal tissue, dysplastic tissue, or adipose tissue were obtained from a total of 17 patients. In addition, four breast carcinomas were obtained, two from two additional patients. One sample of adipose tissue was obtained from the abdomen of a male undergoing surgery for gallstones. An additional slice of each tissue was cut and fixed for tissue histology.

A further 50 samples of tissue (14 breast cancers, 21 benign dysplastic tissues, and 15 adipose tissues) were collected and, after removal of a slice of tissue for determination of oestrogen receptor activity and a second slice for fixation and tissue histology, were stored in liquid nitrogen. These samples were subsequently thawed, homogenised, and used for the determination of oestrone sulphatase activity in whole homogenates.

A single mammary tumour, which had been induced by the intragastric instillation of dimethylbenz(a)anthracene into a random-bred Sprague-Dawley female rat, was also used, after storage in liquid nitrogen, as a control tissue for the determination of oestrone sulphatase activity.

Methods

Four main studies were performed: a. measurements of the levels of oestrone sulphate in biological fluids, b. examination of the capacity of mammary tissues to hydrolyse oestrone sulphate in tissue slices, c. examination of the activity hydrolysing oestrone sulphate in adipose tissue, and d. determination of oestrone sulphatase activity in tissue homogenates.

a. Determination of oestrone sulphate in biological fluids

Oestrone sulphate was determined, after removal of the free oestrogens and fat by extraction with ether and petroleum ether, by enzymic hydrolysis and assay of the oestrone released using a specific antibody generated against 6-oxo-oestrone conjugated to bovine serum albumin. The oestrone content was read from a standard curve derived from

known quantities of oestrone sulphate potassium salt (4–400 pg) which had been similarly processed from enzymolysis onwards. Values were corrected for manipulative losses assessed from the recovery of tracer (^{3}H) oestrone sulphate added initially. Recoveries were 66.1% ± 2.1% SD (n = 21) from milk and cream, 39.5 ± 6.5% (n = 124) from plasma and 80.5 ± 6.4% (n = 81) from cystic fluids. Blanks were 2.9 ± 1.1 (SD) pg ≡ oestrone sulphate (n = 12). All concentrations were expressed as nmol oestrone sulphate/litre.

The accuracy of oestrone sulphate measurements, tested on plasma from a male, was described by the equation y, pg recovered = 1.02× + 3.4 where x = mass added (pg), after correction for the endogenous content of oestrone sulphate.

The specificity of oestrone sulphate determinations was examined in three ways. Firstly, when samples were analysed by the procedure described above plus an additional purification step consisting of chromatography (18) on Sephadex LH-20, the apparent concentration detected was decreased to some extent in plasma (mean −22.1%, n = 3) but only marginally in cyst fluids (mean −4.4%, n = 2). Secondly, since DHA has a low but finite cross-reaction (0.25%) with our antibody against oestrone, the possibility that DHA, derived from the large amount of DHA sulphate which may be present, particularly in cyst fluids (19), might be interfering with the assay was tested. When samples were analysed by a procedure including an additional, alkaline partition step (effective in removing 99.1% of any DHA present with concomitant recovery of 51.3% of the oestrone), the apparent oestrone sulphate concentration was only slightly reduced in plasma (mean −12.3%, n = 4) or cyst fluids (mean −8.8%, n = 19), by comparison with the values found by the standard procedure outlined above. Thirdly, when samples of cyst fluid were analysed by (a) enzymolysis and radio-immunoassay ('RIA', as described above) or (b) enzymolysis, alkaline partition, and gas-liquid chromatography with mass spectrometry ('GLC/MS'), the apparent oestrone sulphate concentrations found by the two techniques were strongly correlated (r = 0.93, n = 8), though the values obtained by GLC/MS were, on average, higher than those found by RIA (mean + 21.5% n = 8), perhaps due to the greater sensitivity of the latter technique. In milk only, oestradiol-17β and oestrone concentrations were also determined by ether extraction, chromatography on Sephadex LH-20, and radioimmunoassy with specific antibodies for each steroid (based on Kelch, Kaplan and Grumbach (18)).

