Studies on Phenolic Steroids in Human Subjects

IX. ROLE OF THE INTESTINE IN THE CONJUGATION OF ESTRIOL

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ABSTRACT In order to compare the enteric circulation of estriol-16α-glucosiduronate (see preceding paper) with that of estriol (E_3), labeled estriol was administered to six women by several routes: both injection and infusion (300 min) into the cubital vein, injection into the portal vein system, ingestion and instillation into the jejunum and ileum. Urine, collected from 0–2, 2–4, 4–8, 8–12, and 12–24 hr, was analyzed by countercurrent distribution for its content of radioactive 3- and 16-glucosiduronate (E_3-3Gl,E_3-16Gl) and sulfoglucosiduronate (E_3-3S,16Gl) of estriol. After peripheral injection of E_3, E_3-16Gl was excreted rapidly and E_3-3S,16Gl at a slower and more constant rate. E_3-3Gl was barely detectable after infusion. After injection of E_3 into the portal vein, the excretion of E_3-3S, 16Gl was greater and quicker than after peripheral injection. Even in a subject with a complete bile fistula, the urinary excretion of E_3-3S,16Gl was essentially unchanged. Ingestion also produced the same result. Only after instillation into the ileum was a large and rapid excretion of E_3-3Gl obtained, whereas the excretion of E_3-3S,16Gl, and E_3-16Gl were depressed. These results together with those of the preceding paper suggest that E_3 does not readily appear in the small intestine except via a hepatoenteric circulation that produces very little E_3-3Gl. When present in the distal segment of the small intestine, however, absorption, conjugation, and elimination proceed readily.

INTRODUCTION

In a previous communication (1) we showed that the metabolic fate of labeled estriol 16α-glucosiduronate[1] was affected by its route of administration. Continuous, prolonged infusion as opposed to a rapid, single injection yielded a 3-glucosiduronate of estriol after a lag period of several hours (2). In order to explain such a lag period, it is necessary to postulate a pool which does not equilibrate readily with the pool in the vascular circulation. One possibility is an enteric circulation via the *succus entericus* (1), together with the formation of E_3-3Gl in the intestine, as has recently been shown by Stoa and Levitz (3). Since a role of the enterohepatic circulation is ruled out by the failure of the labeled steroid conjugate to appear in appreciable amounts in the bile, the experimental results were in accord with this postulate. Doubly labeled esteriol 16α-glucosiduronate injected into the intestinal lumen appeared, after a characteristic and consistent delay, in the urine as both the 3- and 16-conjugate of estriol, labeled primarily in the estriol moiety. Thus, absorption from the small intestine into the circulation and subsequent excretion in the urine apparently require prior hydrolysis. Whether the conjugate appears in the intestine, by passive or active means, has yet to be verified experimentally. To establish this, it became imperative to inject estriol in a fashion analagous to that of E_3-16Gl.

METHODS

E_3-6,7-^{3}H (140 μc/μmole)[2] or E_3-16-^{14}C (6.3 μc/μmole)[3] was administered to six women: three normal women in the

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[1] The following abbreviations and trivial names are used: estriol (E_3), estra-1,3,5(10)-triene-3,16α,17β-triol; estriol-16α-glucosiduronate (E_3-16Gl), 3,17β-dihydroxyestra-1,3,5(10)-trien-16α-yl-β-D-gluco pyranosiduronate; estriol-3-glucosiduronate (E_3-3Gl),16α,17β-dihydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; estriol-3-sulfate-16α-glucosiduronate (E_3-3S,16Gl), 3-sulfo-17β-hydroxyestra-1,3,-5(10)-trien-16α-yl-β-D glucopyranosiduronate; estriol-3,-16α-diglucosiduronate (E_3-3,16Gl), estriol-3,16α-bis-(-D-glucopyranosiduronate); estriol-3-sulfate (E_3-3S),16α-17β-dihydroxyestra-1,3,5(10)-trien-3-yl sulfate; UDPGA, uridine diphospho glucuronic acid; CCD, countercurrent distribution.

[2] The E_3-6,7-^{3}H was purchased from New England Nuclear Corporation.

[3] The E_3-16-^{14}C was synthesized by Dr. Mortimer Levitz from whom it was obtained through the courtesy of the American Cancer Society.

