6. Landy, J., Yerasimides, T. G., Growdon, J. H., Basour, S. C., Surg. Forum, 1960, v11, 425.

7. Herion, J. C., Walker, R. I., Palmer, J. G., J. Exp. Med., 1961, v113, 1115.

8. Stetson, C. A., in Baterial Endotoxins, Landy, M., Braun, W., ed., Inst. of Microbiology, Rutgers, The State University, New Brunswick, N. J., 1964, p. 658.

9. Miler, I., Sterzl, J., Kostka, J., Lanc. A. ibid., p.291.

10. Parant, F., Parant, M., Charlier, H., Sacpuet, E., Chedid, L., Ann. Inst. Pasteur, 1966, v110, Suppl., 198.

11. Sanford, J. P., Noyes, H. E., J. Clin. Invest., 1958, v37, 1425.

Received November 21, 1966. P.S.E.B.M., 1967, v124.

Studies on Biliary Metabolites of Orally Administered Ethynylestradiol (EE) and its 3-Cyclopentyl Ether (EECPE-Quinestrol*). (31989)

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Preliminary experiments showed that measurable amounts of radioactivity appear in the bile of the rat shortly after oral administration of aqueous suspensions of isotopically labeled EE or EECPE. The observation that as much as 45% of the administered dose of EE and 27% of that of EECPE could be recovered from the 2½-hour bile samples indicated that the 2 compounds or their metabolites undergo an active enterohepatic circulation. It seemed, therefore, worthwhile to investigate the nature, distribution and biological activity of the radioactive substances appearing in the bile. The reabsorption of these substances was also studied.

Materials and methods. A) The chromatographically pure compounds tested were ethynylestradiol-6,7 ^{3}H with a specific activity of 0.93 $\mu c/\mu g$ and either ethynylestradiol-6,7 ^{3}H-3-cyclopentyl ether with a specific activity of 0.78 $\mu c/\mu g$ or a mixture of ethynylestradiol-6,7 ^{3}H-3-cyclopentyl ether with a specific activity of 0.78 $\mu c/\mu g$ and ethynylestradiol-3-cyclopentyl-$1^{14}C$ ether with a specific activity of 0.0392 $\mu c/\mu g$.

B) *Rate of biliary excretion and preliminary characterization of biliary metabolites.* Male albino rats, 350-400 g body weight, were used. Under ether anesthesia a polyethylene tubing (Intramedic® PE 10) was inserted into the common bile duct and secured in place by means of silk ligatures.

*Non-proprietary name.

TABLE I. Excretion of Radio-Metabolites of EECPE and EE in Rat Bile.*

	Percentage of administered dose		
	EECPE		EE
Time (hr)	H^3	C^{14}	H^3
2½	27.0	19.8	45.5
5	37.4	22.0	58.0

* Three EECPE-treated rats per group; 2 EE-treated rats per group. Average bile volumes were: EECPE, 2½ hr = 1.85 ml, 5 hr = 2.78 ml. EE, 2½ hr = 2.0 ml, 5 hr = 4.25 ml.

Upon closure of the abdominal incision, this tubing was exteriorized. The animals were thereafter transferred to individual restraining cages as described previously(1). Aqueous suspensions of ^{3}H-labeled EE or ^{3}H-^{14}C-labeled EECPE were administered by gavage at the doses and in the $^{3}H/^{14}C$ ratio (EECPE) as specified in Table I. Bile samples were obtained at 2½ and 5 hours following administration. After determination of total radioactivity present, the bile samples were diluted to 10 ml with HOH and extracted 3× with 20 ml volumes of ether. Ten ml of the pooled ether extracts ("free fraction") were evaporated in a counting vial and 15 ml liquid scintillation cocktail† added for determination of radioactivity on a 3 channel Packard Tri Carb liquid scintillation spectrometer Model 3365 equipped with an external standard for quench correction.

†Formula: 7 g PPO, 0.3 g dimethyl POPOP, 100 g naphthalene in 1 l redistilled dioxane.

