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# Studies on the Metabolism of Oestrone Sulphate

## COMPARATIVE PERFUSIONS OF OESTRONE AND OESTRONE SULPHATE THROUGH ISOLATED RAT LIVERS

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The metabolism of [4-$^{14}$C]oestrone and of [6,7-$^{3}H_2$]oestrone sulphate was studied during cyclic perfusion and once-through perfusion of the isolated rat liver. The following results were obtained. 1. As shown by once-through perfusion, the two steroids are metabolized differently during the first passage through the organ. [4-$^{14}$C]Oestrone was taken up by the liver and partly delivered as oestradiol-17$\beta$ and oestriol into the medium. After uptake of [6,7-$^{3}H_2$]oestrone sulphate, only oestrone, liberated by hydrolysis, was delivered into the medium; no oestradiol-17$\beta$ or oestriol could be detected in the medium after one passage through the organ. This indicates that intracellular oestrone, which was taken up as such, and oestrone, which derived from intracellular hydrolysis, may be metabolized in different compartments of the liver cell. 2. The results of the cyclic perfusion showed that intracellular oestrone is preferentially conjugated with glucuronic acid, and subsequently excreted into the bile. Intracellular oestrone sulphate is preferably reduced to oestradiol sulphate, thus indicating that oestrone sulphate is a better substrate for the 17$\beta$-hydroxy steroid oxidoreductase than is oestrone. 3. Albumin-bound oestrone sulphate acts as a large reservoir, and in contrast with free oestrone is protected from enzyme attack by its strong binding to albumin. 4. Oestrone sulphate is partly converted into the hormonally active oestrone by liver tissue. This suggests that liver not only inactivates oestrogens, but also provides the organism with oestrone, which is subsequently readily taken up by other organs.

In recent years, oestrone sulphate, the major circulating oestrogen in man (Purdy *et al.*, 1961; Brown & Smyth, 1971; Loriaux *et al.*, 1971; Ruder *et al.*, 1972), has received renewed interest. It has been claimed that sulphation of oestrone is a prerequisite of the hormone's stimulatory effect on protein synthesis in rat and rabbit uterine preparations (Brooks *et al.*, 1969). There are still controversies about the physiological role of oestrone sulphate and the reasons for its low metabolic clearance rate. Some authors emphasize that oestrone sulphate is not a precursor for free oestrogens (Longcope, 1972), whereas others regard oestrone sulphate as a transport form of oestrogens in human plasma (Ruder *et al.*, 1972). The low metabolic clearance rate of oestrone sulphate may be due, among other things, to its strong binding to plasma proteins (Rosenthal *et al.*, 1972; Ruder *et al.*, 1972) or to the fact that only a small fraction of the circulating oestrone sulphate is metabolized (Longcope, 1972).

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Previous studies *in vitro* (Dahm & Breuer, 1967) and *in vivo* (Fishman & Hellman, 1973) have revealed that the metabolism of oestrone differs from that of its sulphate ester. The reason for this is not yet known; it was therefore decided to carry out a detailed study with oestrone and oestrone sulphate, by using the isolated perfused rat liver as an organ preparation which approaches physiological conditions. Equimolar amounts of [4-$^{14}$C]oestrone and [6,7-$^{3}H_2$]oestrone sulphate were perfused cyclically at the same time. In addition, once-through perfusions of the isolated rat liver with radioactive oestrone or oestrone sulphate were performed.

The results obtained indicate that the different metabolic fates of oestrone and oestrone sulphate result from differences in the uptake and in the intracellular metabolism of the two steroids. Further, they support the concept of the precursor role of oestrone sulphate as such, rather than its possible role as an entry vehicle to the metabolic site. A preliminary report of this work has been published (Höller *et al.*, 1975).

## Materials and Methods

### *Steroids*

[4-$^{14}$C]Oestrone (specific radioactivity 58.0Ci/mol) and [6,7-$^{3}H_2$]oestrone sulphate (specific radioactivity 54.0Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and New England Nuclear Corp., Waltham, MA, U.S.A. Non-radioactive steroids were purchased from Ikapharm, Ramat-Gan, Israel, and Schering, Berlin-West, or given by Dr. G. Röhle, Institut für Klinische Biochemie, Bonn. All steroids were tested for purity by t.l.c. and g.l.c.

### *Chemicals*

All chemicals used were of reagent grade (Merck, Darmstadt, Germany, and Carl Roth, Karlsruhe, Germany). All reagents used for determination of lactate, pyruvate, ATP and ADP were purchased from Boehringer, Mannheim, Germany. Bovine serum albumin was obtained from Behringwerke, Marburg, Germany. It was twice dialysed before use.

### *Tubes*

Portex nylon tubes were purchased from Medimex, Hamburg, Germany, and polyethylene tubes were from Vogel, Giessen, Germany.