TABLE I

Route of Administration and Amount of E_3-6,7-^3H or E_3-16-^{14}C Administered to Female Subjects

Subject	Age	Route of administration	Radioactivity administered	Diagnosis
1*	43	Cubital vein	1 μc ^{14}C	Normal
2	28	Cont. infusion into cubital vein	10 μc ^3H	Normal
3	48	Portal vein	1 μc ^{14}C	Uterine carcinoma
4*	43	Ingestion	10 μc ^3H	Normal
5	55	Cubital vein	50 μc ^3H	Biliary fistula (T-tube)
6	48	Terminal ileum	10 μc ^3H	Uterine carcinoma

* Same subject.

follicular phase of the menstrual cycle, two with carcinoma of uterus at abdominal operation, and one with a biliary fistula (T-tube drainage). All subjects were in good nutritional status, and none had evident renal or hepatic dysfunction. The route of administration and other data related to these subjects are shown in Table I. The steroids administered were shown to be pure by paper chromatography.

The method of injection, the collection of urine, the extraction of urinary conjugates, the measurement of radioactivity, the solvent systems employed in CCD, and the partition coefficients of estriol conjugates in these systems (Table II) were exactly as those described in our previous paper (1). The ethanol extracts of the urine were submitted to CCD for 49 transfers in solvent system A (1). About 35 mg of sodium sulfate were added to tubes 0 and 1 in order to suppress emulsification of the phases. Two or three fractions were obtained, i.e., fraction I (N = 5–7,where N is the peak tube), fraction II (N = 10–11), and fraction III (N = 31–33). The first two fractions were redistributed in the same solvent system for 99 or 299 transfers and fraction III for 99 transfers in solvent system C. The partition coefficients and percentage of compounds present in the CCD train were computed by means of a computer program developed by Priore and Kirdani (4, 5). The percentage of the E_3 conjugates was calculated on the basis of the radioactivity present in each peak of the first CCD run. The urinary and biliary metabolites were characterized according to techniques described in our previous paper (1).

RESULTS

Injection into the cubital vein. The cumulative radioactivity of urinary conjugates during the 1st 24 hr after injection or continuous infusion of E_3 into two normal women in the follicular phase of the menstrual cycle is shown in Fig. 1.

After a single, rapid injection, the excretion pattern of the urinary radioactivity was identical with that of our previous report (6), i.e., approximately 30% of the radioactivity injected was excreted within 4 hr and nearly 50% within 8 hr. The preponderant portion of the urinary radioactivity was excreted as E_3-16Gl; about 14% of the radioactivity administered was excreted in the urine as E_3-3S,16Gl. In the case of the continuous infusion for 300 min, the excretion patterns of the total radioactivity, E_3-16Gl and E_3-3S,16Gl were almost identical with those obtained after the single injection. A small amount of E_3-3Gl, however, was found in the urine starting 8 hr after cessation of infusion. Since less than 2% of the urinary radioactivity was present as this conjugate, its identification should be regarded with caution. In both experiments, it appears that the excretion of E_3-3S,16Gl was at a relatively constant rate, whereas that of E_3-16Gl was at least at a double rate, i.e., an initial rapid rate followed by a subsequently slower one. Therefore, the relative amount of E_3-3S, 16Gl as compared to E_3-16Gl increased gradually with the passage of time.

The results obtained by analysis of conjugation of the urine show only the net effect of many processes in different metabolic pools. Since such pools may exist in the enteric or enterohepatic circulation, it is important to investigate whether these pools do exist and the characteristic of each pool in the disposition of the E_3 or E_3 conjugates. On the assumption that these pools might exist in the liver and/or in the small intestine (including the lumen), the following experiments were undertaken.

Injection in to the portal vein system. In order to assess the role of the liver in the disposition of E_3, the steroid was injected directly into the portal vein system. Considerable amounts of E_3-3S,16Gl were excreted

TABLE II

Partition Coefficients of Some Estriol Conjugates

Conjugate	System A	System B	System C
E_3-16Gl	2.1	1.1	0.7
E_3-3Gl	0.21	0.1	0.7
E_3-3S, 16Gl	0.09	0.05	0.1
E_3-3S	4.1	1.9	4.8

System A = *n*-butanol:ethylacetate:0.2% ammonium hydroxide (3:1:4); System B = *n*-butanol:ethylacetate:0.2% ammonium hydroxide (1:1:2); System C = *n*-butanol:10% ammonium hydroxide (1:1).

