Studies on Phenolic Steroids in Human Subjects

VIII. METABOLISM OF ESTRIOL–16α–GLUCOSIDURONATE

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A B S T R A C T 6,7-^{3}H-Estriol-16α-glucosiduronate-^{14}C was administered to eight women (nine studies) by several routes: both injection and infusion (300 min) into the cubital vein, injection into the portal vein system, ingestion and instillation into the duodenum, 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$_s$-3Gl,E$_s$-16Gl) and sulfoglucosiduronate (E$_s$-3S,16Gl) of estriol as well as for ^{3}H/^{14}C ratio of each conjugate. After peripheral injection 50–60% of the injected E$_s$-16Gl was excreted unchanged along with about 5% as E$_s$-3S,16Gl with an unchanged ^{3}H/^{14}C ratio, indicating direct sulfation of the injected E$_s$-16Gl. During a 300 min infusion, urinary excretion closely resembled that following injection. But 2–4 hr after the end of the infusion excretion of E$_s$-3S, 16Gl stopped, excretion of E$_s$-3Gl (17%/24 hr) with an elevated ^{3}H/^{14}C ratio started, and excretion of E$_s$-16Gl continued (70%/24 hr), but with a rapidly increasing ^{3}H/^{14}C ratio. This indicated sequestration in a sluggishly metabolizing compartment where two processes occurred: (*a*) extensive hydrolysis of E$_s$-16Gl followed by reconjugation at either C3 or C16 with unlabeled uridine diphosphate glucuronic acid (UDPGA), thereby increasing the ^{3}H/^{14}C ratio; and (*b*) transconjugation from C16 to C3, thereby producing E$_s$-3Gl with finite ^{3}H/^{14}C ratios. Instillation into various segments of the small intestine produced results qualitatively similar to those after intravenous infusion, whereas ingestion and intraportal injection resembled peripheral intravenous injection. Therefore, we have postulated the

possibility of an enteric circulation (in addition to an enterohepatic circulation) in which the steroid or its conjugates are transported into the small intestine in the *succus entericus,* modified, and then reabsorbed and excreted in the urine–a process which requires several hours.

INTRODUCTION

The concept that the 16-glucosiduronate of estriol was destined to be solely a urinary excretory product has undergone revision as a result of studies with this conjugate. Recently, Goebelsmann, Sjoberg, Wiqvist, and Diczfalusy (1) reported the results of experiments in which E$_s$-16Gl[1] was infused intravenously into human subjects for about 5 hr. This conjugate was rapidly excreted into the urine and appeared to be the only conjugate of the labeled estriol found during and for 2–3 hours after cessation of the infusion. Then, E$_s$-3Gl appeared in the urine and increased in *relative* amounts with time. The authors offered no suggestions to account for the delay in appearance of E$_s$-3Gl. The experiments described in this paper were designed to expand the original observations of Goebelsmann et al. and, if possible, to provide a tentative hypothesis which would give a rational explanation for the delay period in the

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[1] The following abbreviations and trivial names are used: estriol (E$_s$), estra-1,3,5(10)-triene-3,16α,17β-triol; estriol-16α-glucosiduronate (E3-16G1), 3,17β-dihydroxyestra-1,3,5-(10)-trien-16α-yl-β-D-glucopyranosiduronate; estriol-3-glucosiduronate (E$_s$-3G1),16α,17β-dihydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; estriol-3-sulfate-16α-glucosiduronate (E$_s$-3S,16G1), 3-sulfo-17β-hydroxyestra-1,3,5-(10)-trien-16α-yl-β-D-glucopyranosiduronate; estriol-3-16α-diglucosiduronate (E$_s$-3,16G1), estriol-3,16α-bis-(β-D-glucopyranosiduronate); estriol-3-sulfate (E$_s$-3S),16α-17β-dihydroxyestra-1,3,5(10)-trien-3-yl sulfate; UDPGA, uridine diphosphoglucuronic acid; CCD countercurrent distribution.

TABLE I
Route of Administration and Radioactivity of Estriol-16α-Glucosiduronate Administered

			Radioactivity administered		
Subject	Age	Route of administration	³H (μc)	³H:¹⁴C	Diagnosis
1	17	Cubital vein	9.8	31	Pregnant, last trimester
2	24	Cubital vein	2.8	2.8	Normal, follicular phase
3*	43	Oral	5.8	2.9	Normal, follicular phase
4	49	Portal vein	4.7	3.4	Carcinoma of uterus
5	48	Portal vein	2.8	2.1	Carcinoma of uterus
6	74	Jejunum	3.1	4.4	Carcinoma of uterus
7	46	Duodenum	3.9	3.9	Carcinoma of uterus
8	45	Ileum	4.0	4.0	Carcinoma of uterus
9*	43	Continuous i.v. infusion	3.7	3.7	Normal, follicular phase

* Same subject.

appearance of E_s-3Gl in the urine. Data will be presented which possibly point to the partial excretion of E_s-16Gl into the *succus entericus,* hydrolysis of the conjugate in the small intestine, and conjugation in the intestine with glucuronic acid at the 3-position, in part through trans-conjugation from the 16-position.

METHODS

6-7-³H-Estriol-16α-glucosiduronate-¹⁴C was administered to eight women. The route of administration and other pertinent data related to these subjects are presented in Table I. All the subjects were in good nutritional status and none had evident renal or hepatic dysfunction.

6,7³H-Estriol-16α-glucosiduronate (794 μc/mg) and estriol-16α-glucosiduronate-¹⁴C (21 μc/mg) were prepared in our laboratory separately by biosynthetic means (2), using, in the latter case, uridine diphosphate glucuronic acid (UDPGA) uniformly labeled in the glucuronic acid moiety with ¹⁴C. The ¹⁴C- and ³H-labeled estriol glucosiduronates were mixed and purified to a constant ratio of ³H to ¹⁴C by countercurrent distribution (CCD). The doubly labeled conjugate was also shown to be pure by paper chromatography. The glucosiduronate was injected by methods described previously (3), except the last subject (Table I) who was infused continuously for 300 min.