The remaining aqueous phase was adjusted to pH 4.5 by adding 0.1 N HCl, and 5000 units β-glucuronidase (Ketodase) and 1 ml pH 4.5 acetate buffer were added. After incubation for 24 hours at 37°C, the pH was checked, and an additional 5000 units β-glucuronidase were added. The mixtures were incubated for an additional 24 hours and then extracted 3× with 20 ml volumes of ether. The ether extracts were pooled ("glucuronide fraction") and radioactivity determined as above. The aqueous residue was saturated with NaCl, adjusted to pH 1 with HCl and extracted 3× with 20 ml volumes of ethylacetate. After pooling the ethylacetate extracts ("sulfate fraction") radioactivity was determined as above. It is realized that this procedure (Ketodase hydrolysis and solvolysis) does not precisely identify the types of conjugates present, but has given reproducible fractions in our laboratory. In one experiment, biliary metabolites were further fractionated according to the method of Brown(2) as applied by Cooke *et al*(3). The radioactivity in the small intestine and plasma was also investigated as described previously(4).

C) *Further characterization of biliary metabolites by thin layer chromatography (TLC) and determination of the biological activity of various fractions.* Cannulation of the common bile duct and bile collection was performed as described under B above. Labeled EE or EECPE (6.5 μc ^{3}H) were adjusted to a dose of 10 mg by addition of unlabeled steroid. Such a dose of either compound was administered by gavage as an aqueous suspension. Bile was collected for 24 hours. Bile samples from each group of EE- or EECPE-treated animals were respectively pooled and subjected to hydrolysis and extraction procedures as described in B and finally to TLC analysis. The primary TLC system employed was chloroform-methanol-water (485:15:1). Preliminary chromatograms of bile extracts indicated the presence of considerable radioactivity with low R_f values in this system. These portions of the chromatograms were therefore eluted and rechromatographed in system J of Lisboa(5) which is more suitable for highly polar steroids. System J employs water-saturated butanol-tert butanol (1:1). The radioactive spots were eluted from the final chromatograms and dissolved in 0.2 ml volumes of benzene-methanol (1:1) and aliquots (20 lambdas) were taken for determination of tritium present. The actual amount (μg) of material present in each fraction was estimated on the basis of radioactivity. This gives only an approximation since the molecular weight of the metabolites is unknown. Whenever possible, each fraction was thereafter diluted with sesame oil to obtain 3 desired concentrations. 0.2 ml of the final dilutions were administered orally for 3 consecutive days to immature female rats 30-35 g body weight. On the morning of the 4th day, the animals were killed and the wet weight of the uterus (after pressing out the intrauterine fluid) determined to the nearest 0.1 mg.

Potency ratios were calculated by comparing these results with those obtained following administration of scalar doses of standard EE or EECPE.

D) *Fate of biliary metabolites following intestinal reabsorption.* The procedures for cannulation of common bile duct and bile collection were similar to those described under B above. In these experiments, however, the bile from a donor animal was shifted directly into the small intestine of a recipient rat by means of a polyethylene tubing (Intramedic® PE 10). An interposed clear, small, air-tight reservoir was used in order to assess constancy of bile flow from one animal to the other. Tritiated EECPE in aqueous suspension was injected directly into the duodenum of the donor rat by means of a polyethylene tubing (Intramedic® PE 90) with a flanged tip inserted into the intestine through a small incision of the intestinal wall. The tubing was secured in place by means of a tobacco purse-type silk ligature of the incision. The bile from the recipient animal was likewise cellected. At the end of the observation period (approx. 2½ hours) plasma and perirenal fat samples were obtained and analyzed. The bile obtained from the recipient rats was analyzed as described under B above.

TABLE II. Distribution of Radioactivity in Bile of Rats Treated with Labeled EECPE or EE.*

Hydrolytic procedure	Solvent extractables as % of total biliary radioactivity					
	2½ hr			5 hr		
	EE	EECPE		EE	EECPE	
	H^3	H^3	C^{14}	H^3	H^3	C^{14}
None†	2	2	8	1	2	4
β-glucuronidase × 48 hr‡	48	50	33	56	51	35
Solvolysis§	32	36	21	32	43	23
Total extracted	82	88	62	89	96	62

* Three EECPE-treated rats per group; 2 EE-treated rats per group.