### *Analytical*

Samples (1ml) of the outflowing venous medium were taken after 5 and 20min as well as at the end of the perfusion and analysed for lactate and pyruvate. The samples were delivered into 7ml of ice-cold 0.3M-$HClO_4$ and immediately analysed. For the determination of lactate, pyruvate, ATP and ADP in liver tissue at the end of the perfusion, liver lobes were rapidly frozen in Wollenberger clamps (6cm×6cm×1cm) at the temperature of liquid $N_2$ (Wollenberger *et al.*, 1960). The frozen tissue was stored under liquid $N_2$ until analysed. Before analysis, the liver pieces were pulverized in a microdismembrator (Braun, Melsungen, Germany) and the powdered liver tissue was extracted with ice-cold 0.3M-$HClO_4$. Substrates were measured spectrophotometrically (PMQ II, Zeiss, Oberkochen, Germany) by modified standard techniques (Bergmeyer, 1974).

### *Thin-layer chromatography*

T.l.c. was carried out on precoated aluminium sheets (silica gel $F_{254}$; Merck; 20cm×20cm). Extracts that were heavily contaminated were prepurified by chromatography on 2mm-thick silica-gel-coated glass plates (Merck). For 'reducing chromatography', thin-layer plates were immersed for impregnation in a solution of 400ml of methanol, 4ml of acetic acid and 3g of ascorbic acid (Gelbke & Knuppen, 1972). After drying at room temperature (20°C) for 60min and loading, plates were developed by ascending technique in pre-equilibrated tanks (Camag, Berlin-West) which were lined with filter paper. Systems for chromatography are listed in Table 1. Areas of radioactivity were located by using a radiochromatogram scanner (model II Berthold, Karlsruhe, Germany). Colour development of authentic steroids used as parallel standards was done by spraying with 5% (v/v) $H_2SO_4$ in methanol and subsequent heating at 120°C. No colour reaction could be applied when 'reducing chromatography' had been carried out. The radioactive areas were eluted with methanol/acetone (3:1, v/v), and radioactivity was determined by liquid-scintillation counting. All results were corrected for losses during elution and chromatography.

### *Gas–liquid chromatography*

G.l.c. was carried out with a Varian 1800 gas chromatograph equipped with a flame ionization detector. Glass columns (1200mm×3mm internal diam.) were packed with 3% Dexsil on 100/120 Chromosorb W-HP or 3% OV-101 on 100/120 Chromosorb W-HP (Günther Analysentechnik, Düsseldorf, Germany). Conditions for g.l.c.: column temperature 230°C; detector temperature 250°C; carrier-gas flow 30ml of $N_2$/min. Before analysis, steroids were converted into their trimethylsilyl ethers by reactions with *N*-methyl-*N*-trimethylsilyl-

Table 1. *Systems for t.l.c.*

Before loading, thin-layer plates were impregnated with ascorbic acid as described in the Materials and Methods section.

| Systems | Solvents | Separation of |
|---|---|---|
| A | Propan-2-ol/chloroform/methanol/water (10:10:5:2, by vol.) | Free steroids, glucuronides, sulphates |
| B | Chloroform/methanol (1:1, v/v) with 0.01M-NaCl | Free steroids/sulphates, glucuronides |
| C | Benzene/ethyl methyl ketone/ethanol/water (3:3:3:1, by vol.) | Free steroids, sulphates |
| D | Toluene/acetic acid/water (10:10:1, by vol.) | Free steroids, glucuronides |
| E | Cyclohexane/chloroform/acetic acid (1:1:1, by vol.) | Free steroids, conjugates |
| F | Cyclohexane/chloroform/acetic acid (2:1:1, by vol.) | Polar free steroids |
| G | Chloroform/methanol (9:1, v/v) | Relatively polar free steroids |
| H | Chloroform/methanol (49:1, v/v) | Non-polar free steroids |

trifluoroacetamide (Machery and Nagel, Düren, Germany). The trimethylsilyl derivatives of authentic standards were injected at a concentration of 50 ng/$\mu$l.

*Measurement of radioactivity*

Radioactivity was assayed in a mark II liquid-scintillation spectrometer (Nuclear–Chicago, Frankfurt-Heusenstamm, Germany). The scintillation mixture consisted of dioxan, containing 100 g of naphthalene, 7 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/litre. Double-label counting was performed by using the discriminating voltages specified by Nuclear–Chicago. Counting efficiency was determined by the external-standard-ratio method. The radioactivity in highly contaminated samples was measured by combustion in a sample oxidizer (Oximat, Deutsche Intertechnique, Mainz, Germany) and subsequent liquid-scintillation counting of the $^{14}CO_2$ and $^3H_2O$ formed.