Urine samples were collected after administration of labeled conjugates as follows: 0-2, 2-4, 4-8, 8-12, 12-24, 24-48, and 48-72 hr. Urine specimens were mixed with an equal volume of 95% ethanol and kept in a cold room. Each specimen was taken almost to dryness under reduced pressure at a temperature not exceeding 40°C and then diluted with 300 ml of 95% ethanol. The precipitates were washed three times with 100 ml portions of 70% ethanol, so that they were essentially free of radioactivity. The ethanol extracts were stored at −15°C and brought to dryness just before analysis by CCD. The solvent systems employed and the partition coefficients of estriol conjugates in these systems are presented in Table II. The initial distribution consisted of 49 transfers in solvent system A. About 35 mg of sodium sulfate were added to tubes 0 and 1 in order to suppress emulsification of the phases. Two and sometimes three peaks were obtained, i.e., an extremely polar conjugate (N = 5, where N is the peak tube), a relatively polar conjugate (N = 10), and the same conjugate as that injected (N = 33).

No detectable amount of material less polar than the injected conjugate was observed.

Radioactivity was measured by a Packard Model 3375 liquid scintillation spectrometer, with a dioxane-ethoxy-ethanol system (4) and an automatic external standard. The efficiency of counting unquenched samples is 29% for tritium and 70% for carbon-14. Therefore, the maximum sensitivity of detection of a compound after CCD is about 1000 dpm, assuming that the entire contents of each tube of the CCD apparatus is analyzed. More conventionally, when 10% of the contents is removed for analysis, 15,500 dpm is required for identification with assurance.

RESULTS

I. Injection of E_3-16Gl into the cubital vein

The cumulative radioactivity and ³H/¹⁴C ratio of the materials excreted in the urine as estriol-16α-glucosiduronate and estriol-3-sulfo-16α-glucosiduronate during the initial 24 hr after a single injection of doubly labeled E_s-16Gl into a normal and a pregnant woman are shown in Fig. 1. In both subjects the ³H/¹⁴C ratio of these two conjugates in the urine did not deviate significantly from that of the injected conjugate, indicating no cleavage of E_s-16Gl before sulfation at the C-3 position. Initially, (0–4 hr) more than 99% of the radioactive products excreted was E_s-16Gl with the same ³H/¹⁴C ratio as that of the E_s-16Gl injected; at 24 hr after injection, approximately 7% of the injected conjugate had appeared as E_s-3S,16Gl. The 24–48 and 48–72 hr samples were processed also and no detectable amounts of E_s-3Gl were found.

Since the results obtained by us after a single, rapid injection of E_s-16Gl differed from those of Goebelsmann et al. (1), i.e. no E_s-3Gl was found in the urine, a prolonged infusion of E_s-16Gl was administered to a normal woman in a manner similar to that used by the above authors. More than 95% of the radioactivity recovered in the urine during the 300 min infusion period was excreted as E_s-16Gl and about 5% as E_s-3S,16Gl (Fig.

TABLE II

Partition Coefficients of Some Estriol Conjugates

Conjugate	System A	System B	System C
E₃-16Gl	2.07	0.76	1.07
E₃-3Gl	0.21	0.64	0.10
E₃-3S-16Gl	0.10	0.25	<0.05
E₃-3S	4.0	—	1.9

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

2). During the 1st 4 hr after cessation of the infusion, E₃-3S,16Gl amounted to 10% of the urinary radioactivity. The ³H/¹⁴C ratio of the two conjugates did not reveal any change from that of the infused E₃-16Gl during the infusion period and during the 1st 2 hr postinfusion.

Estriol-3-glucosiduronate first appeared 4 hr after cessation of the infusion with a ³H/¹⁴C ratio of 22:1. Thereafter, the proportion of E₃-3Gl increased to 16%, whereas E₃-3S,16Gl disappeared. Concomitantly, the ³H/¹⁴C ratio of both E₃-3Gl and E₃-16Gl increased until it was 30–50 times that of the injected conjugate. No detectable amounts of estriol (E₃) or estriol-3-sulfate (E₃-3S) were excreted in the urine of these three subjects.

II. Ingestion

Injection into a peripheral vein and, hence, into the systemic circulation permits all the organs access to the injected material in proportion to their blood flow. In an effort to afford the liver more immediate access to the E₃-16Gl, it was administered orally with the expectation that it might be rapidly absorbed unchanged from the intestine and delivered directly to the liver *via* the portal vein. The results reported below were unexpected.

The excretion pattern and ³H/¹⁴C ratio of the radioactive metabolites excreted in the urine differed from those obtained after intravenous administration even though the urine contained the same two conjugates, i.e., E₃-
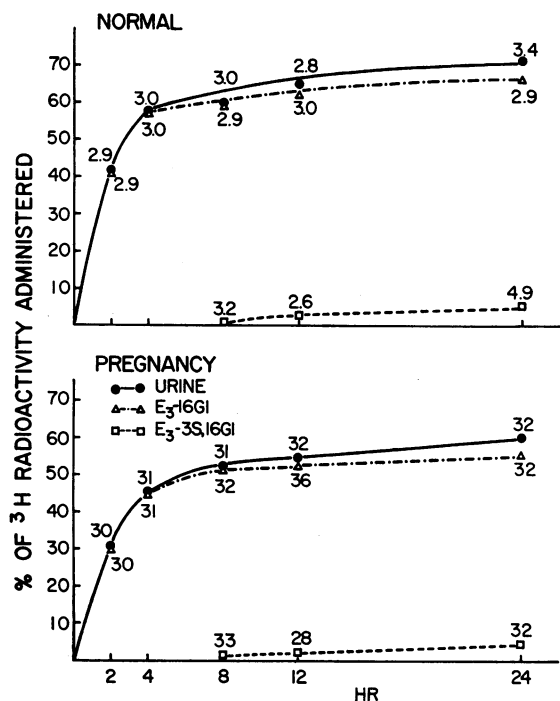

