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The effect of oral contraceptive steroids on bile secretion and bilirubin *Tm* in rats

T. A. J. HEIKEL AND G. H. LATHE

Department of Chemical Pathology, University of Leeds

Summary

1. The effect of oestrogens and progestogens and their 17α-ethinyl derivatives on bile flow, maximum rate of bilirubin secretion, serum and liver bilirubin has been studied.

2. Both 17α-ethinyl substituted oestrogens and progestogens greatly reduced the basal bile flow. The parent compounds, oestradiol-17β and 19-nortestosterone had little or no effect.

3. A much larger dose of progestogens (40 mg/kg) than oestrogens (5 mg/kg) was needed.

4. Between 12 and 48 h were required for 17α-ethinyloestradiol to produce the effect.

5. Bilirubin maximum secretion rate (*Tm*) was little affected, the only significant reduction being produced by the 3-methyl ether of 17α-ethinyloestradiol (mestranol).

6. Rises in serum conjugated bilirubin following infusion of bilirubin were produced by 17α-ethinyloestradiol and mestranol but not by the progestogens.

Introduction

The occasional occurrence of jaundice in women receiving contraceptive steroids has led to studies of their effect on liver functions, especially those thought to reflect interference with biliary secretion. Few studies have included direct measurement of bile flow and of bilirubin secretory capacity, and most have used model substances. From studies of bromsulphthalein (BSP) clearance it has been suggested that oestrogens have a greater effect than have progestogens (Gallagher, Mueller & Kappas, 1966). The effect of 17α-" alkyl " substituted steroids has been emphasized on the basis of clinical observation (Sherlock, 1968). This has led us to examine the effects on bile flow and the maximum rate of bilirubin secretion, and to compare oestrogens and progestogens, and their 17α-ethinyl derivatives. These *in vivo* studies are a step towards the examination of the mechanism of cholestasis at the tissue level, *in vitro*.

Methods

Virgin female Wistar rats weighing 145–290 g were fed rat MRC 41B diet. The rats were not fasted before use.

Bilirubin maximum secretion rate (*Tm*) was determined according to the technique of Weinbren & Billing (1956). Veterinary Nembutal (Abbott) was injected

intraperitoneally in doses of 0·07 ml/100 g body weight. The bile duct was cannulated with a polythene tubing (grade PP 25, Portex, Hythe, Kent) and the bile collected in weighed tubes and kept in ice, or deep frozen, until analysed for bilirubin. Bile was collected for three 10 min periods (basal bile flow), a priming dose of bilirubin was given and after 15 min of continuous bilirubin infusion the rate of flow was measured for three 10 min intervals. Bile bilirubin and bilirubin *Tm* were measured according to Malloy & Evelyn (1937). Methanol was replaced by ethanol. The rate of bile flow was determined by weighing the tubes. The body temperature of the rat was maintained between 37° and 38° C.

Bilirubin (B.D.H.) (24 mg) was dissolved in 6 ml 1% Na_2CO_3 and an equal amount of 1·04% saline was added to give a concentration of 2 mg/ml. This was used for the priming dose (2 mg/100 g body weight during 2 min).

For infusion the priming solution was diluted with 0·85% NaCl to give a concentration of 1·3 mg/ml, and injected into either vena femoralis externa or interna with a continuous slow injector apparatus (Palmer Ltd., London) delivering 1 ml over a period of 4·6 min.

Blood was taken by heart puncture at the end of the experiment and conjugated and total bilirubin of serum were determined according to Malloy & Evelyn (1937).

Conjugated and total liver bilirubin were measured according to Hargreaves (1963) after perfusion of the liver with ice-cold saline. Dry weights of the homogenate were determined.

Steroid solutions for intraperitoneal or intravenous injections were prepared according to Mueller & Kappas (1964) by solution in 10% (v/v) N,N-dimethylacetamide in propylene glycol. The progestogens at the dosage 40 mg/kg were dissolved in 40% (v/v) N,N-dimethylacetamide in propylene glycol. The controls received the same volume of solvent (0·1 ml/100 g body weight).