*Determination of albumin binding of oestrone and oestrone sulphate*

Protein binding of oestrone and oestrone sulphate was determined by equilibrium dialysis (Dianorm BFD, Diachemie, Zürich, Switzerland) with radioactive steroids. The dialysis was carried out with a Visking dialysis membrane (Serva, Heidelberg, Germany). Krebs bicarbonate buffer (Krebs & Henseleit, 1932; 1 ml), containing 35 g of bovine serum albumin/litre, was dialysed against the buffer solution (1 ml) without albumin at 28°C. For each steroid, two different types of dialysis were carried out. In one group the steroid was added to the albumin solution; in the second group it was added to the buffer solution at the beginning of dialysis. The concentration of the steroids at beginning of dialysis was 6.2 $\mu$mol/litre. During dialysis, the radioactivity in each dialysis cell was checked by taking 10 $\mu$l portions every hour. At 2 h after equilibrium had been reached, dialysis was stopped. From the distribution of radioactivity, protein binding was calculated. Recovery of radioactivity at the end of dialysis was 91–95%.

To find out if the steroid was freely diffusible through the membrane, dialysis experiments were carried out with buffer solution or albumin solutions respectively in both dialysis cells.

*Animals and perfusion technique*

Male Wistar rats (280–320 g) were used in all experiments (S. Ivanovas, Kisslegg/Allgäu, Germany). The surgical preparation of the animals has been described (Höller & Breuer, 1974). Two different perfusion models were used: a once-through perfusion (Höller & Breuer, 1975) and a cyclic perfusion (Höller & Breuer, 1974; Höller *et al.*, 1976*a*,*b*). Perfusion temperature was 28°C. The once-through perfusion was carried out with a fixed flow rate of 2 ml/min per g wet wt. (Watson–Marlow roller pump, c/o Erben, Düsseldorf, Germany). The outflowing venous medium was fractionated into 12 ml fractions and analysed for oestrone or oestrone sulphate and their respective metabolites. From each fraction, the hepatic clearance rate of the respective steroid and the clearance rate of the total radioactivity were calculated. The cyclic perfusion was carried out as constant-pressure perfusion with a hydrostatic pressure of about 20 cm of water, which gave a flow rate of 2.0–2.3 ml/min per g wet wt.

The perfusion medium consisted of the Krebs bicarbonate buffer, containing glucose (5.5 mM), lactate (2.0 mM), pyruvate (0.3 mM) and bovine serum albumin (35 g/litre). In cyclic-perfusion experiments, 6.2 $\mu$M-[4-$^{14}$C]oestrone (specific radioactivity 12.9 $\mu$Ci/$\mu$mol) and 6.2 $\mu$M-[6,7-$^3H_2$]oestrone sulphate (specific radioactivity 21.5 $\mu$Ci/$\mu$mol) were added to the perfusion medium (150 ml). The isotope ratio ($^3H/^{14}C$) was calculated to be 1. In once-through-perfusion experiments, radioactive oestrone and radioactive oestrone sulphate were perfused separately with the corresponding molar concentrations and the same specific radioactivities.

During the perfusions, the following parameters of the functioning states of the liver were checked: (*a*) flow rate (cannulating flow transducer with electromagnetic flow-meter, model SP 2202; Statham Instruments, c/o Schubart, Wiesbaden, Germany), (*b*) oxygen pressure and $H^+$ concentration in arterial and venous medium; (*c*) oxygen partial pressure at different areas of the surface of the organ (electrodes and $O_2$-analyser from Eschweiler, Kiel, Germany); and (*d*) oxygen consumption; the last was calculated every 5 min (see the Results section). Organ weight before and after perfusion as well as bile flow were measured. In some perfusion experiments, ATP, ADP, lactate and pyruvate in liver tissue, and lactate and pyruvate in perfusion medium, were determined. Preparation of medium and tissue is described under 'Analytical'.

*Extraction and purification of steroids*

After cyclic perfusion for 40 min, the liver was flushed with 10 ml of fresh ice-cold perfusion fluid, to wash out most of the radioactivity not incorporated; this fluid was added to the perfusion medium. The liver was quickly removed, weighed and homogenized in ethanol (final concentration 85%, v/v) by an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen i. Breisgau, Germany). The homogenate was centrifuged for 10 min at 2000$g_{av.}$ and the pellet washed several times with 85% ethanol. The radioactivity remaining in the residue (protein bound) was determined by a sample oxidizer. After determination of radioactivity, the combined supernatant phases were evaporated to dryness under vacuum, and the