† Bile extracted 3 times with an equal vol ether.

‡ Bile adjusted to pH 4.5 and incubated at 37°C with Ketodase® (500 units/ml) in pH 4.5 acetate buffer for 48 hr extracted 3 times with an equal vol ether.

§ Bile adjusted to pH 1, saturated with NaCl and extracted 3 times with an equal vol ethyl acetate.

Results. A) *Rate of biliary excretion and preliminary characterization of biliary metabolites.* The data are shown in Tables I and II. Total radioactivity of bile samples from EE-treated rats corresponded to 45 and 58% of the administered dose at 2½ and 5 hours respectively following administration. A significantly lower total radioactivity was present in the bile samples from EECPE-treated animals. On the basis of ^3H counts, the distribution of biliary metabolites as expressed in per cent of the total radioactivity was fairly similar for the two compounds from both a quantitative and qualitative standpoint as far as free, glucuronide and sulphate fractions are concerned. In each case the glucuronide fraction contained the greater amount of tritium (approx. 50%), followed by the sulphate fraction (30-40%) with very little tritium present in the free fraction (1-2%). In the case of EECPE, 33-35% of the ^{14}C became ether extractable after glucuronidase hydrolysis and an additional 21-23% after solvolysis. The glucuronide and sulphate fractions of pooled bile of EE- or EECPE-treated rats were further fractionated by the method of Brown (2). About twice as much radioactivity appeared in the glucuronide-phenolic fraction of bile of EE- than EECPE-treated animals where the reverse was true of the glucuronide-neutral fraction (Table III). The sulphate fractions of each group contained predominantly highly acidic metabolites. In the case of EECPE, the ^{14}C was associated primarily with the glucuronide-neutral fraction and the acidic fraction of the sulphates.

The distribution of radioactivity in plasma of bile-cannulated rats treated with EECPE or EE is shown in Table IV. The patterns observed in plasma were very different for the two compounds and different from those observed in bile. In the case of EE, most of the tritium counts appeared in the sulphate fraction. EECPE treatment resulted in high levels of tritium in the free and glucuronide fractions although the sulphate fraction again contained most of the

TABLE III. Distribution of Biliary Metabolites of EECPE and EE when Fractionated According to Brown(2).

Treatment	Sub-fraction	Distribution of radioactivity as percentage of radioactivity in each major fraction			
		Glucuronide		Sulfate	
		H^3	C^{14}	H^3	C^{14}
EECPE	Phenolic	13.6	0	9.2	0
	Neutral	22.0	46.0	5.2	0
	"Estriol"	12.4	14.3	6.9	0
	"Acid"	7.9	11.0	25.6	39.3
	Total extracted	55.9	71.3	46.9	39.3
EE	Phenolic	24.8	—	13.0	—
	Neutral	10.2	—	4.1	—
	"Estriol"	6.4	—	5.9	—
	"Acid"	6.8	—	28.9	—
	Total extracted	48.2		51.9	

TABLE IV. Distribution of Radioactivity in Plasma of Rats (with Bile Ducts Cannulated) Treated with EECPE or EE.

Compound administered	Time hr	Total plasma radioactivity (dpm/ml)		% of plasma radioactivity in fraction							
				Free		Glucuronide		Sulfate		Non-extractable	
		H^3	C^{14}	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}
EECPE	2.5	9478	681	31.7	33.2	20.6	23.3	39.1	14.1	8.6	29.4
	5	5651	266	22.4	34.6	28.8	43.6	33.8	21.8	15.0	<1
EE	2.5	9459	—	6.5	—	6.2	—	79.2	—	7.9	—
	5	2089	—	3.9	—	10.0	—	86.0	—	<1	—

^{3}H. Although total radioactivity in plasma was similar at 2½ hours, the drop observed at 5 hours was much more precipitous in the case of EE-treated animals.