FIGURE 1 The cumulative urinary excretion of radioactive estriol conjugates after peripheral intravenous and rapid injection of ³H-E₃-16Gl-¹⁴C into a normal woman (³H/¹⁴C = 2.9) and into a woman in the third trimester of pregnancy (³H/¹⁴C = 31). The number associated with each point is the ³H/¹⁴C ratio of the material isolated at the indicated time period. In this and in the following four figures the curves have been arbitrarily drawn through the zero point, but it should be understood that the points of the curves between 0–2 hr have no significance or meaning regarding the rate of excretion of radioactivity during that period.
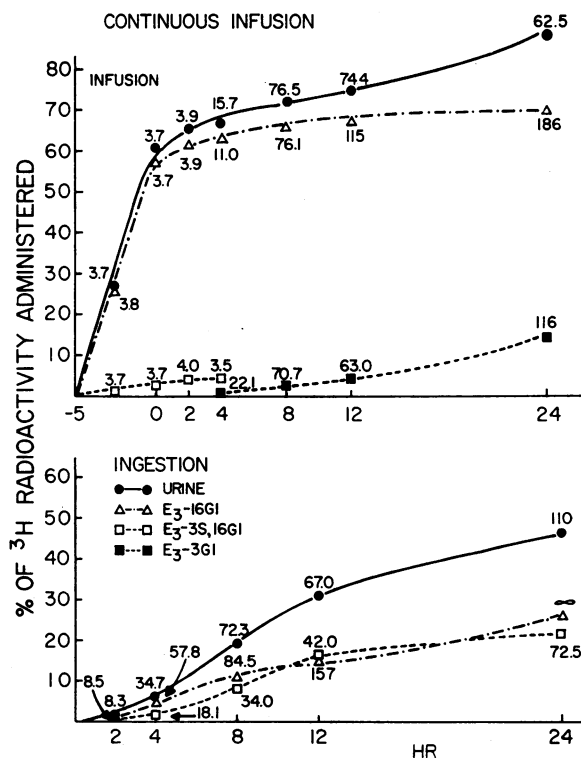

FIGURE 2 The cumulative urinary excretion of radioactive estriol conjugates after continuous infusion (³H/¹⁴C = 3.7) and after ingestion (³H/¹⁴C = 2.9) of ³H-E₃-16Gl-¹⁴C.
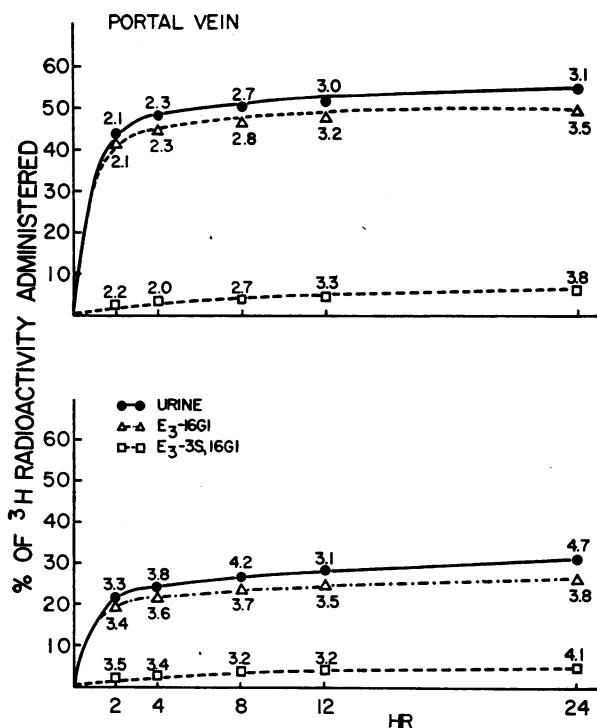
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FIGURE 3 The cumulative urinary excretion of radioactive estriol conjugates after injection of ^{3}H-E$_s$-16Gl-^{14}C (^{3}H/^{14}C) of 2.1 (top) and 3.4 (bottom) into the portal vein system.

16Gl and E$_s$-3S,16Gl (Fig. 2). After a latent period of 2 hr, a slow excretion and a rapid elevation of ^{3}H/^{14}C ratio of the two conjugates were observed, indicating that more than 90% of the ingested E$_s$-16Gl had been hydrolyzed, reconjugated, and excreted as the two conjugates. No significant amounts of E$_s$, E$_s$-3S, or E$_s$-3Gl were found in the urine.

III. Injection into the portal vein system

Since extensive hydrolysis of E$_s$-16Gl occurred after ingestion, injection into the portal vein was performed. At abdominal operation, E$_s$-16Gl was injected in 20 ml of saline in a period of a few minutes into the portal veins of two subjects, with cystic ovaries and carcinoma of the uterus, respectively. Their postoperative courses were uneventful. Unlike the results after the single injection into the cubital vein, E$_s$-3S,16Gl was excreted immediately (0–2 hr) (Fig. 3). The ^{3}H/^{14}C ratio of both estriol conjugates was the same as that of the injected E$_s$-16Gl. E$_s$-3S,16Gl accounted for about 12% of the urinary radioactivity excreted during the initial 24 hr after injection. In these experiments, as well as in the one after ingestion of the E$_s$-16Gl, it is possible that E$_s$-3Gl may have appeared in the urine after 24 hr, since the recovery of the total radioactivity ranged about 50%. It was felt, however, that the radioactivity

was being excreted at such a slow rate after 24 hr that it would have precluded the detection and identification of any compounds.

IV. Instillation into intestine

Intestinal instillation was tried next for two reasons. First, the failure to find E$_s$-3Gl in the preceding experiment made it unlikely that the liver was involved in the conjugate transformation. Second, the 4–8 hr delay in appearance of E$_s$-3Gl eliminated the entire vascular compartment and the liver from consideration. Among the more sluggishly exchanging compartments, such as the lymphatic circulation, spinal fluid and small intestine, the last appeared most attractive. It is commonly known to be both metabolically active and to require several hours to complete the process of digestion and absorption. As will be pointed out in the Discussion, there is also a substantial inward flow into the intestine. In addition, the excretion of substantial amounts of estriol conjugates in the bile raised the possibility that the hepatoenteric circulation may play a part in the metabolic transformation of E$_s$-16Gl.

The excretion pattern and ^{3}H/^{14}C ratio of the urinary radioactive materials after injection of doubly labeled E$_s$-16Gl into the small intestinal lumen of three subjects at abdominal operation are shown in Figs. 4 and 5. After injection into the duodenum, jejunum, and ileum urinary radioactivity was excreted slowly and the ^{3}H/^{14}C ratio remained unchanged for 8, 12, and 24 hr postinjection, respectively, with the next collection in each instance showing a sudden elevation of the ^{3}H/^{14}C ratio (Fig. 4).