dry residue was dissolved in 50ml of water (tissue extract). About 100mg of ascorbic acid was added to the tissue extract, to the medium and to the bile to prevent oxidative decomposition of labile steroids. The radioactivities of the tissue extract, of the medium and of the bile, which was diluted to 10ml with water, were determined. The solutions were passed through 30g columns of Amberlite XAD-2 (Bradlow, 1968; Osawa & Slaunwhite, 1970). Each column was rinsed with 100ml of water and 50ml of water/methanol (3:1, v/v) at a rate of 1ml/min. The wash phases that contained less than 10% of the radioactivity of the fraction were discarded. The steroids were eluted from the resin by 100ml of methanol, 50ml of acetone and 100ml of methanol/0.1M-NaOH (9:1, v/v) buffered to pH10.5 with ascorbic acid (Höller *et al.*, 1976*a*). The eluates were neutralized and evaporated to dryness under vacuum; the residues were dissolved in a small volume of methanol. After determination of radioactivity, the methanolic solution was applied to a column (20cm × 1cm) of Sephadex LH-20, by using the solvent system chloroform/methanol (1:1, v/v), containing 0.01M-NaCl (Sjövall *et al.*, 1968). Fractions containing radioactivity were combined, evaporated to dryness under vacuum and the residues dissolved in a small volume of methanol. The steroids were chromatographed on silica-gel-coated glass plates (2mm). The free steroids were eluted and further characterized by t.l.c. and g.l.c. The conjugates were eluted and purified by column chromatography on Sephadex G-10 (20cm × 1cm), eluted with water. The fractions containing radioactivity were combined and evaporated to dryness under vacuum; the residues were dissolved in a small volume of methanol and the conjugates characterized by t.l.c. The fractionated venous medium of the once-through perfusions was worked up in the same way as described for cyclic perfusions.

*Chromatography and hydrolysis of steroids*

The steroid sulphates and glucuronides were separated from each other by t.l.c. They were eluted and subjected to solvolysis (cf. Höller *et al.*, 1976*a*; Hellström *et al.*, 1969) or hydrolysis with bacterial β-glucuronidase (cf. Höller *et al.*, 1976*a*) respectively. At the end of solvolysis, the ethyl acetate phase was washed several times with water, evaporated to dryness and the residue taken up in a small volume of methanol. The incubation mixture for the hydrolysis of the glucuronides was applied to an Amberlite XAD-2 column as described under 'Extraction and purification of steroids'. The eluate was evaporated to dryness under vacuum and the residue dissolved in a small volume of methanol.

*Characterization of steroids*

The free steroids and the steroids liberated during hydrolysis were characterized by t.l.c. Single, distinct peaks of radioactivity were obtained. After t.l.c., the radioactive peaks were eluted and the radioactivity of each peak was determined. The steroids were converted into their trimethylsilyl ethers and characterized by g.l.c.

## Results

*Functional state of the liver*

Oxygen consumption of the liver was calculated from the flow rate, the differences in oxygen partial pressure in arterial and venous medium and the absorption coefficient [$\alpha_{O_2(28°C)} = 0.026$]; it was found to be 1.1–1.2μmol/min per g wet wt. at the beginning of the perfusion and between 0.8 and 1.0 after 40min perfusion. No anoxic regions were detected by measurement of the oxygen partial pressure at the surface of the organ. The pH in the venous medium was 0.04–0.06 unit lower than in the arterial medium; in the latter, the pH was stable at 7.38–7.40 during perfusion. The average bile flow during 40min perfusions was 0.8–1.0μl/min per g wet wt.; no increase of organ weight was observed at the end of the perfusion. The concentrations of ATP, ADP, lactate and pyruvate in liver tissue, and of lactate and pyruvate in the perfusion medium, were similar to those observed earlier (Höller *et al.*, 1976*a*,*b*); this indicated a sufficient supply of oxygen to the liver cells. After 40min of perfusion, the mean ratio (±S.E.M.) of ATP to ADP in liver tissue was 3.0±0.22 ($n = 8$); the mean ratio (±S.E.M.) of lactate to pyruvate was 11.2±0.79 in liver tissue and 11.0±0.40 ($n = 8$) in perfusion medium.

*Cyclic perfusion of [4-$^{14}C$]oestrone and [6,7-$^{3}H_2$]-oestrone sulphate*

*Distribution of radioactivity between liver tissue, perfusion medium and bile.* The perfused $^{3}H$ radioactivity was distributed differently from the perfused $^{14}C$ radioactivity between liver tissue, perfusion medium and bile after cyclic perfusion (Höller *et al.*, 1975). Whereas in liver tissue the $^{3}H/^{14}C$ ratio of the steroids was near unity (0.92), it differed significantly from unity in perfusion medium and bile.

The ratio of about 1.7 in the perfusion medium suggests a slower uptake of [6,7-$^{3}H_2$]oestrone sulphate than of [4-$^{14}C$]oestrone. The ratio of about 0.5 in the bile indicates that oestrone and its metabolites were preferentially secreted into the bile (Höller *et al.*, 1975).