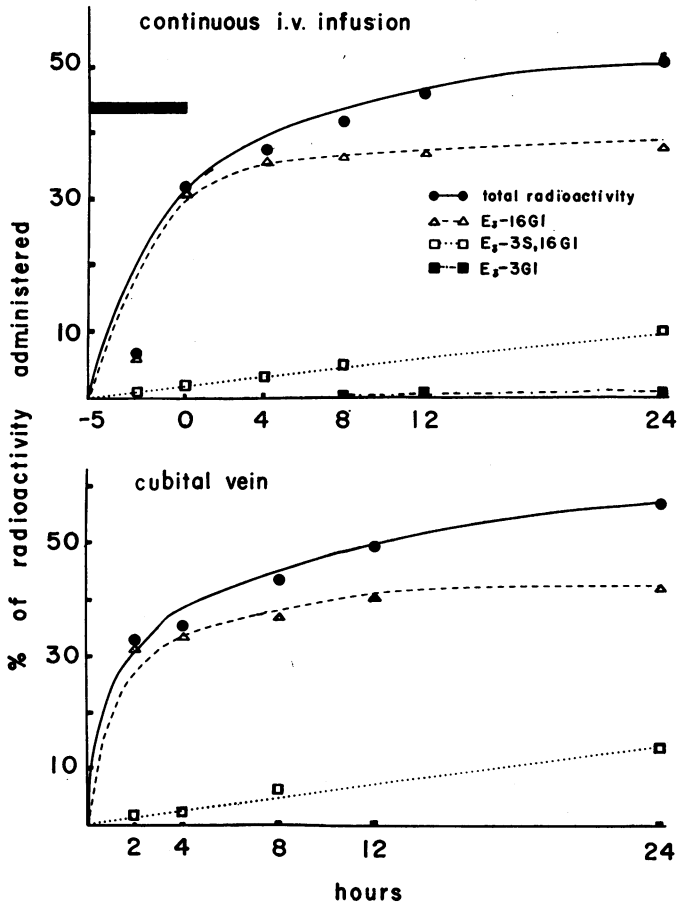

FIGURE 1 The cumulative urinary excretion of radioactive estriol conjugates following a 300 min (solid bar) infusion (TOP) and following the rapid injection (BOTTOM) into peripheral veins of two normal women of 10 μc of 6,7-^{3}H-estriol and of 1 μc of 16-^{14}C-estriol, respectively. As was pointed out in the previous paper, even though the lines in this and in the following figures have been extrapolated to 0, the segment between 0–2 hr has no meaning regarding the rate of excretion of radioactivity during that period.

immediately in the urine (Fig. 2). The percentage of E_3-3S,16Gl excreted in the urine during 0–4 hr after injection into the portal vein was much higher than that obtained after a single injection into the cubital vein (9.12% vs. 5.5%). Thereafter, the proportion of E_3-3S,16Gl to E_3-16Gl increased gradually in the same manner as that following injection into the cubital vein. No E_3-3Gl or E_3-3S was detected.

Peripheral injection into a subject with T-tube drainage of bile. In order to further clarify the hepatic disposition of E_3, the steroid was injected into a subject with a T-tube, whose bile was continuously drained during the time of the study so that no enterohepatic circulation could occur. After injection into the cubital vein (Fig. 2), nearly 30% of the radioactivity administered was excreted in the urine within 24 hr; approximately 20% was excreted in the bile.[4] Nearly 8% of the radioactivity injected was excreted in the urine as E_3-3S, 16Gl, whereas after intraportal injection about 11% was present as this conjugate. These amounts are also comparable with those obtained after injection into the cubital vein, 10.6 and 14.0% for single injection and continuous infusion, respectively. No detectable amounts of E_3-3Gl and E_3-3S were excreted in the urine.

In summary, under experimental conditions in which the results should reflect hepatic disposition, the urine contained mostly E_3-16Gl and some E_3-3S,16Gl. The rate of urinary excretion of E_3-3S,16Gl did not change significantly with or without enterohepatic circulation. No detectable amount of E_3-3Gl was excreted in the urine.

Ingestion. The findings that a small amount of E_3-3Gl may have been present in the urine after the infusion of E_3 for a 300 min period and that hepatic disposition does not appear to be concerned with E_3-3Gl formation led us to study the enteric disposition of E_3.