Initially, nearly 90% of the estriol conjugates excreted was as E$_s$-16Gl and about 10% as E$_s$-3S,16Gl with an unchanged ^{3}H/^{14}C ratio. The preponderant part of the tritium radioactivity was excreted after the break in the ^{3}H/^{14}C ratio, and it was during this period that E$_s$-3Gl was excreted. As in the case of continuous infusion, excretion of E$_s$-3Gl was accompanied by an abrupt cessation of E$_s$-3S,16Gl excretion (Figs. 4 and 5). Nearly 50% of the urinary ^{3}H excreted during 72 hr after injection was E$_s$-3Gl with a ^{3}H/^{14}C ratio 10–20 times that of the injected conjugate. The ^{3}H/^{14}C ratio of the E$_s$-16Gl closely approximated that of the E$_s$-3Gl formed during the same period.

V. Characterization of the urinary estriol conjugates

A. E$_s$-3S,16Gl

1) Partition Coefficient

A separation of the two more polar conjugates was to some extent incomplete whenever both polar conjugates occurred in the same extract. Fortunately, this
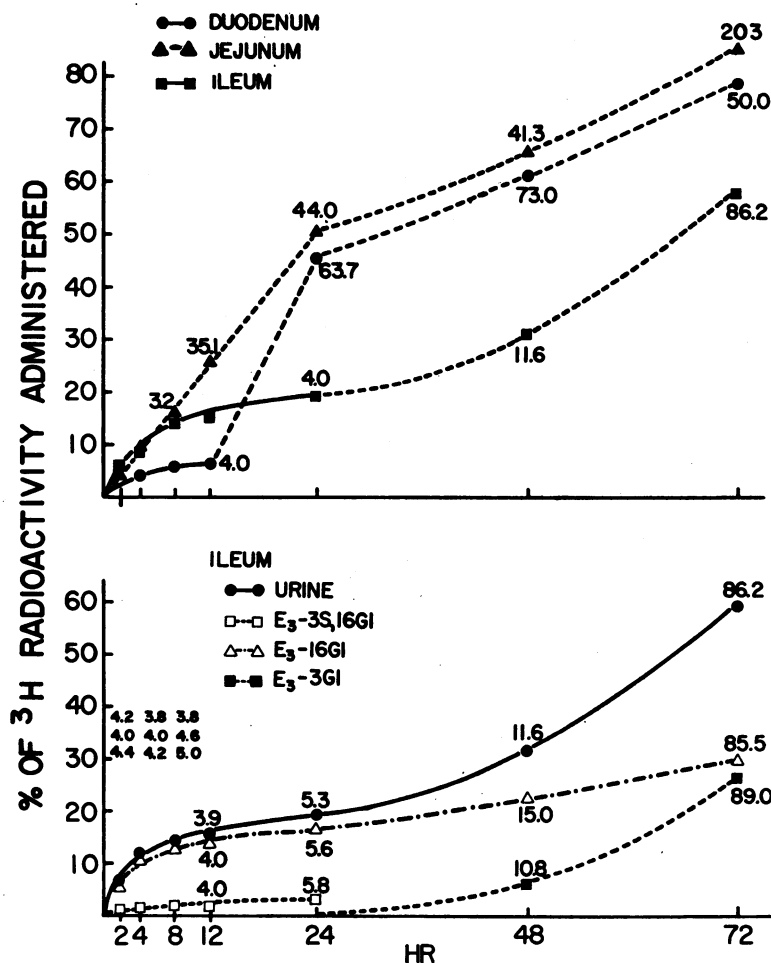
FIGURE 4 The cumulative urinary excretion of tritium after instillation of ³H-E₃-16G1-¹⁴C into the duodenum, jejunum, and ileum (³H/¹⁴C = 3.7, 4.4, and 4.0, respectively). In this case, the solid line indicates the time during which the urinary ³H/¹⁴C ratio did not deviate significantly from that of the injected E₃-16G1. Bottom: the cumulative urinary excretion of radioactive estriol conjugates after instillation into the ileum.

did not happen frequently. To assure complete purity, a pool of each polar conjugate (N = 5 and N = 10) was made by pooling a 10 tube region around the peak from all CCD's. Each pool was then submitted to CCD for 99 transfers in solvent system B (Table II). Authentic E₃-3G1 was biosynthesized in our laboratory by the method of Goebelsmann et al. (5) with rabbit or guinea pig liver.[2]

2) ENZYMATIC HYDROLYSIS

(*i*) *Bacterial β-glucuronidase*.[3] A portion of the E₃-3S,16G1–like material, dissolved in 15 ml of 0.1 M acetate buffer at pH 7.0, was incubated with bacterial β-glucuronidase (100 U/ml) for 20 hr at 37°C. After incubation, the mixture was evaporated to dryness and the residue, dissolved in the lower phase, was submitted to CCD for 29 transfers in solvent system C with 5 mg

of authentic estriol-3-sulfate (E₃-3S)[4] (Fig. 6). The distribution of tritium matches that of the carrier, measured as Kober chromogen, which produced a specific activity that remained nearly constant over the entire distribution. All of the ¹⁴C in tube 0 is presumably free glucuronic acid-¹⁴C (or a metabolite). More than 95% of the incubated material was hydrolyzed by β-glucuronidase and converted to E₃-3S.

(*ii*) *Phenolsulfatase (Mylase-P)*.[5] A portion of the E₃-3S,16G1–like material was incubated with Mylase-P (2 mg/ml) in 15 ml of 0.1 M phosphate buffer (pH 6.0) for 20 hr at 37°C and was submitted to CCD in solvent system C (Fig. 6). More than 95% of the E₃-3S,16G1–like material was hydrolyzed by phenolsulfatase and converted to E₃-16G1.

(*iii*) *Sequential enzymatic hydrolysis*. The material from tubes 13–25 (Fig. 6, β-glucuronidase) was pooled

[2] The ³H-E₃-3S,16G1 was kindly supplied by Dr. M. Levitz (24).