*Separation of free and conjugated steroids in liver tissue, perfusion medium and bile.* Oestrone, oestrone sulphate and their respective metabolites were separated from each other as described in the Materials and Methods section. Three fractions were obtained: free steroids, steroid glucuronides and

Table 2. *Distribution of radioactivity in liver tissue, perfusion medium and bile after perfusion of [4-$^{14}C$]oestrone and [6,7-$^{3}H_2$]oestrone sulphate*

The following three fractions were separated from each other: free oestrogens, oestrogen glucuronides and oestrogen sulphates. The $^{3}H/^{14}C$ ratio was calculated from the individual values of the six perfusions, and not simply by dividing the mean values of $^{3}H$ and $^{14}C$ as shown in the Table for each fraction. At the beginning of perfusion, the $^{3}H/^{14}C$ ratio of the total radioactivity in the medium was 1.0. The values are expressed as % of perfused radioactivity (=100%) and represent the means±S.E.M. for six perfusion experiments. For further details, see the Materials and Methods section.

| Fraction | $^{3}H$ | $^{14}C$ | $^{3}H/^{14}C$ ratio |
|---|---|---|---|
| Liver tissue | | | |
| Free oestrogens | 10.2±1.2 | 8.3±0.9 | 1.2±0.06 |
| Glucuronides | 10.6±0.8 | 10.9±1.3 | 1.0±0.07 |
| Sulphates | 17.1±1.3 | 18.8±1.7 | 0.9±0.02 |
| Perfusion medium | | | |
| Free oestrogens | 8.5±0.7 | 6.2±0.4 | 1.4±0.08 |
| Glucuronides | 3.2±0.4 | 3.6±0.6 | 0.9±0.06 |
| Sulphates | 20.3±1.3 | 8.6±0.8 | 2.4±0.12 |
| Bile | | | |
| Free oestrogens | 1.0±0.1 | 1.5±0.1 | 0.7±0.05 |
| Glururonides | 13.3±1.1 | 24.6±1.6 | 0.5±0.02 |
| Sulphates | 1.2±0.2 | 0.8±0.1 | 1.4±0.19 |

Table 3. *$^{3}H/^{14}C$ ratios of the various oestrogens in liver tissue, perfusion medium and bile after cyclic perfusion of [4-$^{14}C$]oestrone and [6,7-$^{3}H_2$]oestrone sulphate*

The values represent the means of the individual $^{3}H/^{14}C$ ratios of six perfusion experiments (±S.E.M.). Tr, Trace.

| Oestrogen | Liver tissue | Perfusion medium | Bile |
|---|---|---|---|
| Free steroid | | | |
| Oestrone | 1.6±0.1 | 1.7±0.3 | Tr |
| Oestradiol-17$\beta$ | 1.9±0.1 | 1.8±0.5 | Tr |
| 2-Methoxyoestrogens | 1.9±0.2 | — | Tr |
| Oestriol fraction | 0.8±0.1 | 1.3±0.1 | 0.6±0.1 |
| Steroid glucuronide | | | |
| Oestrone | 0.9±0.1 | 0.9±0.1 | 0.4±0.1 |
| Oestradiol-17$\beta$ | 0.8±0.1 | 1.0±0.1 | 0.5±0.1 |
| 2-Methoxyoestrogens | 0.8±0.1 | 0.8±0.2 | 0.6±0.1 |
| Oestriol fraction | 1.2±0.1 | 0.8±0.1 | 0.6±0.1 |
| Steroid sulphate | | | |
| Oestrone | 1.6±0.3 | 29.9±3.6 | Tr |
| Oestradiol-17$\beta$ | 0.9±0.1 | 2.5±0.1 | Tr |
| 2-Methoxyoestrogens | Not investigated | Not investigated | 1.7±0.2 |
| Oestriol fraction | 0.6±0.1 | 0.7±0.1 | 1.4±0.1 |

steroid sulphates. The distribution of the three fractions in liver tissue, perfusion medium and bile and their respective $^{3}H/^{14}C$ ratios are shown in Table 2.

In liver tissue, the $^{3}H/^{14}C$ ratios of the various fractions were near unity, which indicated that after 40min of cyclic perfusion, [4-$^{14}$C]oestrone and [6,7-$^{3}H_2$]oestrone sulphate had entered the same metabolic pathways.

In bile, most of the metabolites were glucuronides, which were mainly $^{14}C$-labelled. This indicated that oestrone taken up by the liver was mainly metabolized to conjugated phenolic steroids, which were then secreted into bile.

The $^{3}H/^{14}C$ ratio of 1.4 for the free steroids in perfusion medium indicated that after desulphation a significant proportion of the liberated oestrogens diffused into the medium. The high $^{3}H/^{14}C$ ratio (about 30) of the sulphate fraction in perfusion medium may be due to oestrone sulphate, which was not taken up by the liver even after 40min of perfusion.