b. Assessment of the capacity of tissue slices to hydrolyse oestrone sulphate

Slices of benign, normal, or adipose tissue (two per flask approx 100–500 mg each) were cut and incubated in duplicate, for 2 hours at 37° C in 2 ml bicarbonate Ringer with (^{3}H) oestrone sulphate (approx 10,000cpm, i.e. 170 fmol, purified (7) before use). In addition, slices from four breast cancers known to contain oestrone sulphatase activity were also incubated as 'positive controls'. After incubation, the medium and slices were separated and stored, each with one drop of concentrated aqueous ammonia solution, at −20° C until processing, separately. The stored slices were cut up finely with scissors and homogenised in a total of 4 ml water. The homogenate was shaken twice with 10 ml diethyl ether and the phases were left to separate. The combined ether extract (20 ml) was backwashed with 1ml of water, evaporated to dryness and counted. The aqueous phase and backwash were pooled and an aliquot (1/10) was counted. The medium from each flask was thawed and similarly partitioned between water and ether, and the two phases were counted. Samples were counted in 10ml 'Picofluor 15' scintillator and a correction was made for quenching (considerable in the extracts from adipose tissue) by addition of internal standard to each vial and recounting. Where possible, three kinds of control experiment were performed with each tissue:

1. 'Incubated substrate' control in which (^{3}H) oestrone sulphate was incubated alone for 2 hours at 37° C, cooled in ice, then added to tissue slices on ice and re-separated, after 5 minutes, into tissue and medium, for storage and processing.

2. 'Homogenisation control' in which unincubated tissue was stored at −20° C and to which (^{3}H)

oestrone sulphate was added immediately prior to homogenisation (no 'incubation medium' fraction in this control).

3. 'Boiled tissue control' in which tissue slices were boiled for 30 minutes at 100° C prior to incubation with (^{3}H) oestrone sulphate and storage for processing as above. These control experiments showed that approximately 0.1–5.0% of the substrate could break down during processing or under non-physiological conditions (mean values + 1SD) – incubated substrated controls: tissue 0.55 ± 0.18%, medium 1.89 ± 1.52% (n = 28); boiled tissue controls: tissue 0.25 ± 0.16%, medium 0.61 + 0.14% (n = 11); homogenisation controls: tissue 0.43 + 0.25% (n = 27). After correction for the apparent hydrolysis using the appropriate control for each tissue, the capacity for hydrolysis was expressed as fmol oestrone sulphate cleaved per gram tissue per hour incubated.

c. Additional investigations of the hydrolysis of oestrone sulphate by adipose tissue

In two experiments, slices of mammary adipose tissue were incubated with increasing concentrations of (^{3}H) oestrone sulphate over the range 10^{-9} to 10^{-3} molar, and processed as described above.

In a third experiment, two sets of slices of mammary adipose tissue were incubated with 100,000 cpm (^{3}H) oestrone sulphate, one set in the presence, the other in the absence, of serum albumin (4 g/100 ml), to examine the effect of this binding protein on hydrolysis. In a fourth experiment, slices of mammary adipose tissue were similarly incubated with 100,000 cpm (^{3}H) oestrone sulphate either in Krebs-Ringer or in plasma from a male.

In a fifth experiment, adipose tissue was fractionated in an attempt to localise and further identify the nature of the hydrolytic activity. Portions of adipose tissue were homogenised in Krebs bicarbonate buffer, with or without detergent (sodium dodecyl sulphate 1% v/v, or Miranol[20] 1% v/v). The homogenate was centrifuged at 1460 × g to yield floating lipid, soluble protein and insoluble (pellet) fractions. Each fraction was incubated with approximately 100,000 cpm (^{3}H) oestrone sulphate and the products were partitioned as described above.

d. Determination of oestrone sulphatase activity in whole homogenates

The method used was based on those of other workers (20, 21, 22). Tissue (50 mg for cancers, 100mg for benign tissues, 200mg for adipose tissue) was cut up finely with scissors and homogenised, on ice, in 1.5 ml tris-monothioglycerol-glycerol buffer (tris 10mM, sucrose 0.25M EDTA 1 mM, pH 8.0, containing monothioglycerol 1% v/v and glycerol 10% v/v) once for 20 seconds and once for 15 seconds with an interval of 45–60 seconds for cooling, using a Silverson homogeniser. A further 0.5 ml of buffer containing 4% (v/v) Miranol was added and a short burst (10 seconds) of re-homogenisation was effected. The concentration of homogenate was adjusted to 25 mg/ml (cancers), 50 mg/ml (benigns) or 100 mg/ml (adipose) by the addition of buffer containing 1% (v/v) Miranol.