The generous gifts from B.D.H. Pharmaceuticals Ltd., London, of 17α-ethinyl-oestradiol (estra-1,3,5(10)-trien-17α-ethinyl-3,17β-diol); from Organon Laboratories Ltd. of mestranol (3-methoxyestra-1,3,5(10)-trien-17α-ethinyl-17β-ol) and lynoestrenol (estren-4-17α-ethinyl-17β-ol); from Syntex Pharmaceuticals Ltd. of norethisterone (estren-4-17α-ethinyl-17β-ol-3-one) and its acetate; and from Searle & Co. Ltd. of norethandrolone (17-hydroxy-19-nor-17α-pregn-4-3-one) are gratefully acknowledged.

Oestradiol-17β (estra-1,3,5 (10)-trien-3-17β-diol) and 19-nor-testosterone (estren-4-17β-ol-3-one) were from Steraloids Ltd.

Results

We first sought to determine which of the two steroid components of contraceptive tablets, the oestrogen or the progestogen, affected bile and bilirubin secretion and whether 17α-ethinyl substitution was important. Rats were treated daily for 5 days, intraperitoneally, with 5 mg/kg oestradiol-17β and the ethinyl derivatives, 17α-ethinyl-oestradiol and its 3-methyl ether (mestranol). Following cannulation of the bile duct of each rat the basal bile flow and bilirubin *Tm* were determined. Table 1 compares the oestrogen-treated animals with untreated controls and those receiving only the solvent. At the conclusion of each experiment, total and conjugated bilirubin were estimated in the liver and serum. Only the conjugated

bilirubin is given in the tables because the amounts of total bilirubin did not differ significantly between the groups (except in two experiments). Progestogens (lynoestrenol and norethisterone acetate) were tested for their effects on bile flow, bilirubin *Tm* and serum and liver bilirubin (Table 2). Experiments with high dosage (40 mg/kg) were included, as progestogens are combined with oestrogens in contraceptive pills at a ratio 10/1 to 80/1. The low solubility of progestogens made it impossible to increase the dose above 40 mg/kg. 17α-ethinyl derivatives of both oestrogens and progestogens reduced basal bile flow, and bile flow during infusion by about 30–45%, with small or negligible effect on bilirubin *Tm*. A higher dose of progestogen was required to reduce bile flow markedly. The conjugated bilirubin in serum and liver were not significantly changed by progestogens, but the 17α-ethinyl oestrogens consistently raised the serum conjugated bilirubin.

TABLE 1. *Effect on bile flow, bilirubin* Tm, *and conjugated serum and liver bilirubin of oestradiol and derivatives at a daily dose of 5 mg/kg for 5 days*

Treatment	No. of rats	Basal bile flow (mg/min per 100 g body weight)	Bile flow during bilirubin infusion (mg/min per 100 g body weight)	Bilirubin *Tm* (μg/min per 100 g body weight)	Serum conjugated bilirubin (mg/100 ml)	Liver conjugated bilirubin (mg/100 g)
None	4	8·0±2·1	9·2±2·4	81·3± 6·9	5·8±3·3	15·3±5·0
Solvent	4	9·4±1·3	10·6±0·75	73·0± 7·2	6·5±0·74	18·1±2·1
Oestradiol	5	7·5±2·1	8·4±2·0*	76·2±11·3	11·9±4·8*	16·0±1·87
Oestradiol 17α-ethinyl	4	5·2±0·76**	4·9±1·2***	62·5±11·5	19·3±7·2*	18·5±3·1
Oestradiol 17α-ethinyl 3-O-methyl	5	4·6±0·45***	4·6±1·2***	53·1± 8·3***	15·4±3·6***	21·5±3·3

Values in table are means and standard deviations. Steroids were given in 10% dimethylacetamide in propylene glycol. *P* values have been calculated for the difference between oestrogen and solvent-treated animals. They are marked * for $P<0·05$, ** for $P<0·01$ and *** for $P<0·001$.