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

[4] The chemically synthesized E₃-3S was kindly supplied by our colleague, Dr. R. Y. Kirdani.
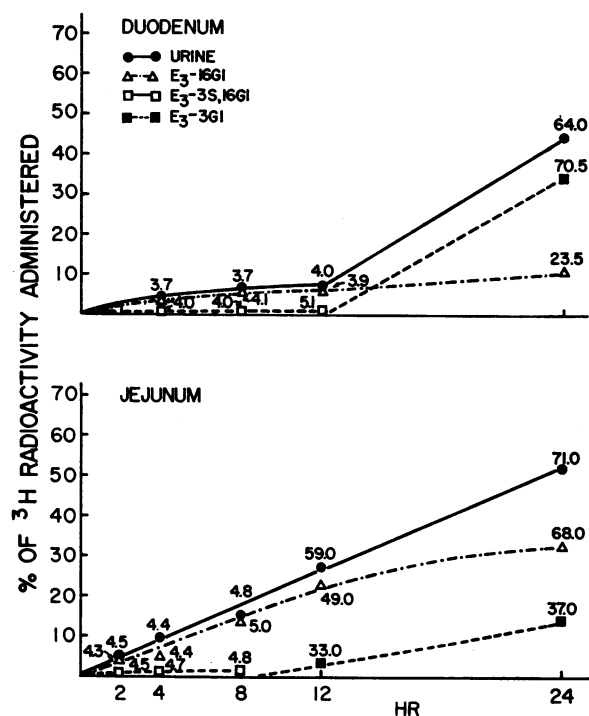
[5] Mann Research Lab., *Aspergillus oryzae*.

FIGURE 5 The cumulative urinary excretion of radioactive estriol conjugates after instillation into the duodenum and jejunum. See Fig. 4 for details.

and hydrolyzed with phenolsulfatase and the material from tubes 11–21 (Fig. 6, phenolsulfatase) was pooled and hydrolyzed with β-glucuronidase. The combined extracts of these two incubations were submitted to CCD for 49 transfers in solvent system D (methanol: water: chloroform: carbon tetrachloride, 70:30:80:20). More than 90% of the tritium radioactivity and all of the carrier showed a single peak, K, 1.04, consistent with that of estriol. The specific activity of the distributed mixture was constant.

TABLE III

Recrystallization of Estriol Formed by Hydrolysis of the Urinary Es-3S,16Gl–Like and Es-3Gl–Like Materials with Phenolsulfatase and Bacterial β-glucuronidase*

Solvent of crystallization	Es-3S,16Gl		Es-3Gl	
	Crystals	Mother liquor	Crystals	Mother liquor
Starting material	3885		2985	
Acetone-n-hexane	3619	3836	3005	2970
Methanol-water	3607	3707	3106	3200
Ethanol-water	3381	3745	2960	3100

* Specific activity of crystals and mother liquor are in dpm/mg.

3) REVERSE ISOTOPIC DILUTION

The estriol-like material, which was formed by sequential enzymatic hydrolysis (see preceding paragraph), was further identified by reverse isotopic dilution from three different solvents to constant specific activity as shown in Table III.

In summary, upon partial hydrolysis of the Es-3S, 16Gl–like material with β-glucuronidase or phenolsulfatase, more than 95% of the radioactivity was released as Es-3S and Es-16Gl, respectively. Upon sequential hydrolysis with both enzymes, more than 90% of the radioactivity was released as estriol, which was characterized by reverse isotopic dilution.

B. Es-3Gl

A portion of the combined Es-3Gl–like material was submitted to CCD for 99 transfers in three different solvent systems (A, B, and C) (Table II). In each instance, radioactivity followed a theoretical distribution with a constant $^3H/^{14}C$ ratio as shown in Fig. 7. The ratio was also the same as that of the undistributed material.

The Es-3Gl–like material was incubated with bacterial β-glucuronidase (200 U/ml) in 15 ml of 0.1 M acetate buffer (pH 7.0) for 20 hr at 37°C and was then submitted to CCD for 49 transfers in solvent system D with 5 mg of authentic estriol. More than 90% of the radioactivity distributed as a single peak with a constant specific activity. The partition coefficient (1.04) was identical with that of estriol. No hydrolysis occurred during incubation with phenolsulfatase.

The estriol-like material from the CCD was recrystallized from three different solvents to a constant specific activity (Table III).

DISCUSSION

The metabolic disposition of estriol in human subjects is characterized by a number of features which include rapid conjugation of estriol with glucuronic acid and primary excretion of the conjugate in the urine, substantial excretion of estriol conjugates in the bile (about 20%), the preponderant excretion of Es-3S,16Gl in the bile, negligible metabolic transformation of the aglycone and an enterohepatic circulation of estriol and its conjugates. Even though Es-16Gl has been identified and rigorously proven as the major conjugate in pregnancy urine (6), the presence of small amounts of Es-3Gl (7, 8), as well as Es-3S,16Gl (7), has been reported. We have previously published data on the metabolic disposition of Es-16Gl (labeled only in the steroid moiety) and concluded that the conjugate was excreted almost exclusively in the urine (0% in the bile) (3), but recent investigations with Es-16Gl performed by
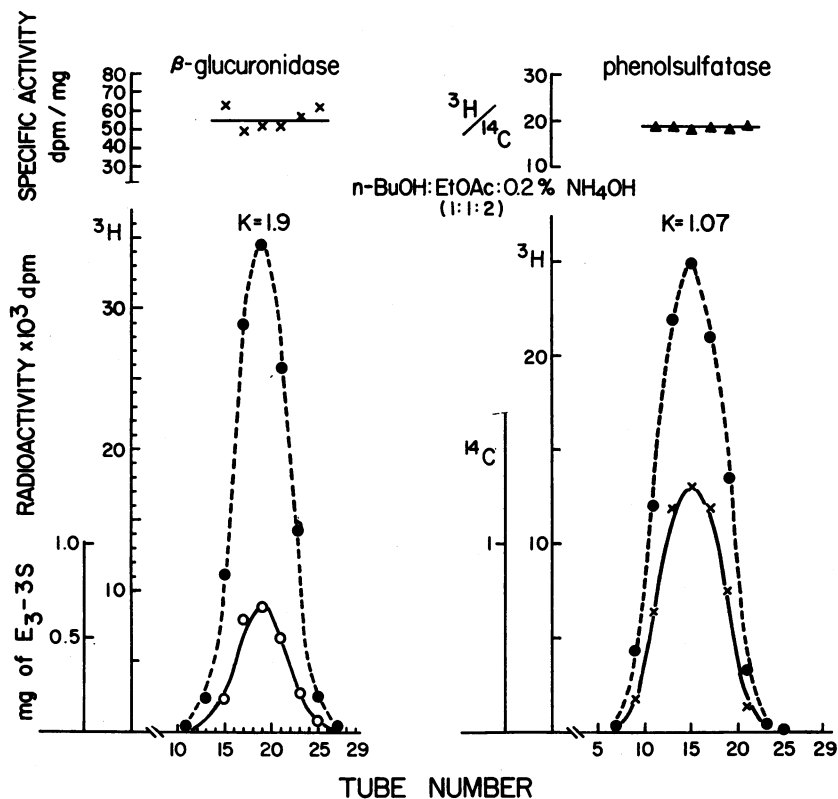
FIGURE 6 Characterization of ³H-E₃-3S-16G1-¹⁴C by partial enzymatic hydrolysis. Left: CCD of H³-E₃-3S formed by β-glucuronidase hydrolysis and of carrier E₃-3S. Right: CCD of ³H-E₃-16G1-¹⁴C formed by phenolsulphatase hydrolysis. In both cases 29 transfers were performed in system C (see Table II).