*Separation and identification of steroids in liver tissue, perfusion medium and bile.* The steroids were separated and characterized as described in the Materials and Methods section. In each fraction (free steroids, steroid glucuronides, steroid sulphates) the the following metabolites were found; oestrone, oestradiol-17$\beta$ and two groups of metabolites that

were designated as 'oestriol' fraction and '2-methoxyoestrogen' fraction. Whereas the oestrone and oestradiol-17$\beta$ fractions were homogeneous, the oestriol fraction comprised various different metabolites, the two main ones of which were identified as 2-hydroxyoestradiol-17$\beta$ and oestriol; only small amounts of 2-hydroxyoestrone were detected. The fraction of the 2-methoxyoestrogens contained mainly 2-methoxyoestrone; 2-methoxyoestradiol-17$\beta$ was present in measurable amounts only in the sulphate fraction obtained from bile and liver tissue.

After 40min of cyclic perfusion, the $^3H/^{14}C$ ratio of free oestrone in liver tissue and perfusion medium was above 1 (Table 3); of the initially perfused [4-$^{14}$C]oestrone only 2.8±0.73% in liver tissue and 1.9±0.3% in perfusion medium (±S.E.M.; $n = 6$) were found to be unchanged. As indicated by the $^3H/^{14}C$ ratios, most of the free steroids in liver tissue and perfusion medium were derived from [6,7-$^3H_2$] oestrone sulphate.

Most of the glucuronides were found in bile, and only small amounts were found in the perfusion medium. The $^3H/^{14}C$ ratios of the glucuronides in bile indicated that [4-$^{14}$C]oestrone, rather than [6,7-$^3H_2$]oestrone sulphate, was preferentially metabolized to oestrogens, which were then available for biliary secretion. In contrast with the steroids of the bile, the $^3H/^{14}C$ ratios of most of the steroid glucuronides in liver tissue were only slightly below unity.

The role of the sulphate esters in the metabolism of oestrogens is stressed by the results shown in Tables 2 and 3. At the end of cyclic perfusion, large amounts of unmetabolized [6,7-$^3H_2$]oestrone sulphate were still present in the perfusion medium. This may be explained by a slow uptake of [$^3$H]oestrone sulphate as compared with [4-$^{14}$C]oestrone. The $^3H/^{14}C$ ratio of oestrone sulphate in perfusion medium was 29.9, but in liver tissue it was 1.6. The large amounts of unchanged oestrone sulphate in perfusion medium (13.9±1.3% of perfused radioactivity) indicated that this oestrogen was protected from enzymic attack by the liver because of its strong binding to albumin, which decreased its availability for the liver. Of the total $^{14}$C radioactivity, 0.5% was found as oestrone sulphate in the perfusion medium and 4.6% in the liver tissue. This means that only small amounts of the newly formed oestrone sulphate were delivered to the medium as such; intracellular oestrone sulphate was preferentially further metabolized to other sulphate esters. In fact most of the oestradiol-17$\beta$ found during analysis was in the sulphate fractions of liver tissue and perfusion medium (Table 4).

*'Once-through' perfusion of oestrone and oestrone sulphate*

These perfusions lasted for only 8min and therefore indicated only the early steps in the metabolism of the respective steroid. The clearance rates of oestrone, oestrone sulphate, total $^{14}$C radioactivity and total $^3$H radioactivity were calculated from each fraction of the outflowing venous medium. The clearance rate of oestrone was three to four times that of oestrone sulphate. There were differences between the clearance rates of total $^{14}$C-labelled steroids and [$^{14}$C]oestrone on the one hand, and the clearance rates of total $^3$H-labelled steroids and [$^3$H]oestrone sulphate on the other. It appears that, in perfusions with oestrone, more metabolites were secreted into the medium than during perfusion of oestrone sulphate; this was indicated by the higher differences between the clearance rates of total $^{14}$C radioactivity and the respective steroid (Höller *et al.*, 1975). Further investigation of the outflowing venous medium of the once-through perfusion revealed qualitative differences of the secreted metabolites of oestrone and oestrone sulphate. The rates of formation of the various metabolites which appear in the venous medium are presented in Fig. 1. [4-$^{14}$C]Oestrone was taken up by the liver and partly delivered as oestradiol-17$\beta$ and oestriol to the medium. After uptake of [6,7-$^3H_2$]oestrone sulphate, oestrone was liberated by intercellular hydrolysis, and oestrone and 2-methoxyoestrone were delivered to the medium; no oestradiol-17$\beta$ or oestriol was detected after one passage of oestrone sulphate through the liver. The difference in metabolic pattern leads to the assumption that oestrone is metabolized differently, according to its origin.