TABLE 2. *Effect of progestogens on bile flow, bilirubin* Tm, *conjugated serum and liver bilirubin*

Treatment	No. of rats	Basal bile flow (mg/min per 100 g body weight)	Bile flow during bilirubin infusion (mg/min per 100 g body weight)	Bilirubin *Tm* (μg/min per 100 g body weight)	Serum conjugated bilirubin (mg/100 ml)	Liver conjugated bilirubin (mg/100 g)
Experiment 1						
Solvent	4	6·7±0·71	7·1±0·69	63·0± 7·2	8·0±1·2	11·2±0·79
19-Nortestosterone (5 mg/kg per day for 5 days)	5	6·9±1·4	7·3±1·1	73·9±11·7	8·4±2·4	11·6±3·6
19-Nortestosterone (40 mg/kg per day for 5 days)	5	8·7±1·8	9·0±2·4	72·3±19·0	5·5±1·6	9·9±1·9
Norethisterone (5 mg/kg per day for 5 days)	4	5·8±1·7	5·8±0·85	59·2± 8·1	9·9±1·1	13·9±0·79
Experiment 2						
Solvent	4	11·5±2·6	11·6±2·2	59·1±10·6	6·9±2·7	12·4±0·72
Lynoestrenol (40 mg/kg per day for 5 days)	4	6·3±1·7*	6·0±1·5**	48·8±11·8	9·9±4·7	14·3±4·9
Norethisterone acetate (40 mg/day for 5 days)	5	7·7±1·3*	7·6±1·0**	47·5± 5·4	5·4±3·3	13·9±2·9

Values in table are means and standard deviations. In experiments 1 and 2 steroids were given in 10% and 40% dimethylacetamide in propylene glycol respectively. *P* values have been calculated for the difference between progestogen and solvent-treated animals. They are marked * for $P<0·05$, ** for $P<0·01$ and *** for $P<0·001$.

In view of the effect of the 17α-ethinyl oestrogens on bile flow at a low dosage, we attempted to determine the time required to produce this inhibitory effect. In two experiments (Table 3, experiments 1 and 2) animals were examined 2 days after treatment with 5 mg/kg per day and compared with solvent-treated controls. Only the bile flow was affected by this treatment in both experiments. In one of the two experiments the serum and liver conjugated bilirubin was elevated. The effect of larger intraperitoneal doses (25 mg/kg per day) was also studied in two experiments at 2 days (experiments 1 and 2, Table 3). Again the bile flow was reduced, both in the basal period and during infusion, without affecting the bilirubin *Tm*. In both experiments the serum conjugated pigment was significantly elevated. Four animals (two test and two controls) were studied 24 h after an intraperitoneal dose of 17α-ethinyloestradiol 25 mg/kg. The basal bile flow and bile flow during infusion were reduced in one treated rat and in the other bile flow during infusion and bilirubin *Tm* were reduced. The conjugated pigments of serum and liver were elevated in both animals. Two rats were studied 24 h after a single 5 mg/kg dose of 17α-ethinyloestradiol and showed no response.

In order to determine the effect of 17α-ethinyloestradiol at shorter time intervals, rats were injected with the steroid (25 mg/kg) intraperitoneally and tested after 2–12 h. No effect on bile flow and bilirubin *Tm* was noted. Two animals which received 50 mg/kg also had a normal bile flow and normal bilirubin *Tm* when tested 2 and 3 h after intraperitoneal injection.

In a further attempt to elicit an early effect of 17α-ethinyl-oestradiol on bile flow and bilirubin *Tm*, rats were cannulated, the basal bile flow determined, and the steroid (25 mg/kg) given intraperitoneally. The animals were allowed to recover from anaesthetic, under restraint. Six to 24 hours later a second dose of pentobarbitone was given in order to determine basal bile flow and bilirubin *Tm*. Twelve rats were studied. Two had very low bile flow before and during infusion, and the bilirubin *Tm* was also very low. In two the bile flow almost stopped with the second dose of pentobarbitone and the bile became colourless. Six treated animals

TABLE 3. *Effect of* 17α-*ethinyloestradiol on bile flow, bilirubin* Tm, *conjugated serum and liver bilirubin*