In order to know the net effect on the urinary E_3 conjugates formed during passage through the gut, liver, and kidneys, E_3 was administered orally. The results are shown in Fig. 3. Approximately 30% of the radioactivity administered was excreted in the urine during the initial 24 hr after ingestion. The preponderant part of the radioactivity was excreted as E_3-16Gl; E_3-3S,16Gl

[4] This bile and an aliquot of this urine was analyzed and reported separately by Kirdani, Priore, and Sandberg (23). A sepecially constructed T-tube assured complete collections of bile.

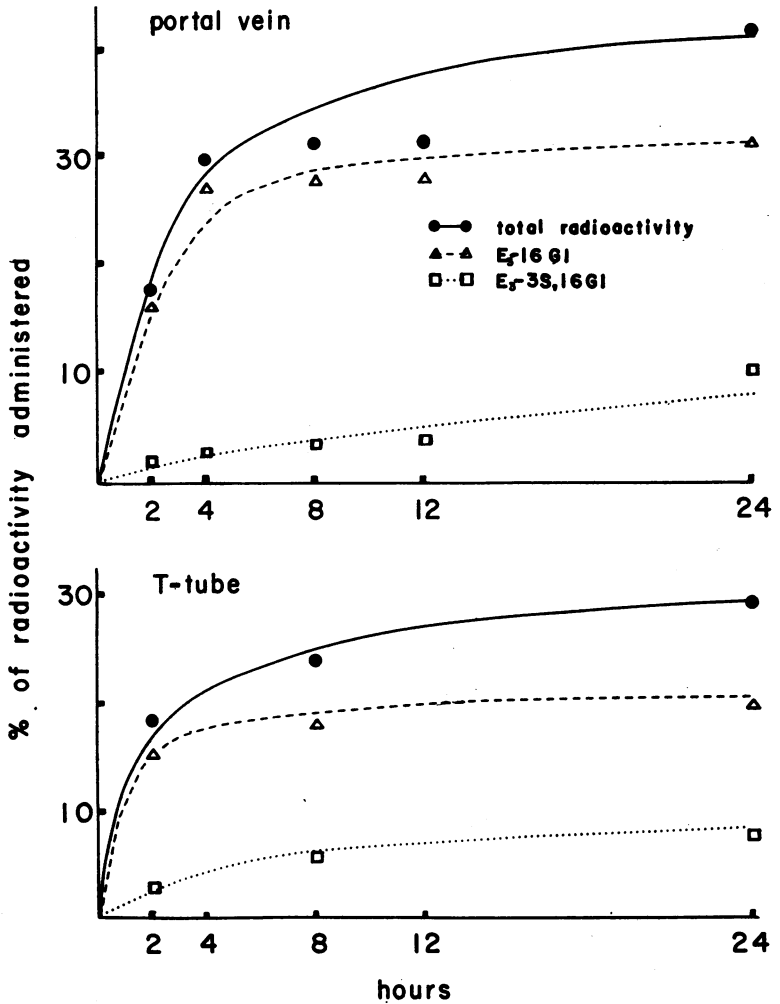

FIGURE 2 The cumulative urinary excretion of radioactive estriol conjugates following injection of 1 μc of 16-^{14}C-estriol into the portal vein system of a woman with uterine carcinoma (TOP) and following peripheral injection of 50 μc of 6,7-^{3}H-estriol into a woman with a complete biliary fistula (BOTTOM).

appeared in the urine in the first period (0–2 hr). The proportion of E_3-3S,16Gl to E_3-16Gl increased gradually with time. No detectable amount of E_3-3Gl was excreted in the urine, indicating that E_3-3Gl was not formed at that part of the gastrointestinal tract where the steroid was absorbed, not in the liver or in the kidney.

Instillation into the terminal ileum. Another approach to determine the site of E_3-3Gl formation was made by injecting E_3 into the terminal ileum at abdominal operation. During the 24 hr after injection of E_3, the rates and patterns of urinary excretion of total radioactivity and various conjugates were considerably different from those obtained after administration of E_3 by other routes (Fig. 3). The rapid excretion of E_3-3Gl was the most characteristic feature; almost equal amounts of E_3-16Gl were excreted in the urine, whereas E_3-3S, 16Gl constituted only 6.4%.