The residual radioactivity extracted from the lumen of the small intestine at 2½ and 5 hours was low in the case of both EE- and EECPE-treated, bile duct-cannulated animals (Table V). However, some water soluble radioactivity was found in each intestinal washing suggesting that some alteration of EE and EECPE may occur in the small intestine prior to absorption.

B) *Further characterization of biliary metabolites by TLC and determination of the biological activity of various fractions.* The extracts of bile obtained from rats treated with a 10 mg dose of EE or EECPE were divided into 2 aliquots. One aliquot was bioassayed as such ("A" series), whereas the second aliquot ("B" series) was subjected to TLC in one or two systems. The radioactive spots were eluted from the chromatograms and then diluted for the bioassay. The biological activity of each fraction was compared with that obtained following administration of standard EE or EECPE (Fig. 1). The R_f values, uterine weights and estimated potencies (in terms of EE) of the various radioactive fractions are shown in Table VI. The free fraction obtained from bile of EECPE- or EE-treated animals exhibited similar uterotrophic activity which amounted to 15-20% that of EE standard. One "free" metabolite of EE (F_1B_2, R_f 0.38) had approximately the same mobility but only 48% of the activity of EE. The glucuronide fractions of bile of EECPE- or EE-treated animals

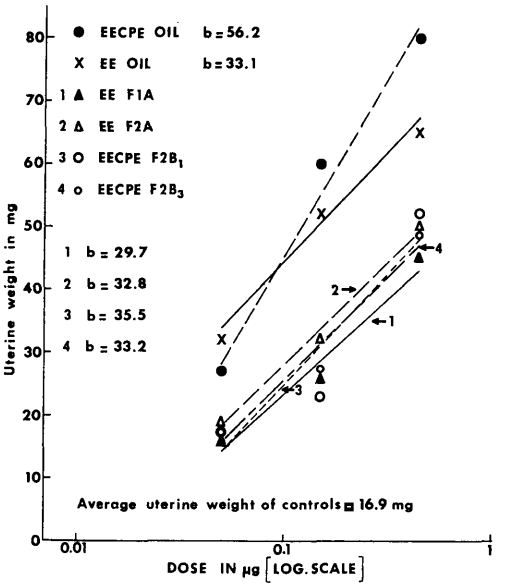

FIG. 1.

exhibited definite uterotrophic activity. The sulphate fraction of EECPE- but not of EE-treated animals likewise stimulated uterine growth. Some of the glucuronide subfractions

TABLE V. Influence of Intestine on Labeled EECPE and EE in Rats with Cannulated Bile Ducts.

Compound administered	% of dose in small intestine							
	2½ hr				5 hr			
	Ether sol		Water sol		Ether sol		Water sol	
	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}
EECPE	1.44	1.21	2.82	1.77	.36	.24	1.96	1.57
EE	2.56	—	3.00	—	2.55	—	2.56	—

exerted uterotrophic activity (notably F_2B_1 and F_2B_3 from EECPE-treated rats) but none of the sulphates were active at the doses tested. While it is impossible to cross-compare metabolites on the basis of R_f in only 1 or 2 TLC systems, the general pattern of R_f values was remarkably similar between the various metabolites of the two compounds. With the exception of F_1B_2 (EE) noted above and F_2B_3 (EE), none of the metabolites had R_f values comparable to EE or EECPE. F_2B_3 (EE) had only 8% of the uterotrophic activity of EE so that it cannot be the same compound.

C) *Fate of biliary metabolites following intestinal reabsorption.* It was assumed on the basis of the results reported under A above that 27% of the ^3H dose and 20% of the ^{14}C dose would appear in the bile of the donor animals during the 2½-3 hours of transfusion, and these figures were used to estimate the doses of radioactivity received by the recipient rats. If these assumptions are valid, the recipient animals excreted only 7-8% of the administered radioactivity in their bile (Table VII). However, the distri-

TABLE VI. Biological Activity of Biliary Metabolites of EECPE and EE.