Treatment	No. of rats	Basal bile flow (mg/min per 100 g body weight)	Bile flow during bilirubin infusion (mg/min per 100 g body weight)	Bilirubin *Tm* (μg/min per 100 g body weight)	Serum conjugated bilirubin (mg/100 ml)	Liver conjugated bilirubin (mg/100 g)
Experiment 1						
Solvent	4	9·0±2·1	9·4±1·8	104·5±35·9	6·4±0·9	15·0±2·1
Oestradiol 17α-ethinyl (5 mg/kg per day for 2 days)	4	5·9±1·3*	5·9±0·55**	92·4± 9·6	10·3±1·4**	21·4±3·7*
Oestradiol 17α-ethinyl (25 mg/kg per day for 2 days)	5	6·1±0·8*	5·7±0·55**	72·9± 3·6	11·6±1·3***	19·2±4·8
Experiment 2						
Solvent	5	7·1±1·1	8·1±0·71	68·6± 3·9	5·3±1·3	17·6±1·9
Oestradiol 17α-ethinyl (5 mg/kg per day for 2 days)	5	5·3±1·2*	5·5±0·73***	63·6± 5·3	7·8±3·1	18·9±1·5
Oestradiol 17α-ethinyl (25 mg/kg per day for 2 days)	5	5·3±1·4*	5·4±1·9*	64·2±18·1	11·9±4·8*	18·2±4·6

Values in table are means and standard deviations. 17α-Ethinyloestradiol was given in 10% dimethylacetamide in propylene glycol. *P* values have been calculated for the difference between oestrogen and solvent-treated animals. They are marked * for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$.

showed no important change, as did two at 6 h and two controls receiving solvent only. This series was not extended because of the difficulty of knowing whether the variable effects were due to cumulative loss of bile salts through the fistula, to the second dose of pentobarbitone, or to poor absorption of steroid. In order to avoid the latter uncertainty rats were anaesthetized, cannulated, and the basal bile flow was determined. Steroid was then given intravenously and the bile flow studied for the next 30 min, following which bilirubin *Tm* was measured as usual. Pairs of animals were studied in this way with 17α-ethinyloestradiol (25 mg/kg) and lynoestrenol (25 mg/kg) without any marked changes being produced. Hargreaves & Lathe (1963) have noticed large immediate effects on indocyanine green secretion by intravenous norethandrolone, and so the above experiment was repeated with 25 mg/kg norethandrolone. Only minor changes in bile flow or bilirubin *Tm* were seen.

The possibility that the liver had to be stimulated by an oestrogen for some days before an inhibitory effect of 17α-ethinyloestradiol could be evoked was examined. Rats were given oestradiol-17β (5 mg/kg) intraperitoneally for 5 days following which a single dose of 17α-ethinyloestradiol (5 mg/kg) was given. The test and control rats were examined 6 hours later (Table 4). Only the total bilirubin of the liver was altered.

It was noted that in all the control groups of rats which received no treatment, steroid solvent, oestradiol-17β, or 19-nortestosterone, the bile flow during bilirubin infusion was higher than during the basal period. Although the increase in individual experiments was small and never significant, it seemed possible that bilirubin plus infused solvent might be having a choleretic effect, and that this might be altered by ethinyl substituted oestrogens and progestogens. This led us to determine the effect of 17α-ethinyloestrogens on bile flow during infusion with the solvent (Na_2CO_3 and NaCl) used for bilirubin. Basal bile flow was determined in the usual way, following which the solvent alone was given. This was done with a group of five controls and four mestranol-treated (5 mg/kg for 5 days) rats. The control animals had a basal bile flow of $7{\cdot}9 \pm 1{\cdot}3$ mg/100 g, compared with $9{\cdot}2 \pm 1{\cdot}6$ mg/100 g during infusion. In the mestranol-treated group the basal bile flow ($4{\cdot}7 \pm 1{\cdot}5$ mg/100 g) was very similar to that ($4{\cdot}5 \pm 1{\cdot}3$ mg/100 g) during infusion of solvent.

Weight loss was a feature of most of the experiments and occurred whenever oestrogens or 17α-ethinyl progestogens were given. The solvent alone was without

TABLE 4. *Effect on bile flow, bilirubin* Tm, *conjugated serum and liver bilirubin of* 17α-*ethinyloestradiol (5 mg/kg) or solvent as a single dose after pre-treatment with oestradiol at a daily dose of 5 mg/kg for 5 days*

Treatment	No. of rats	Basal bile flow (mg/min per 100 g body weight)	Bile flow during bilirubin infusion (mg/min per 100 g body weight)	Bilirubin *Tm* (μg/min per 100 g body weight)	Serum conjugated bilirubin (mg/100 ml)	Liver conjugated bilirubin (mg/100 g)
Solvent	5	$6{\cdot}4 \pm 0{\cdot}88$	$6{\cdot}5 \pm 0{\cdot}87$	$68{\cdot}7 \pm 13{\cdot}9$	$8{\cdot}5 \pm 3{\cdot}0$	$18{\cdot}2 \pm 0{\cdot}81$
Oestradiol 17α-ethinyl	5	$5{\cdot}9 \pm 0{\cdot}67$	$6{\cdot}1 \pm 0{\cdot}76$	$68{\cdot}4 \pm 13{\cdot}9$	$8{\cdot}9 \pm 4{\cdot}6$	$21{\cdot}6 \pm 3{\cdot}9$