Characterization of the urinary estriol conjugates. Primary identification was based on partition coefficients in two solvent systems. Previous experience (1) combined with better methods of quantitation of CCD (4, 5) have increased our confidence in the methodology. As a result, only the E_3-3S,16Gl fractions from all the experiments were combined and treated further as previously described (1). In summary, E_3-3S,16Gl moved identically with authentic standard[5] in CCD (N = 299), in high voltage paper electrophoresis, and on paper chromatography. Partial and sequential enzymatic[6, 7] hydrolyses yielded products consistent with the assignment made. Thus our confidence appears to be justified.

DISCUSSION

Recent tracer studies on the fate of E_3 in normal or pregnant women revealed that whereas no significant

[5] The E_3-15-^{3}H-3S,16Gl was kindly supplied by Dr. M. Levitz (22).

[6] Sigma Chemical Co., Bacterial Type II.

[7] Mann Research Lab, *Asperigillus oryzae*.

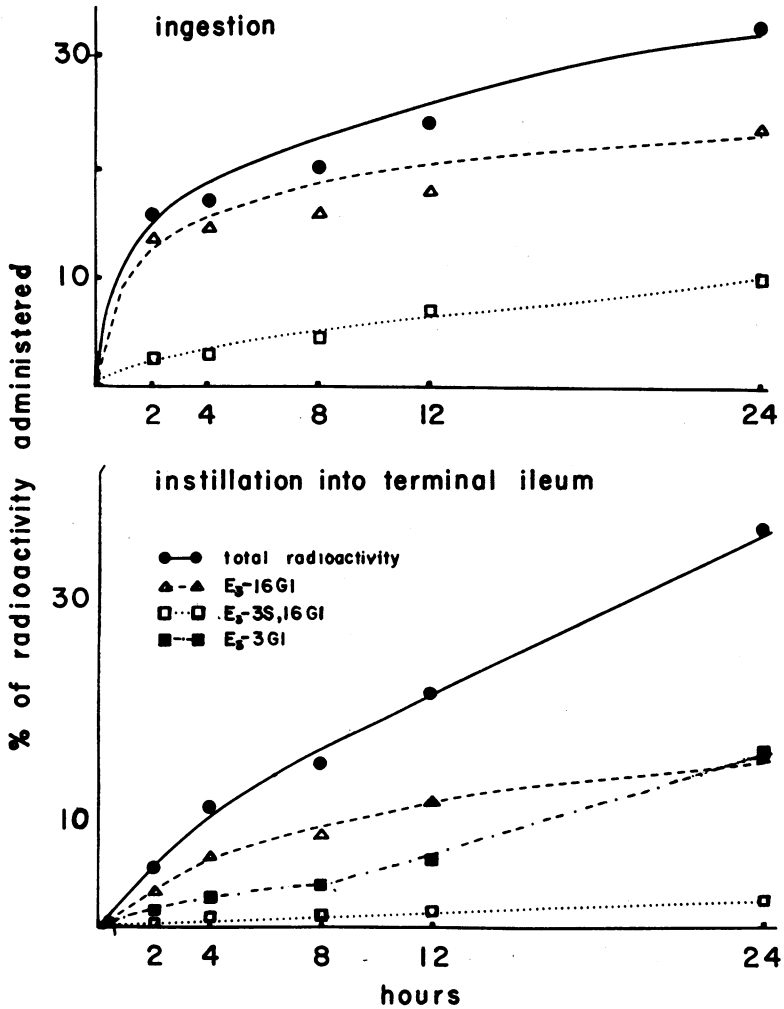

FIGURE 3 The cumulative urinary excretion of radioactive estriol conjugates following ingestion of 10 μc of 6,7-^{3}H-estriol by the same subject who received the rapid intravenous injection (see Fig. 1, bottom) (TOP) and following instillation of 10 μc of 6,7-^{3}H-estriol into the terminal ileum of a woman with uterine carcinoma (BOTTOM).

metabolic alteration of the steroid molecule itself occurs (6, 7), E_3 conjugates exist which can undergo re- and transconjugation (2, 8). None except E_3-16Gl (9), has been isolated and identified in chemically pure form. Of these E_3 conjugates, it has been shown that E_3-16Gl, the major urinary metabolite of estriol, is formed efficiently by human liver (10) and human small intestine (11, 12), whereas E_3-3S,16Gl, the major biliary metabolite of estriol (13), is presumably made in the liver. The interesting observation that significant amounts of E_3-3Gl are excreted in the urine after infusion of either E_3 or E_3-16Gl into normal or pregnant women has recently been presented (1, 2, 14). E_3-3Gl is also an important constituent of pregnancy urine (15, 16). The enzyme responsible for 3-glucuronide formation is located in some part of the small intestine (12, 17). Stoa and Levitz (3) have convincingly demonstrated that E_3-3Gl is an intestinal metabolite of estriol in vivo.