Table 4. *Distribution of oestradiol-17$\beta$ in liver tissue, perfusion medium and bile among the fractions of free steroids, steroid glucuronides and steroid sulphates*

The values are expressed as % of perfused radioactivity and represent the means±S.E.M. for six perfusion experiments (cyclic perfusion). Tr, Trace.

| | Liver tissue | | Perfusion medium | | Bile | |
|---|---|---|---|---|---|---|
| | $^3$H | $^{14}$C | $^3$H | $^{14}$C | $^3$H | $^{14}$C |
| Free steroid | 1.7±0.2 | 0.8±0.1 | 1.6±0.1 | 1.0±0.2 | Tr | Tr |
| Glucuronides | 0.8±0.1 | 0.9±0.1 | 0.3±0.1 | 0.3±0.1 | 2.2±0.5 | 4.3±1.1 |
| Sulphates | 5.2±0.2 | 5.6±0.9 | 2.4±0.2 | 1.0±0.1 | Tr | Tr |

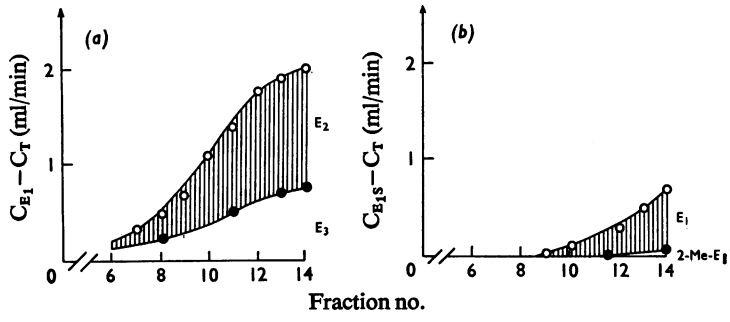


Fig. 1. *Rates of formation of metabolites formed during once-through perfusions of* [4-$^{14}$C]*oestrone and* [6,7-$^3H_2$]*oestrone sulphate through isolated rat liver*

The outflowing venous medium was fractionated (each fraction 12ml). The metabolites from each fraction were analysed. For further details, see the Materials and Methods section. The total formation of metabolites was calculated from the differences of the clearance rates of total radioactivity ($C_T$) on the one hand and the clearance rates of oestrone ($C_{E_1}$) and oestrone sulphate ($C_{E_1S}$) respectively on the other hand (Höller *et al.*, 1975). The values are mean values for six perfusion experiments. $E_1$, Oestrone; 2-Me-$E_1$, 2-methoxyoestrone; $E_2$, oestradiol-17$\beta$; $E_3$, oestriol.

*Protein binding of oestrone and oestrone sulphate*

The binding of oestrone and oestrone sulphate to bovine serum albumin was determined as described in the Materials and Methods section. It was found that 10.4±1.1% of oestrone and 6.0±0.6% of oestrone sulphate was unbound (±S.E.M.; $n = 10$) in the albumin solution (3.5%, w/v). If both dialysis cells contained buffer solution, about 50% of the added steroid (oestrone or oestrone sulphate respectively) was found at each side of the membrane after 3h of dialysis. This indicates that oestrone as well as oestrone sulphate are freely diffusible through the membrane. The same equilibrium was reached, if both cells contained albumin solution.

## Discussion

The results of metabolic studies with the perfused liver can only be adequately interpreted if the relations of the three compartments (liver, medium and bile) to each other are taken into account. This relation has an influence on the metabolism and is different in once-through perfusions and cyclic perfusions. Whereas once-through perfusions are suitable for the measurement of uptake of a substrate into the perfused organ, cyclic perfusion may be used for studies of the metabolic pathways of a substrate. Both models yield specific results, on the basis of which the metabolism of a substrate can be advantageously interpreted.

The uptake of a substrate into a perfused organ can be determined by several factors: (1) protein binding in perfusion medium; (2) factors affecting liver cell membrane (e.g. permeability, transport); (3) metabolizing capacity of the organ, and (4) blood flow through the organ (Nagashima & Levy, 1968). If the metabolizing capacity of the liver is not rate-limiting, the velocity of metabolism can be determined by the blood flow through the liver (Whitsett *et al.*, 1971). Because of the influence of blood flow on the metabolism, the continuous and careful control of flow rate is a prerequisite to obtain comparable results from perfusion to perfusion. This is an important point affecting the reproducibility of metabolic perfusion studies.