Source of metabolities	Fraction	TLC sub-fraction	R_f system 1	R_f system 2	Daily dose (μg/rat)*	Uterine wt (mg)	Biological activity % EE
EECPE-6,7-H^3 (R_f system 1 = .81)	Free (F1A)	—	—	—	.5	39.0	15
		$F1B_1$	.08	—	.15	14.0	<9 if any
	Glucuronide (F2A)	—	—		1.0	48.8	14
		$F2B_1$	.17	.80	.45	52.3	
					.15	23.2	25
					.05	18.5	
		$F2B_2$	.22	—	.15	17.8	<9 if any
		$F2B_3$	.58	—	.45	49.9	
					.15	27.0	26
					.05	18.3	
	Sulfate (F3A)	—	—	—	1.0	34.8	6
		$F3B_1$	.18	.84	.15	17.9	<9 if any
EE-6,7-H^3 (R_f system 1 = .40)	Free (F1A)	—	—	—	.45	44.7	
					.15	25.7	20
					.05	16.4	
		$F1B_1$	.10	—	.15	14.0	<9 if any
		$F1B_2$	.38	—	.15	44.5	48
					.05	19.3	
	Glucuronide (F2A)	—	—	—	.45	50.1	
					.15	32.5	31
					.05	19.0	
		$F2B_{1a}$	.17	.53	.45	23.0	
					.15	15.6	9?
					.05	15.6	
		$F2B_{1b}$	.17	.82	.45	22.5	
					.15	15.1	9?
					.05	15.3	
		$F2B_2$	.30	—	.45	17.6	<6 if any
		$F2B_3$	.43	—	.45	30.1	8
					.15	17.2	
		$F2B_4$	.58	—	.45	17.2	<6 if any
	Sulfate (F3A)	—	—	—	.45	18.7	<6 if any
		$F3B_{1a}$	.20	.55	.45	20.9	<6 if any
		$F3B_{1b}$	.20	.85	.45	17.9	<6 if any
		$B3B_2$	.52	—	.45	16.2	<6 if any

* 5 rats per group. Doses only approximate as they are based on specific activities of originally administered EE and EECPE. Obviously the molecular weights of the metabolites would be different.

TABLE VII. Absorption and Distribution of Radioactivity in Bile of Rats Receiving an Intraduodenal Infusion of Bile from EECPE-Treated Rats.

Source of bile	Doses administered (μc)	% of dose in bile		% distribution of total bile radioactivity					
				Free		Glucuronide		Sulfate	
		H^3	C^{14}	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}
EECPE-treated rats (2.8 ml)	5.43 H^3 .79 C^{14}	27.0	19.8	2	8	50	33	36	21
Rats infused with bile of EECPE-treated rats (2.3 ml)	6.5 H^{3*} .58 C^{14*}	7.1*	8.6*	3	8	56	36	37	23

* These values estimated on basis of percentage of dose found in bile of EECPE-treated rats. Thus in this case the bile donor rats were dosed with 24.2 μc H^3 and 2.93 μc C^{14}. It was therefore calculated that the recipient rats when infused with the bile of the donor rats received approximately 27% and 20% of the original H^3 and C^{14} doses respectively.

bution of metabolites between the free, glucuronide and sulphate fractions of bile of recipient animals was very similar to that observed in bile of other rats treated orally with EECPE (Table VII).

The perirenal fat of the recipient animals had little or no radioactivity (less than 1.5%) as compared with their corresponding donors although they had received approximately ¼ of the tritium dose (Table VIII). Low but significant amounts of radioactivity were found in the plasma of the reecipient animals.

Discussion. Present results show that following administration of radio-labeled EE or EECPE to rats, a large percentage of the radioactivity is found in the bile between 2½ and 5 hours. In each case, biliary metabolites were predominantly glucuronides but some sulphates were also present. Very little radioactivity was ether extractable (free fraction) prior to hydrolytic procedures.

Some of the biliary metabolites of either compound had oral biological activity (uterotrophic) but none of them was as potent as the original steroids. Thin layer chromatographic analysis likewise indicated the absence of unaltered EE or EECPE in bile. When it was possible to test 3 dose levels of the bile metabolites from EECPE-treated animals, it was obvious that the dose-response curves paralleled that of EE rather than EECPE. These findings suggest that these metabolites are not stored in body fat and therefore do not have prolonged activity.