continuous infusion of the material indicate that the conjugate may, in fact, undergo cleavage and re-conjugation, including glucuronidation at the 3-position (1). In addition, the methods used in our previous studies (3) would not have detected the small amounts of E₃-3G1 or E₃-3S,16G1 possibly present in the urine, particularly following a single, rapid injection. The studies of Goebelsmann et al. (1) and the data of the present investigations further indicate that the metabolism of E₃-16G1 is significantly modified by the duration of the infusion. Prolonged infusion intravenously apparently allows the conjugate sufficient time to enter a compartment not accessible to it following a rapid, single injection.

The investigations reported in this paper utilize a conjugate labeled with tritium in the estriol moiety and carbon-14 in the glucuronic acid portion. Thus, the ratio of the two radioactivities serves as a measure of the metabolism of the conjugate. If the ratio is unchanged from that of the injected material, no metabolism has occurred. If, as frequently happened, urinary E₃-16G1 with a changed ratio was isolated, the labeled glu-

curonic acid must have been removed and replaced by an unlabeled glucuronic acid. If ¹⁴C-labeled E₃-3S,16G1 is obtained, the original injected molecule of E₃-16G1 was sulfated; otherwise, the injected molecule was hyhydrolyzed before reconjugation (9). If carbon-14-labeled E₃-3G1 is obtained, transconjugation must have occurred. Thus, the use of the double label has allowed us to make several conclusions which would have been impossible with the use of a single label.

After a single, rapid injection into the cubital vein of a normal and a late-pregnant woman, most of the urinary conjugate was E₃-16G1 accompanied by a small amount of E₃-3S,16G1 after a delay of 4–8 hr (Fig. 1). The ³H/¹⁴C ratio of these two conjugates did not show any significant deviation from that of the injected conjugate, indicating no cleavage of E₃-16G1, even during sulfation at the C-3 position. The fact that E₃-3S,16G1 was formed from E₃-16G1 without cleavage is in accord with conclusions by Emerman, Twombly and Levitz (9). It is interesting to note that there was no difference in the results obtained in a pregnant and a nonpregnant woman, indicating that the foeto-placental
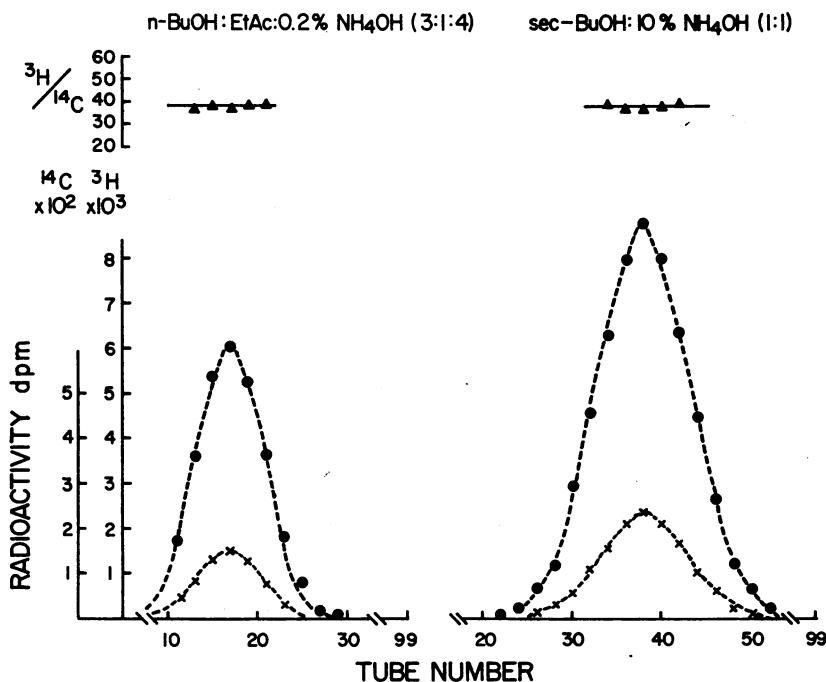
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n-BuOH:EtAc:0.2% NH₄OH (3:1:4)   sec-BuOH:10% NH₄OH (1:1)

³H/¹⁴C   60 50 40 30 20

¹⁴C ³H
x10² x10³

RADIOACTIVITY dpm

8
7
6
5   5
4   4
3   3
2   2
1   1

10   20   30   99   20   30   40   50   99
TUBE NUMBER

FIGURE 7   Characterization of ³H-E₃-3Gl-¹⁴C by CCD in two systems.

unit does not play a significant role in the above reaction.

After continuous infusion, most (60%) of the infused conjugate was excreted unchanged accompanied again by a small amount of E₃-3S,16Gl with unchanged ³H/¹⁴C ratio. Levitz and coworkers also observed the excretion of E₃-3S,16Gl in nearly identical amounts after either the infusion of ³H-E₃-3³⁵S (9) or the intravenous or intraduodenal administration of labeled estriol (10). Starting 4 hr after cessation of the infusion, however, three things happened: (1) a slow excretion of E₃-16Gl continued, but with a rapidly increasing ³H/¹⁴C ratio; (2) cessation of E₃-3S,16Gl excretion; and (3) commencement of E₃-3Gl excretion with a ³H/¹⁴C ratio much like that of the E₃-16Gl. The delay in excretion of E₃-³Gl is in accord with the observation of Goebelsmann et al. (1).