As shown in the present paper, the metabolites of oestrone and oestrone sulphate were differently distributed between the three compartments (liver, medium and bile). A ratio of 1.7 ($^3$H/$^{14}$C) for the total radioactivity in the perfusion medium suggests a slower uptake of oestrone sulphate than of oestrone. Further analysis of the sulphate fraction of the perfusion medium after cyclic perfusion revealed that this fraction consists mainly of [$^3$H]oestrone sulphate; only small amounts of newly formed [$^{14}$C]oestrone sulphate were delivered to the medium. A slow uptake of oestrone sulphate was also demonstrated by the results of the once-through perfusions, which showed that the hepatic clearance rate of oestrone was three to four times that of oestrone sulphate. One of the reasons for this phenomenon may be that more oestrone sulphate is bound to albumin than is bound to oestrone, as shown by equilibrium dialysis. Comparing the nearly quantitative hepatic extraction of oestrone at the beginning of once-through perfusions and considering the fact that about 90% of oestrone is bound to albumin, it is evident that the metabolic clearance rate of oestrone and oestrone sulphate do not simply depend on the extent to which the two steroids are bound to albumin.

When dextran was used as macromolecular component in once-through perfusions, oestrone sulphate was almost quantitatively extracted by the liver out of the medium (M. Höller & K. Dengler, unpublished work). This indicates that the liver cell membrane is permeable for oestrone sulphate. A low hepatic clearance rate of oestrone sulphate has also been suggested by other authors, who found that orally applied oestrone sulphate shows no first-pass effect and reaches peripheral circulation without significant metabolism (Ruder *et al.*, 1972).

At the beginning of the cyclic perfusion, the free steroid was exclusively $^{14}$C-labelled ($^{3}$H/$^{14}$C ratio for free steroids = 0); at the end, the $^{3}$H/$^{14}$C ratios of the free steroids in perfusion medium and in liver tissue were above unity. Although after 40min of cyclic perfusion, almost all of the [$^{14}$C]oestrone had been metabolized, a large pool of unmetabolized [$^{3}$H]oestrone sulphate was still in the medium. It seems appropriate to consider the close relation of the results of the cyclic and the once-through perfusion. The metabolism shown in once-through perfusion may also take place at the beginning of cyclic perfusion, before the first recirculation starts. The results of the once-through perfusion with [4-$^{14}$C]oestrone indicate that free oestradiol-17$\beta$ and free oestriol are delivered into the medium; they may be taken up during recirculation in cyclic perfusion and further metabolized. Probably these metabolic pathways are dominating at the beginning of cyclic perfusion. At the end of cyclic perfusion, the [$^{14}$C]oestrone has been totally metabolized, whereas [$^{3}$H]oestrone, as was shown by the results of the once-through perfusion, is still delivered to the medium after hydrolysis of oestrone sulphate by the liver. The [$^{3}$H]oestrone is taken up during recirculation and metabolized like primarily unconjugated oestrone as described above. In fact, free oestrone, oestradiol-17$\beta$ and oestradiol in the perfusion medium are mainly $^{3}$H-labelled after 40min of cyclic perfusion.

This leads to the conclusion that the initial metabolism of free oestrone, which was taken up as such by the liver, differs from the initial metabolism of free oestrone, which was derived by hydrolysis of oestrone sulphate. The finding that free oestrone, depending on its origin, was metabolized in a different way suggests that the metabolism occurs in different compartments of the liver cell. It is evident that the metabolism of oestrone and oestrone sulphate depends on the interaction between albumin and these steroids.

The bile is a major route for the excretion of steroids (Sandberg *et al.*, 1967); therefore it is not surprising that after 40min of cyclic perfusion 16% of the $^{3}$H radioactivity and 27% of the $^{14}$C radioactivity were excreted into the bile.

The low $^{3}$H/$^{14}$C ratio of the steroid glucuronides in bile is in good agreement with results of Fishman & Hellman (1973), who found that oestrone was converted into oestrone glucuronide and oestradiol-17$\beta$ glucuronide to a much greater extent than was oestrone sulphate. The $^{3}$H/$^{14}$C ratio of the different glucuronides in bile was still fairly low after 40min, but it was near unity in liver tissue. This indicates that an equilibrium between chemically identical but differently labelled steroids is quickly reached in the metabolizing organ.

A $^{3}$H/$^{14}$C ratio for oestrone sulphate in liver tissue of 1.7 indicated that at least 38% of this conjugate in liver was newly formed. The large amounts of $^{14}$C-labelled sulphates in liver tissue point to the important role of the steroid sulphotransferase in oestrogen metabolism (Gugler *et al.*, 1973). The low amounts of $^{14}$C-labelled oestrone sulphate in perfusion medium and the fact that the largest pool of oestradiol-17$\beta$ was found in the sulphate fraction of liver and perfusion medium indicated that intracellular oestrone sulphate was not delivered to the medium as such but was reduced to oestradiol-17$\beta$ sulphate. Similarly, Fishman & Hellman (1973) found *in vivo* that oestrone contributed mainly to biliary glucuronides, whereas oestrone sulphate was excreted by the kidney as such or as oestradiol-17$\beta$ sulphate.

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