The present study was designed to estimate primarily the role of the liver and of the gastrointestinal tract in the conjugation of estriol in vivo. After the direct injection of E_3 into the portal vein, so that practically all of the steroid passes directly through the liver, E_3-3S, 16Gl and E_3-16Gl were immediately excreted in the urine in a slightly higher ratio than that ratio obtained from peripheral administration. The fact that the urinary excretion of E_3-3S,16Gl continued for at least 24 hr at a relatively constant rate, whereas the amount of E_3-16Gl in the urine decreased with time, may indicate that a small portion of the E_3 or E_3-16Gl was retained in the liver for a longer period of time. It was then excreted in the urine as well as in the bile as E_3-3S,16Gl in confirmation of the results of Stoa and Levitz (3). Another possibility is the enterohepatic circulation of E_3-3S,16Gl, which is known to be the major estriol conjugate in bile (13). However, the comparable amounts of E_3-3S, 16Gl excreted in the urine after injection of E_3 into a patient with complete bile collection would favor the

former possibility. These findings are in accord with those of Emerman Twombly, and Levitz (13), who observed that, following the intravenous infusion of E_3-3S into patients whose bile was continuously drained via a T-tube, only E_3-16Gl and E_3-3S,16Gl were excreted in the urine.

Another approach to the study of the hepatic metabolism of E_3 was made by ingestion. Although this experiment was probably unphysiological and the urinary recovery of radioactivity was poor, it is interesting that the urine contained E_3-16Gl and E_3-3S,16Gl in almost the same relative amounts as those obtained after injection into the portal vein. Thus, it appears most likely that most of the E_3-16Gl and all of the E_3-3S,16Gl are formed by the liver and excreted in the urine. The amount of urinary F_3-3S,16Gl is small and may or may not be related to that involved in the enterohepatic circulation. Most striking was the constancy of urinary excretion of E_3-3S,16Gl. In five subjects receiving estriol intravenously, including one with complete bile collection,, E_3-3S,16Gl excretion averaged 26% (range 21–29%) of the urinary radioactivity. (In contrast, this value was 6% with injection into the ileum.)

No remarkable difference between the results obtained after single injection and continuous infusion of E_3 was observed. This is in marked contrast to the results with E_3-16Gl (1) and indicates a difference in the handling of the two molecules. There are at least two alternative explanations. First, E_3 does not participate in an enteric circulation because its hepatic clearance may be more rapid than that of E_3-16Gl, thus giving the latter more opportunity to diffuse across the intestinal wall. Alternatively, there may be a specific structural requirement that tends to transport the conjugate across intestinal membranes more rapidly than its aglycone. This does not necessarily imply active transport since there is a difference in charge.

Our failure (see Fig. 1) to detect E_3-3Gl in the urine of subjects given intravenous E_3 is in contrast to the results of Goebelsmann, Sjoberg, Wiquist, and Diczfalusy (2) in which a considerable amount of E_3-3Gl (10%) was excreted in the urine during 24 hr after infusion of E_3. There is no apparent reason that can readily account for this discrepancy, but it may be related to a remarkable viariability exhibited by different subjects in the metabolism of E_3, to differences in intestinal flora of the patients, or to differences in amounts of metabolites excreted in the bile and/or *succus entericus*. Substantial variations in the excretion of estriol or its conjugates have been reported, e.g., the failure of Levitz and Katz (8) to detect E_3-3S,16Gl in the urine of their subjects after injection of the double conjugate into the duodenum, the impressively low conversion of intraduodenally instilled E_3 to E_3-3Gl reported by Stoa and Levitz (3), and the differences in the biliary excretion of E_3 or its conjugates (6, 13). Thus, it is possible, though not likely, that none of our subjects excreted significant amounts of the injected E_3 into the bile, even though the excretion of significant amounts of E_3-3S, 16Gl in the urine indicates that the subjects were capable of forming the major conjugated metabolite of E_3 excreted under normal concentrations in the bile. It is also possible that substantial amounts of E_3 were excreted in the bile, but that the subjects' intestinal tract was lacking in the enzyme system necessary for hydrolysis of the E_3-conjugates and reconjugation at position-3. Even though any of the above speculations may explain the discrepancies between our results and those of others, we are, nevertheless, perplexed by our failure to find E_3-3Gl following the intravenous administration of E_3, since a substantial biliary excretion of E_3 (about 20%) has been adequately demonstrated (6), in contrast to the seeming lack of such excretion after the administration of E_3-16Gl. Yet, the 3-glucuronide was observed in the urine of subjects given the latter but not the former compound. It is hoped that future studies will shed light on this complex picture.