One hundred microlitres of whole homogenate was added to each of duplicate tubes containing 300 μl tris buffer and 100 μl of (^{3}H) oestrone sulphate (specific activity adjusted to 9.1 mCi/mmole) to yield final concentrations of substrate 2, 5, 10, 15, 20, and 25 μM (final incubation volume 500 μl). The tubes, along with 6 additional 'blank' tubes containing aliquots of boiled homogenate (boiled previously 1 hour) and 3 selected substrate concentrations (2, 15, and 25 μM) were incubated at 37° C for 30 minutes. After incubation, the reaction was arrested by the addition of 0.1 ml 1M Na_2CO_3 solution to each tube. The contents of each tube were extracted twice with 5 ml organic scintillator (toluene 240: 'Scintol-7' 10: ethanol 5, v/v) and the combined scintillator extract was washed with 0.1 ml water before transfer to a counting vial and determination of the ^{3}H content (oestrone produced) at an efficiency of approx. 56.4%. The ^{3}H activity of the substrate (oestrone sulphate) was determined by counting in aqueous scintillant ('Picofluor-15', Packard Instrument Co.) at an efficiency of approx. 49.5%. The recovery of (^{3}H) oestrone by this extraction with organic scintillant was approx. 97% whilst that of the substrate was only 0.15%. After correction for the apparent hydrolysis found in the presence of boiled homogenate (values derived by interpolation of a linear

plot of the 3 selected blanks), the cpm of oestrone released by hydrolysis were converted to μmoles oestrone produced per litre and the data were analysed by the Hanes plot (23) (s against s/v). From this plot, the Km and V_{max} for the enzyme were read and expressed ultimately in μmol/l and nmol/hr/g or p mol/min/mg protein respectively, the protein concentration in each homogenate being determined by the method of Bradford (24), using bovine serum albumin in Miranol (1% v/v) solution as standard.

Results

Oestrone sulphate concentrations in biological fluids

Relatively low and constant concentrations of oestrone sulphate were found in the samples of domestic milk and cream (Table 1, Figure 1). The levels of oestrone and oestradiol in these fluids were still lower, being of the order of 0.08–0.29 and 0–0.09 nmol/l respectively (not shown). The levels of oestrone sulphate found in the plasmas of postmenopausal women, either normal or hospitalised for conditions other than breast disease, were comparable to those found in milk and cream,

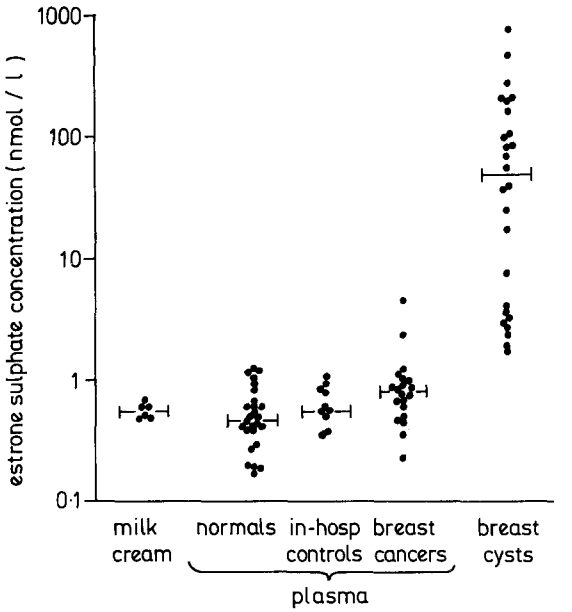

Fig. 1. The concentrations of oestrone sulphate in 3 types of biological fluid. For the plasmas, the concentrations in patients with breast cancer were significantly higher ($p<0.05$, Wilcoxon rank test) than those found in the normal patients. Horizontal bars indicate the median values.

whilst the level in women with breast cancer was slightly elevated. This increase in plasma oestrone sulphate concentration was significant when compared with the level found in normal subjects but

Table 1. Oestrone sulphate concentrations in biological fluids.

Fluid	Number analysed	Oestrone sulphate (nmol/l)	
		Range	Median
Human plasma			
– men	10	1.18–3.19	1.66
– premen. women	10	0.91–4.45	2.54
– postmen. women			
normal	27	0.18–1.27	0.46
br. cancer	20	0.23–4.63*	0.81*
hosp. controls	10	0.34–1.05	0.55
Bovine milk	4	0.49–0.67	0.57
Bovine cream	2	0.49–0.51	0.50
Human breast cyst fluid	26	1.74–77.51	47.5

br = breast, hosp. = hospitalised for non-breast diseases. Each sample was estimated in triplicate or quadruplicate.
* value significantly higher ($p<0.01$) than that found in normal subjects, but not significantly different from that in hospitalised controls. (Wilcoxon Rank test)

not when compared with the value found for patients hospitalised for other, non-breast diseases. In normal men and premenopausal women, concentrations of the conjugate were higher, and in breast cyst fluid, a wide range of concentrations from low to extremely high was detected.