Values in tables are means and standard deviations. Oestradiol and 17α-ethinyloestradiol were given in 10% dimethylacetamide in propylene glycol. Animals were tested 6 h after administration of 17α-ethinyloestradiol, or solvent. Both groups were pre-treated with oestradiol-17β.

effect. The weight loss during 5 day experiments with oestradiol (5 mg/kg), 17α-ethinyloestradiol (5 mg/kg), mestranol (5 mg/kg) and lynoestrenol (40 mg/kg) was about 20–30 g. Norethisterone acetate was somewhat less effective and the nortestosterone produced negligible loss even at 40 mg/kg. These losses did not appear to be correlated with the effects on bile flow and bilirubin *Tm*.

Discussion

The experiments were planned to define *in vivo* conditions for producing cholestasis with components of contraceptive pills. We have compared the effects of 17α-ethinyl oestrogens and progestogens, with that of their parent compounds, especially in regard to bile flow and bilirubin *Tm*. While the parent compounds, oestradiol-17β and 19-nortestosterone had little or no effect, both 17α-ethinyl substituted oestrogens (Table 1) and progestogens (Table 2, experiment 2) inhibited basal bile flow and bile flow during infusion of bilirubin, and to about the same extent (40%). However, the dosage required to produce these effects was much higher with progestogens (40 mg/kg) than with oestrogens (5 mg/kg).

Only one compound, the oestrogen, 3-O-methyl ether of 17α-ethinyl-oestradiol (mestranol) significantly reduced bilirubin *Tm*. Whether the effect of mestranol is due to this compound itself, or ethinyl-oestradiol after loss of the methyl group, is uncertain. Jungblut, Neumann, Smith, Collucci, de Sombre & Jensen (1965) reported that the 3-O-methyl group of mestranol is removed in the rat uterus. Quinestrenol, a cyclic 3-O derivative of ethinyloestradiol, is partly cleaved, probably in the liver (Steinetz, Meli, Giannina, Beach & Manning, 1967a).

The effect of the progestogens may be an indirect one through conversion to oestrogens. This may be the reason a higher dosage (40 mg/kg) was required. Brown & Blair (1960) showed that norethisterone and its acetate were partly secreted as a compound which yielded ethinyloestradiol on acid hydrolysis. Norethisterone and lynoestrenol differ in the absence of a 3-oxo group in the latter. There does not appear to be any information about aromatization of compounds lacking a 3-oxo group. However, Kamyab, Fotherby & Klopper (1968) showed that labelled lynoestrenol gave rise to 1·75% phenolic substances in the urine in man. Paper chromatography indicated the presence of two hydroxyl groups in the molecule. Norethisterone acetate (Table 2, experiment 2) was slightly less active than lynoestrenol. This difference was probably not due to the ester linkage, which is hydrolysed *in vivo* in humans (Brown & Blair, 1960). Our studies with a limited number of compounds do not make it possible to say what are the essential features of a cholestatic steroid. All the compounds tested had a 17β-OH. This group is not oxidized when there is a 17α-ethinyl group, and the latter has been shown to be very resistant to metabolism (Fotherby, Kamyab, Littleton & Klopper, 1966; Kamyab, Fotherby & Steele, 1969; Williams, Nilsen & Blahey, 1967). Our studies emphasize the importance of 17α-ethinyl substitution but leave open the question whether 3-hydroxyl, aromatic A ring and 17β-OH are necessary for inhibition of bile secretion.

There are few indications of the way in which 17α-ethinyl steroids might affect bile flow. Sperber (1959) has suggested that bile secretion is initiated by the active transport of bile acids and that water and salts flow towards the higher osmotic pressure in the bile canaliculi. An alternative explanation is the active transport of sodium ions. Emmelot & Bos (1966) showed that bile added *in vitro*, as well as

deoxycholate, activated the sodium potassium ATPase of the plasma membranes of liver. This might be the way in which bile acids affect the bile flow.