The long lag period associated with E₃-3Gl formation may be related either to a slow excretion of a metabolite into the intestine, either via the bile or *succus entericus*, or to a slow hydrolysis of estriol conjugates and glucuronidation at position-3 in the intestine. It is possible that during prolonged infusion of E₃-16Gl, sufficient amounts of the conjugate are excreted in the bile, in contrast to an apparent absence of such excretion after a single injection (3), as to result in ultimate formation of E₃-3Gl. The hydrolysis of E₃-3S,16Gl and E₃-16Gl in the intestine with subsequent formation of E₃-3Gl appear to be rather slow reactions in the intestinal tract, as is evident from the results obtained after instillation

of E₃-16Gl into various parts of the intestine (see Figs. 5–7).

Even though the above explanation for the time lag in the excretion of E₃-3Gl is likely and plausible, the sequestration of estriol (or one of its conjugates) in an anatomical pool with an extremely slow turnover should not be lost sight of. Such a possible pool may be the *succus entericus,* which flows at the rate of about 3 liters/day. Nearly all of this fluid and presumably most of its solutes are reabsorbed, although not necessarily unchanged, during passage along the intestine. Therefore, E₃-16Gl was administered by us by different gastroenteric routes in order to ascertain whether an enteric route might exist. After oral administration, most of the E₃-16Gl was hydrolyzed, reconjugated, and excreted in the urine at a slow rate as E₃-3S,16Gl with a greatly elevated ³H/¹⁴C ratio. Extensive hydrolysis may have been due primarily to gastric juice. Regardless of the mode of hydrolysis, however, it is clear from this result that neither the stomach nor the liver is the site of E₃-3Gl formation.

After injection into the portal vein system a significant amount of the injected E₃-16Gl was converted to E₃-3S, 16Gl without cleavage of the glucuronic acid moiety. The prompt excretion of E₃-3S,16Gl indicates that the sulfation reaction is rapid in the liver. We failed, however, to detect E₃-3Gl during the time of this study, indicating, again, that the liver is not the site of transconjugation from E₃-16Gl to E₃-3Gl.

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After instillation into the duodenum, jejunum, and ileum, the urine showed a sudden elevation of the $^3H/^{14}C$ ratio at 8, 12, and 24 hr postinjection, respectively. After the break in the $^3H/^{14}C$ ratio, E$_s$-3Gl was excreted in the urine, while before that time the urine contained E$_s$-3S,16Gl as the sole metabolite. Nearly 50% of the urinary ^3H excreted during 72 hr after injection was E$_s$-3Gl with a $^3H/^{14}C$ ratio 10–20 times that of the injected conjugate. The $^3H/^{14}C$ ratio of E$_s$-3Gl was different from those of injected E$_s$-16Gl and of E$_s$-3S,16Gl and was quite similar to the results after prolonged intravenous infusion.

These results strongly suggest that the intestine or the content of the intestinal lumen is the site of transconjugation from E$_s$-16Gl to E$_s$-3Gl. The mechanism is unknown. One possibility is hydrolysis by the β-glucuronidase present in various bacteria (e.g., *E. coli*) in the intestinal lumen followed by absorption by the mucosal cells and reconjugation at C3 and at C16 before release into the bloodstream. This is in accord with the observations of Stoa and Levitz (10) and Levitz and Katz (11) in vivo and of Dahm and Breuer (12) in vitro.

Another possibility, which probably is operating to some extent, is direct transconjugation. Fishman and Green (13, 14) demonstrated that purified mammalian or crude bacterial (*E. coli*) β-glucuronidase can act as a transferase, i.e., transferring glucuronic acid from a number of phenols or menthol to 18 different alcohols and glycols in yields varying from 7–89%. Crucial to the success of this in vitro reaction was a high concentration (about 1 M) of acceptor. (Even so, the concentration of water for the competing reaction of hydrolysis was about 56 M.) Phenols and terpene alcohols could not be used as acceptors due to a combination of insolubility and inhibitory action on the enzyme. Such limitations may not exist in vivo.

The metabolic disposition of estriol and its conjugates presents a complex situation with several reactions and processes proceeding simultaneously. Some E$_s$-16Gl is absorbed unchanged. This definitely occurs because the E$_s$-16Gl excreted shortly after intestinal injection has the same $^3H/^{14}C$ ratio as the injected material. Some E$_s$-16Gl is hydrolyzed. The released E$_s$ competes with other aglycones, mostly nonsteroidal, in a transferase reaction which produces E$_s$-3Gl. Presumably E$_s$-16Gl would not be the sole source of glucuronic acid. The transfer reaction would tend to conserve the glucuronic acid-^{14}C and produce a lower $^3H/^{14}C$ ratio in the product than a de novo synthesis employing unlabeled UDPGA. Fig. 5 shows that the E$_s$-3Gl did have a lower $^3H/^{14}C$ ratio than the E$_s$-16Gl isolated in the same time period. Finally, the rest of the E$_s$ would be absorbed and reconjugated in the intestinal mucosa (or liver), mostly as E$_s$-16Gl (15, 16).

The difference between a rapid injection and a prolonged infusion of E$_s$-16Gl into a peripheral vein may be one of time. We would like to stress that we do not propose that there is a qualitative difference between the metabolism of injected vs. infused E$_s$-16Gl. The difference may be merely quantitative and related to the relative amounts of the administered E$_s$-16Gl that are sulfated in the 3-position and, thus, may be excreted in the bile and further metabolized in the intestine and the relative amounts available to the intestine via the general circulation or in the *succus entericus*. It is entirely possible that E$_s$-3Gl was present in the urine of the subjects given a single injection, e.g., Levitz and Katz (11) found that about 3% of E$_s$-16Gl appeared in the urine as the E$_s$-3Gl in one of their subjects, but the amounts present were too small to be detected by our methods. It has been shown that E$_s$-16Gl (3) as well as E$_s$-3Gl (17) are cleared from the blood with great rapidity. On the other hand, the flow of *succus entericus* is very slow–125 ml/hr or 2.5% of the blood volume/hr. The rapid clearance of injected E$_s$ from the blood (3), the delayed appearance of E$_s$-3Gl in the urine after instillation of E$_s$-16Gl into the intestine, and the rate of flow of the *succus entericus* result in insufficient secretion of the labeled conjugate to permit detection after a rapid injection. After a 5 hr infusion about ⅛ of the infused conjugate should have entered the intestine. This number is in approximate agreement with the excretion of 16% of the infused tritium radioactivity as E$_s$-3Gl.