Capacity of slices of breast tissues to hydrolyse oestrone sulphate

The results of the studies with tissue slices are shown in Figure 2 and summarised in Table 2. The four breast cancers examined were found to show a high rate of oestrone sulphate cleavage: the considerable quantity of oestrone produced was present in both tissue and incubation medium (Table 2). The extent of hydrolysis in these tissues corresponded to metabolism of 25.5–55.4% of the substrate present over a 2 hour period. In normal breast or benign dysplasia, a much lower rate of cleavage was observed, with the bulk of the oestrone remaining in the tissue slice. In adipose tissue, whether from biopsy of patients with benign disease or from the mastectomy specimen of patients with breast cancer, higher rates of cleavage were observed. In two benign tissues, which upon histological examination were found to contain an appreciable proportion of fat, an intermediate rate of hydrolysis was observed (Table 2). In a single specimen of abdominal adipose tissue from a male (not shown), a rate of hydrolysis of 45 fm/g/hr, similar to that seen in mammary fat, was observed.

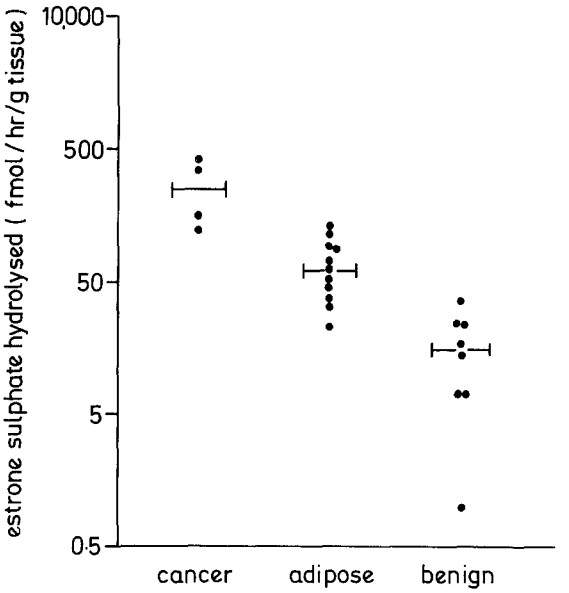

Fig. 2. The capacity of slices from three types of breast tissue to hydrolyse [^{3}H] oestrone sulphate (85 pM) at 37° C: cancers (n = 4), adipose (n = 11), and benign (n = 8). Each value represents the sum of hydrolysis in both tissue slice and incubation medium, and is derived from 1–5 determinations. The hydrolysis in slices of adipose tissue was significantly greater ($p<0.01$, Wilcoxon rank test) than that seen in slices of benign breast. Horizontal bars indicate the median values.

Table 2. The capacity to hydrolyse oestrone sulphate in slices of human breast tissue.

Breast tissue	Number studied	fmol oestrone sulphate hydrol/g tissue/hour		
		Medium	Tissue	Total
cancers	4	128	107	235
adipose				
– ex-benign	2	3	122* *	125
– ex br. ca.	9	3	50* *	53
adipose + benign	2	38	38	76
benign				
– ex-benign	4	7	13	20
– ex br. ca.	4	3	8	11

ex benign = from patients with benign breast disease; ex br. ca. = from patients with breast cancer.

* Median value. Each value was derived from 1–5 estimations, and has been corrected for spontaneous breakdown of substrate (0.1–5.0%).

* * Hydrolysis in the adipose tissue slices was significantly greater than that found in the benign slices by Wilcoxon Rank test, $p<0.01$.

Additional studies on oestrone sulphate hydrolysis by mammary adipose tissue

When, in two experiments, adipose tissue was exposed to increasing levels of (^{3}H) oestrone sulphate, the amount of oestrone produced by hydrolysis increased proportionately until about 10^{-4} molar sulphate, when some evidence of saturation was obtained.

Incubation of slices of adipose tissue with (^{3}H) oestrone sulphate either in medium containing serum albumin, or in pure human plasma (Table 3), lead to the abolition of sulphate cleavage by comparison with the values found in other slices, incubated with substrate in buffer only.