In attempting to explain the mode of action of steroids considerable importance is attached to the time required to produce cholestasis. Our experiments with oestrogens suggest that cholestasis is a late phenomenon. It appeared after 2 days at a dosage of 5 mg/kg (Table 3, experiments 1 and 2) but no greater effect was produced with 25 mg/kg (Table 3, experiments 1 and 2). At 24 h the results with the larger dose were variable (one out of two animals) and 5 mg/kg was without effect in this time. Although this may be partly due to dose dependence it seems unlikely that the steroid does not reach the liver during this time as Steinetz, Meli, Giannina & Beach (1967b) showed that 58% of a tracer dose of labelled ethinyloestradiol, given orally to rats, was secreted into the bile in 5 h, 45% being accounted for in the first 2·5 h. Moreover, when ethinyloestradiol and lynoestrenol were given intravenously to assure absorption, bile secretion was also unimpaired. In summary, between 12 and 24 h (or longer) are required to produce an effect with ethinyloestradiol. We have established that norethandrolone (25 mg/kg at 45 min) has no immediate large effect on bile flow or bilirubin *Tm*, in contrast to its marked effect on indocyanine green secretion (Hargreaves & Lathe, 1963). There remains the possibility that it is not the steroid secreted in the bile that produces cholestasis but that retained in the liver. Steinetz *et al.* (1967a) have emphasized that much more 17α-ethinyloestradiol than oestradiol is retained in the liver at 24 h. A study of the movement of ethinyloestradiol during the hours before it produces cholestasis might be worth while.

The means by which 17α-ethinyl oestrogens affect bile secretion might have two phases: first, a change in liver metabolism, possibly by alteration in membrane permeability or in the amounts of some enzymes; second, the ethinyl steroid might compete for a transport mechanism, or inactivate an enzyme. The first of these two effects would probably be time dependent, the latter less so. Further, it was possible that cholestasis could be produced immediately by 17α-ethinyl oestrogens, provided that the time-requiring metabolic or membrane changes, due to their oestrogenic properties, had already been induced. We were able to exclude this by treating rats with oestradiol for 5 days, following which the animals were tested 6 h after administration of 17α-ethinyloestradiol 5 mg/kg. Bile secretion was unaffected.

The finding that ethinyl steroids inhibited bile flow more than bilirubin secretion indicates that the two processes are partly independent. Further, Gallagher *et al.* (1966) found that oestradiol-17β inhibited BSP secretion, which indicates that bilirubin and BSP are secreted in different ways.

The findings in the literature regarding the effect of bile flow on the secretion of substances are somewhat contradictory. Increase in bile flow without change in BSP and bilirubin secretion has previously been demonstrated in the dog (Bradley, 1960). This is contradictory to the findings of O'Maille, Richards & Short (1966), who showed that the *Tm* of BSP could be increased in dogs by inducing cholestasis with taurocholate but not with secretin. Goresky & Kluger (1969) found that the bilirubin *Tm* was partly dependent on the bile flow. Billing (personal communication, 1969) was unable to increase bilirubin *Tm* in the rat with taurocholate. In general our experiments suggest that the bile flow during bilirubin infusion had to

be depressed to about 4 mg/min per 100 g body weight before the reduction of bilirubin *Tm*, which was about 25%, became significant.

Our findings are complementary to those of Javitt & Harkavy (1969) which appeared in abstract form during the course of this work. Ethinyloestradiol decreased bile flow. The *Tm* of taurocholate and of phenoldibromphthalein disulphonate, which is secreted without conjugation (Javitt, 1964) was depressed. Bilirubin was not studied.

Although the 17α-ethinyl progestogens did not have a significant effect on bilirubin *Tm*, there is a suggestion (Table 2, experiment 2) that they, like oestrogens, may reduce bilirubin *Tm*. This is supported by the significant rise in serum conjugated bilirubin in many experiments and even of liver conjugated bilirubin (Table 3, experiment 1). These rises indicate that the steroid does not act by inhibiting conjugation. The rise in serum must be due either to increased escape from the liver cells, a diminished storage capacity or reduced transport into bile canaliculi. The latter is probably the main mechanism, although others may contribute.

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