The pattern of plasma metabolites was quite different from that seen in bile. Following EECPE, large amounts of free radioactivity were found in addition to glucuronide and sulphate conjugates. In the case of EE, the only significant radioactivity was found in the sulphate fraction.

The presence of significant amounts of

TABLE VIII. Distribution of Radioactivity in Perirenal Fat and Plasma of Rats Receiving an Intraduodenal Infusion of Bile from EECPE-Treated Rats.

Rat	Radioactivity in perirenal fat (dpm/g)		Plasma radioactivity							
			Free (dpm/ml)		Water sol (dpm/ml)		Glucuronides (dpm/ml)		Sulfates (dpm/ml)	
	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}	H^3	C^{14}
Donor* 1	16029	2137	1903	146	16598	763				
Recipient 1 (bile infused)	59	nsc†	nsc	nsc	208	nsc				
Donor* 2	23000	2720	1580	102	—	—	960	85	2070	100
Recipient 2 (bile infused)	359	nsc	80	nsc	—	—	281	nsc	274	nsc

* EECPE dose for donors = 24.2 μc H^3; 2.93 μc C^{14}. It is estimated from other data that recipients received 27% of the H^3 and 20% of the C^{14} doses.

† nsc = no significant counts.

water soluble radioactivity in the intestine of rats with cannulated bile duct suggests that a certain amount of either compound undergoes chemical transformation prior to absorption.

When bile from rats treated with EECPE was infused into the small intestine of the recipient animals, only 7-8% of the calculated administered radioactivity appeared in the bile of the recipients. Since little or no radioactivity could be found in body fat depots or plasma (free fraction) of the recipients, it appears that most of the metabolites excreted *via* the bile into the small intestine are either eliminated *via* the feces and/or reabsorbed from the large intestine prior to final disposition. In the dog, biliary conjugated metabolites of testosterone appear to be reabsorbed in the lower region of the gut and require a longer period for absorption than do free steroids(6). Thus, a period longer than 2½ hours may also be required for peak absorption of biliary metabolites of EECPE in rats. The pattern of radioactivity in the bile of the recipient rats was, however, similar to that observed in the bile collected directly from animals treated with EECPE. These findings show that metabolites of EECPE may undergo enterohepatic circulation.

Summary. Large amounts of radioactivity appeared in the bile at 2½ and 5 hours after a single oral dose of radiolabeled EECPE or EE. Conjugated metabolites (glucuronides and sulfates) predominated in bile although their pattern of distribution differed markedly from that observed in plasma of the same rats. Intestinal washings obtained from treated rats with bile duct cannulas suggested that some alteration of both EECPE and EE may occur in the gut. Thin layer chromatograms of extracts of bile obtained from EE- or EECPE-treated rats did not reveal the presence of unaltered EE or EECPE. However, several of the TLC fractions exerted uterotrophic activity when administered orally to immature rats. The dose response curves were similar to those of EE, suggesting loss of lipophilic properties in the case of EECPE metabolites. None of the fractions were as potent as EE or EECPE. When bile from EECPE-treated donor rats was infused into the small intestine of recipient rats, radioactivity appeared in the plasma and bile of the recipients providing direct evidence of active enterohepatic circulation of metabolites. Little or no radioactivity accumulated in body fat confirming the loss of lipophilic properties following biliary cycling.

1. Giannina, T., Steinetz, B. G., Meli, A., Proc. Soc. Exp. Biol. & Med., 1966, v121, 1175.

2. Brown, J. B., Biochem. J., 1955, v60, 185.

3. Cooke, A., Rodgers, A., Thomas, G., J. Endocrinol., 1963, v27, 299.

4. Steinetz, B. G., Meli, A., Beach, V. L., Giannina, T., Proc. Soc. Exp. Biol. & Med., 1966, v123, 163.

5. Lisboa, B., Acta Endocrinol., 1963, v43, 47.

6. Martin, R. P., Loriaux, D. L., Farnham, G., Steriods, 1965, Suppl. 11, 149.

Received December 2, 1966. P.S.E.B.M., 1967, v124.