Tissue fractionation experiments suggested that the sulphatase activity could be detected in all three fractions; fat, soluble protein, and the pellet. Attempts to extract the activity with sodium dodecyl sulphate solution were complicated by the fact that the activity was almost completely (87%) inhibited by the detergent. However, with the addition of Miranol at both homogenisation and rehomogenisation of the fat and insoluble fractions, part of the activity (56%) became soluble. Further fractionation[20] of this soluble fraction showed that the sulphatase-like activity could be concentrated and partially purified in a manner similar to that used to purify the oestrone sulphatase of rat liver. Using this partially purified extract, an apparent K_m of approximately 10–20 μM was found for the oestrone sulphatase activity.

Measurement of oestrone sulphatase activity in whole homogenates of breast tissues

In view of the presence of sulphatase activity in several different subcellular fractions, whole homogenates were used to determine the maximum activity which could be detected at saturating levels of substrate. At 37° C, the rate of sulphate cleavage was approximately linear for at least 30 minutes in homogenates from a breast cancer, a benign tissue, mammary adipose tissue, and a DMBA-induced rat mammary tumour (Figure 3), and in a breast cancer, hydrolysis was proportional to the mass of homogenate present up to approx. 3 mg tissue (not shown). The rate of hydrolysis approached a maximum at substrate concentrations of 20–25 μM for each type of tissue, whilst the apparent hydrolysis in boiled tissue blanks was linearly related to substrate concentration (Figure 4). Typical Hanes plots of these assays are shown in Fig. 5.

Sulphatase activity was detectable in all the tissues examined. Per unit weight of tissue, activity was higher in breast cancers than in benign tissues which, in turn, contained higher levels than those found in fat (Figure 6). The difference between the

Table 3. Effect of serum albumin or plasma on the cleavage of (^{3}H) oestrone sulphate by slices of mammary adipose tissue.

Pat. no.	Incubation medium	fmol oestrone sulphate cleaved/g/hour		
		Medium	Tissue	Total
1 (Age 46)	KRB	0	504	504
		9	617	626
	KRB + albumin (4 g%)	2	1	3
		7	8	15
		1	1	2
2 (Age 75)	KRB	25	499	524
		18	408	426
	human plasma	5	4	9
		0	3	3

KRB = Krebs' bicarbonate Ringer.
Slices were incubated in 2 ml medium containing 1700 fmoles of (^{3}H) oestrone sulphate. Incubations were carried out in duplicate or triplicate; the result for each flask is shown separately. Values are corrected for spontaneous substrate breakdown (0.1–5.0%).

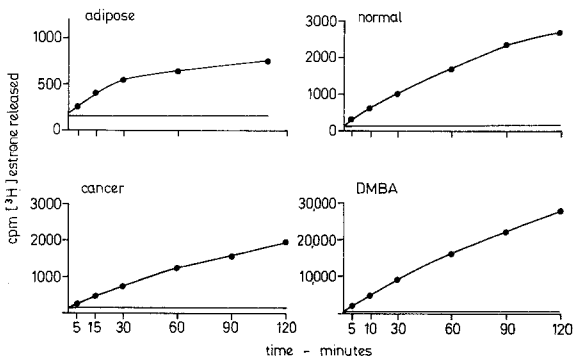

Fig. 3. The time course of hydrolysis of (^{3}H) oestrone sulphate (10 μM) at 37° C in homogenates from 4 different types of tissue: 3 human breast tissues and one rat mammary carcinoma (DMBA). The lower line in each group represents spontaneous hydrolysis in boiled control tissues.

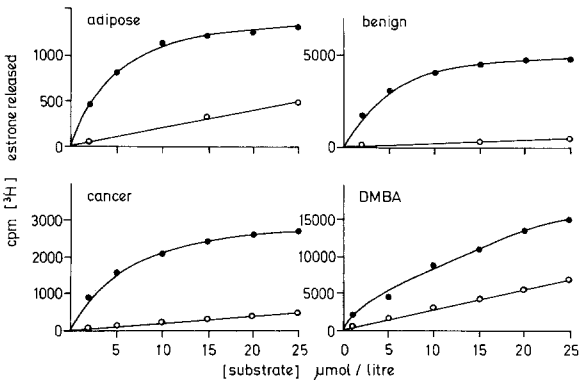

Fig. 4. Oestrone sulphatase activity in homogenates from 4 types of tissue: 3 human breast tissues and one rat mammary carcinoma (DMBA). Homogenates (25–100 mg/ml) were incubated with each of 5 concentrations of (^{3}H) oestrone sulphate for 15 or 30 mins. at 37° C. Boiled homogenate served as a control. Each point represents the mean of duplicate determinations.

levels of sulphatase activity in fat and those found in either benign or cancerous tissues was statistically significant ($p<0.01$, Wilcoxon rank test), whilst the difference between cancers and benigns was insignificant. The highest sulphatase activity observed was found in a benign tissue from the youngest pätient examined (fibroadenoma from a 14-year-old girl).