E_3 conjugates usually remain intact in the upper part of the small intestine (11) although low glucuronidase activity in the bile has been observed (18). Assuming that reabsorption of E_3 or E_3 conjugates is a passive process, E_3-3S,16Gl, which is an extremely water-soluble compound of larger molecular size[8] than the effective pore radius of the mucosal membrane (3A–6.5A) (19), is not easily reabsorbed. Free E_3, however, may be absorbed by diffusion through the lipoidal face of the mucosa in the same manner as cortisol or progesterone (20, 21). Therefore, even though the biliary E_3-3S,16Gl may be absorbed to a variable extent in the upper small intestine, it is more likely that it reaches the terminal ileum where it is hydrolyzed, probably under the influence of bacterial flora, and reconjugated. The finding that a considerable amount of E_3-3Gl (16%) (Fig. 3) was excreted in the urine after injection of E_3 into the terminal ileum is in accord with this view.

However, rates and patterns of urinary excretion of the total radioactivity and E_3-3Gl after injection of E_3 into the terminal ileum were so different from those obtained from other routes of administration that this is probably not the major physiological route of estriol metabolism. In addition, the results obtained from in vitro experiments in our laboratory showed that the upper small intestine mostly formed E_3-16Gl and the lower small intestine formed E_3-16Gl and E_3-3Gl in relative amounts of 2.5:1 (Table III). Therefore, it may be

[8] Estriol occupies a space roughly equivalent to a cylinder 6 A in diameter by 13 A long (24). A model of E_3-3S,16Gl shows that the carbohydrate moiety attached to the C16α oxygen can fit under the α-surface of the steroid, thereby more than doubling its diameter.

TABLE III

*E_3 Conjugates Formed by Different Parts of the Human Small Intestine**

	No. of cases	Per cent conjugation	Per cent of conjugate formed			
			E_3-16Gl	E_3-3Gl	E_3-3S	E_3-double conjugate‡
Jejunum	2	87.2	88.2	7.3	4.6	
Ileum	6	59.6	63.4	26.5	4.4	2.9

* All incubations were performed for 1 hr in 3.0 ml of 0.1 M phosphate buffer, pH 7.3, containing 100 mg of the mucosa homogenized in 10 volumes of phosphate buffer, 50 μg of E_3, 0.5 μg of E_3-6,7-^{3}H, and 200 μg of UDPGA. The amounts of the E_3 conjugates formed are shown as their relative amounts after CCD.

‡ Due to the small amount of this very polar conjugate, rigorous identification could not be made.

plausible to presume that the lower portion of the small intestine is the most probable site of E_3-3Gl formation.

On the basis of our present study and the data available in the literature, particularly the recently published results and concepts formulated by Stoa and Levitz (3), our suggestion for the fate of E_3 can be formulated as follows: (*a*) Most of the circulating E_3 is extracted by the liver and converted to E_3-16Gl and E_3-3S,16Gl. (*b*) Nearly all of the E_3-16Gl is released into the blood stream and most of it is excreted in the urine, but some enters the small intestine possibly via the *succus entericus*. (*c*) E_3-3S,16Gl is excreted in the bile where it is the major conjugate of estriol. (*d*) In the intestine, part of the biliary E_3-3S,16Gl may be transported intact through the mucosa of the upper small intestine; the rest moves to the lower small intestine where it is hydrolyzed and reconjugated in the mucosa at the C-16 or C-3 position with glucuronic acid. Any E_3-16Gl in the lumen is largely hydrolyzed and reconjugated in the mucosa at C-16 or C-3 position with glucuronic acid. The newly formed estriol conjugates are then transported by the blood to the kidney for excretion.

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