When sulphatase levels were expressed relative to the total concentration of protein in the homoge-

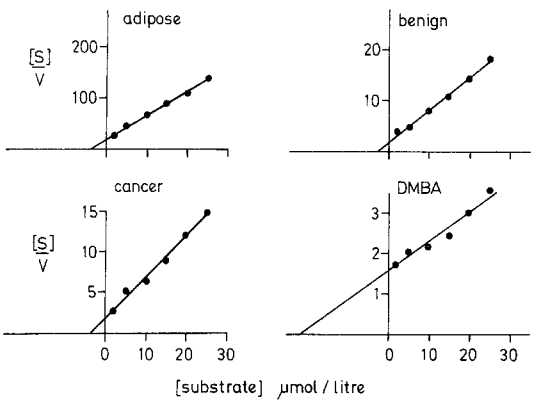

Fig. 5. Oestrone sulphatase activity in homogenates from 4 types of tissue: Hanes' plots of the data from Figure 4, after correction for spontaneous substrate breakdown seen in boiled controls; s = substrate, v = reaction velocity.

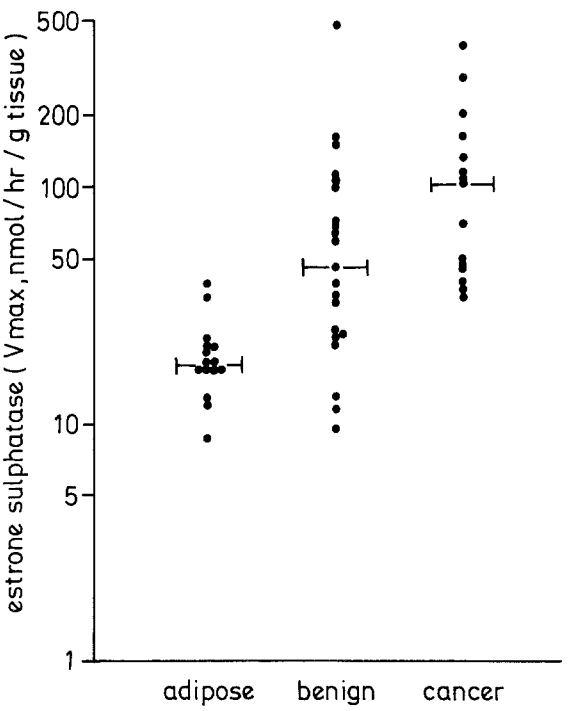

Fig. 6. The oestrone sulphatase activities (V_{max}) per gram tissue in homogenates from three types of breast tissue: adipose (n = 16), benign (n = 21), and cancers (n = 14). Each value was derived by assay at 5 substrate concentrations and analysis according to Hanes. Sulphatase activity in the benign or cancerous tissues was significantly higher ($p<0.01$, Wilcoxon rank test) than that found in adipose tissue. Horizontal bars indicate median values.

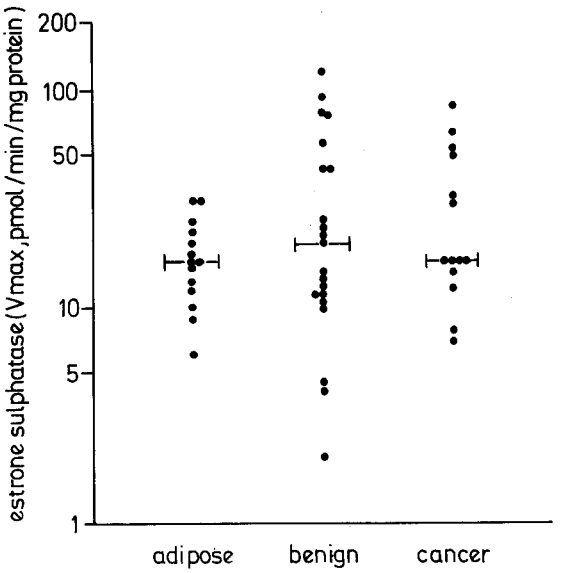

Fig. 7. The oestrone sulphatase activities (V_{max}) per mg protein in homogenates from 3 types of breast tissue: the same data as shown in Figure 6 have been re-expressed relative to total homogenate protein. There were no significant differences between the groups (Wilcoxon rank test). Horizontal bars indicate median values.

nate, all 3 types of tissue contained comparable levels of enzyme activity (Figure 7).

The Km values for the enzyme ranged from 2.5–16.5 μM in breast cancers (median 5.9), 3.4–16.1 μM in benign breast (median 6.8), and 2.0–8.4 μM in fat (median 4.4). These data are summarised in Table 4.

Discussion

These studies confirm an earlier report (9) that some breast cystic fluids contain high levels of oestrone sulphate (1–775 nmol/l) by comparison with the levels found in plasma (approx. 0.1–4.6 nmol/l). A more recent study (25) by the same group has shown that the most dramatic differences between cyst fluid and plasma are seen in oestriol sulphate amongst the sulphates of the 3 classical oestrogens, and has suggested that these compounds may derive from synthesis by the epithelial cyst lining.

The values found here for the concentration of oestrone sulphate in plasma are in good agreement with those found by previous workers (21, 26–29). In plasma, oestrone sulphate levels were slightly higher in patients with breast cancer than in normal women ($p<0.05$), or in subjects hospitalised for non-breast diseases (N.S.). This slight difference may be associated with a greater degree of stress in the patients with breast cancer, but has also been reported by some other workers (21).

Although oestrone sulphate has been reported to be present in the milk of domestic animals (8, 30), derived, at least in the goat, by both production and concentration in the mammary gland (30), the levels detected in commercially available samples of milk and cream were low and similar in magnitude to those found in the plasmas from postmenopausal women (Figure 1). The levels of

Table 4. Oestrone sulphatase activity in homogenates of human breast tissues.

Tissue	Number studied	Km (μM)	Activity (V_{max})	
			nmol/hr/g tissue	pmol/min/mg protein
cancers	14	5.9	84	16
		(2.5–16.5)	(34–394)	(7–82)
benigns	21	5.8	46	19
		(3.4–16.1)	(9–485)	(2–119)
adipose	15	4.4	18	16
		(2.0–8.4)	(9–39)	(6–30)

Values represent the medians and in parentheses, range.
Homogenates (25 mg/ml for cancers, 50 mg/ml for benign, or 100 mg/ml for adipose) were incubated with 5–25 μM (^{3}H) oestrone sulphate, each in duplicate, at 37° C for 30 minutes. Values were corrected for the spontaneous breakdown of substrate in controls containing boiled homogenate.

the non-conjugated oestrone and oestradiol, which might be formed upon metabolism of the conjugate in milk, were also low. It can be calculated that for persons consuming 1–2 pints of milk per day, the intake of oestrogen by this means constitutes about 0.09–0.18 μg equivalents, compared with plasma production rates of 77 and 95 μg/day in men and women (follicular phase) respectively (31). It seems unlikely, therefore, that dietary intake of oestrogen by this means would be of significance.

The potential to cleave oestrone sulphate was found in to be present in tissue slices from the four malignant and 21 nonmalignant mammary tissues examined. The finding that malignant breast can cleave the sulphate is in agreement with previous reports (14, 17, 21, 22), and one report (17) indicates that this activity may constitute the major pathway for the production of oestrogen in postmenopausal breast cancers. Both adipose and non-adipose components of breast had the capacity of hydrolyse the conjugate, but in slices the fat tissue was more active than the other non-malignant tissues, having a capacity approaching that of the four established breast cancers examined. The hydrolytic activity in adipose tissue appeared to be enzymatic since it was (1) destroyed by boiling, (2) inhibited by sodium dodecyl sulphate, (3) extracted with Miranol, (4) concentrated and purified by precipitation with ammonium sulphate and ethanol (20), and (5) half-saturated with substrate at a concentration of 10–20 μM in partially purified extracts, or at 2–8 μM in whole homogenates. In these properties, it resembles the sulphatase derived from rat liver (20).

When sulphatase activity was determined by standard enzymological techniques in whole homogenates, rather than in tissue slices, a much higher (10^6 times) capacity for sulphate cleavage was apparent in all three types of breast tissue studied. In homogenates, breast cancers again showed higher rates of activity than those seen in benign tissues, but in contrast to the situation seen in tissue slices, benign tissues showed higher activity levels than adipose tissue. When, however, the specific enzymic activities (sulphatase/total homogenate protein) were compared, the levels were similar in all three tissues. The levels of sulphatase activity found in the present work agree with those reported by others (17, 21, 22), though being perhaps slightly lower than those observed by one group (17).

The large difference in magnitude of sulphatase activity detected by the slice and homogenate techniques reflects the very considerable differences in substrate availability and structural integrity in the two preparations. The level of substrate used for the slice technique (85 pM) is subphysiological in relation to plasma levels in postmenopausal women (approx. 180–1270 pM), whereas that used for saturation of the enzyme in homogenates (5–25 μM) is grossly non-physiological. It seems likely that the real capacity for sulphatase activity lies somewhere between these two extremes.

To our knowledge, this sulphatase activity of adipose tissue has not been studied previously. Adipose tissue has thus now been reported to contain three enzymes which may affect the oestrogenic environment: aromatases converting androgen to oestrogen (32–34), an oxido-reductase which interconverts oestrone and oestradiol (35), and sulphatase capable of releasing oestrone from its conjugate. The presence of these enzymes, and in particular aromatase, in adipose tissue is in accord with the finding that in obesity, the circulating levels of oestrogen are increased in proportion to excess body weight (29, 36, 37).

In subjects consuming a diet adequate in calories, the bulk of dietary fat after digestion and absorption is released by lipoprotein lipase as fatty acids for storage in the depots of adipose tissue (38). The amount of fat deposited in this way in the depots of the mammary gland in women increases markedly with age (39), as does the risk of developing breast cancer. A high dietary intake of fat also enhances carcinogenesis, at least in experimental animals, but the nature of this enhancement is complex. Thus, in animals, tumour induction is promoted particularly by unsaturated fats (41, 42), yet in humans, breast cancer incidence is correlated strongly with intake of animal, not vegetable, fat (4), and Eskimos, who have a high intake of unsaturated fat, have a relatively low incidence of breast cancer (43). In the light of these considerations, some workers (44) consider that adipose

tissue may have a special role in the aetiology of breast cancer, either by acting as a store for carcinogens, or perhaps by providing, via the enzymic mechanisms described above, a promoting, oestrogen environment. However, other workers (45) dispute such a special role for adipose tissue, and it is generally held that obesity, though possibly an indicator of poor prognosis in established disease (46), is not a strong risk factor for breast cancer (47–50). Although obesity or *total* body fat may be unrelated to risk of breast cancer, it may still be that *mammary* fat content is of significance. It is thus tempting to speculate that the increasing deposition of fat in the breast with age, irrespective of total body fat or size, could contribute to risk, for example, by leading to increased local hydrolysis of oestrone sulphate from the circulation or from cystic fluid. This, in turn, could provide a promoting oestrogenic environment in which carcinogens or mutagens such as those described by Petrakis (51) might act. Whether or not local hydrolysis of oestrone sulphate occurs in fat or other breast tissues *in vivo* is uncertain. *In vitro,* in the presence of albumin (4 g/dl) or plasma (100%), oestrone sulphate fails to enter mammary adipose tissue and is not hydrolysed (Table 3), presumably due to the tight binding of the conjugate by albumin (52). In the absence of albumin, however, or in the presence of only 5% serum, others (14, 15) have shown that the conjugate does enter mammary cancer cells and is hydrolysed. Probably none of these *in vitro* conditions accurately reflect the true situation, and further studies will be required to establish the extent to which hydrolysis of oestrone sulphate occurs in breast tissues *in vivo*.

In summary, we have demonstrated that (1) oestrone sulphate is present at high levels in breast cysts by comparison with the levels in plasma or milk, (2) some patients with breast cancer may have slightly elevated levels of the hormone, (3) the dietary contribution from milk to the endogenous oestrone sulphate pool is small, and (4) normal, benign, malignant, and adipose tissue can cleave oestrone sulphate, but the magnitude of this capacity, assessed *in vitro*, depends on the technique used and the hydrolysis is abolished in the presence of high levels of serum albumin or plasma. These findings raise the possibility that hydrolysis of oestrone sulphate *in vivo* might contribute in certain types of breast tissue to the production of an oestrogenic local environment, a milieu which it has been proposed may promote the formation of breast cancer.

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