In support of intestinal secretion of estrogen conjugates are a number of unrelated observations. We found that injected E$_s$-16Gl was not excreted in the bile (3) and Emerman et al. (9) demonstrated that E$_s$-3S was nearly excluded from the bile. A significant amount (9%) was, however, hydrolyzed, reconjugated, and excreted in the bile as E$_s$-3S,16Gl (9). Others have shown that unconjugated estrogens (18, 19), fat (20), and manganese (21) can be found in the intestinal lumen in the absence of bile. Furthermore, recent studies on intestinal absorption in man have revealed that there are functional differences at different levels of the human small intestine (22, 23): It is most likely that E$_s$-16Gl is partly absorbed intact, presumably at the proximal segment, while the unabsorbed E$_s$-16Gl reaches the distal segment where it is subjected to further metabolism. Thus, it appears that intestinal secretion should be considered as an additional parameter of estrogen metabolism. In addition, we have shown that the glucuronic acid moiety of E$_s$-16Gl may be reutilized and we have postulated that the ubiquitous enzyme, β-glucuronidase, acting as a transferase is responsible.

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REFERENCES

1. Goebelsmann, U., K. Sjoberg, N. Wiqvist, and E. Dicz-falusy. 1965. Oestriol-3-glucosiduronate, a major urinary metabolite of estriol and estriol-16(17?)-glucosiduronate. *Acta Endocrinol.* **50:** 261.

2. Slaunwhite, W. R., Jr., M. A. Lichtman, and A. A. Sandberg. 1964. Studies on phenolic steroids in human subjects. VI. Biosynthesis of estriol-glucosiduronic acid-16-C^{14} by human liver. *J. Clin. Endocrinol. Metab.* **24:** 638.

3. Sandberg, A. A., and W. R. Slaunwhite, Jr. 1965. Studies on phenolic steroids in human subjects. VII. Metabolic fate of estriol and its glucuronide. *J. Clin. Invest.* **44:** 694.

4. Plager, J. E., R. Knopp, W. R. Slaunwhite, Jr., and A. A. Sandberg. 1963. Cortisol binding by dog plasma. *J. Clin. Endocrinol. Metab.* **73:** 353.

5. Goebelsmann, U., E. Diczfalusy, J. Katz, and M. Levitz. 1965. Biosynthesis of radioactive estriol-3-glucosiduronate by guinea pig liver homogenate. *Steroids.* **6:** 859.

6. Hashimoto, Y., and M. Neeman. 1963. Isolation and characterization of estriol 16α-glucosiduronic acid from human pregnancy urine. *J. Biol. Chem.* **238:** 1273.

7. Smith, E. R., and A. E. Kellie. 1967. Oestrogen conjugates of human late pregnancy urine. *Biochem. J.* **104:** 83.

8. Hahnel, R. 1967. The quantitative relationship of oestrogen-3-glucosiduronates and oestrogen-16(or 17)-glucosiduronates in human late pregnancy urine. *J. Endocrinol.* **38:** 417.

9. Emerman, S., G. H. Twombly, and M. Levitz. 1967. Biliary and urinary metabolites of estriol-15-^{3}H-3-sulfate-^{35}S in women. *J. Clin. Endocrinol. Metab.* **27:** 539.

10. Stoa, K. F., and M. Levitz. 1968. Comparison of the conjugated metabolites of intravenously and intraduodenally administered oestriol. *Acta Endocrinol.* **57:** 657.

11. Levitz, M., and J. Katz. 1968. The enterohepatic metabolism of estriol-3-sulfate-16-glucosiduronate in women. *J. Clin. Endocrinol. Metab.* **28:** 862.

12. Dahm, K., and H. Breuer. 1966. Enzymatische Untersuchungen ueber die Glucuronidieren von Oestriol beim Menschen. *Z. Klin. Chem.* **4:** 153.

13. Fishman, W. H., and S. Green. 1956. Glucosiduronic acid synthesis by β-glucuronidase in a transfer reaction. *J. Amer. Chem. Soc.* **78:** 880.

14. Fishman, W. H., and S. Green. 1957. Enzymic catalysis of glucuronyl transfer. *J. Biol. Chem.* **225:** 435.

15. Diczfalusy, E., C. Franksson, and B. Martinsen. 1961. Oestrogen conjugation by the human intestinal tract. *Acta Endocrinol.* **38:** 59.

16. Dahm, K., and H. Breuer. 1966. Biogenese von oestriol-16α-monoglucuronid und oestriol-17β-monoglucuronid. *Acta Endocrinol.* **52:** 43.

17. Goebelsmann, U., I. Cooke, N. Wiqvist, and E. Diczfalusy. 1966. Comparison of the metabolism of oestriol-3-glucosiduronate and estriol-16-glucosiduronate women. *Acta Endocrinol.* **52:** 30.

18. Albert, S., R. D. H. Heard, C. P. Leblond, and J. Saffran. 1949. Distribution and metabolism of iodo-α-estradiol labeled with radioactive iodine. *J. Biol. Chem.* **177:** 247.

19. Pearlman. W. H., A. E. Rakoff, K. E. Paschkis, A. Cantarow, and A. A. Walkling. 1948. The metabolic fate of estrone in bile fistula dogs. *J. Biol. Chem.* **173:** 175.

20. Shapiro, A., H. Koster, D. Rittenberg, and R. Schoenheimer. 1936. The origin of fecal fat in the absence of bile, studied with deuterium as an indicator. *Amer. J. Physiol.* **117:** 525.

21. Bertinchamps, A. J., S. T. Miller, and G. C. Cotzias. 1966. Interdependence of routes excreting manganese. *Amer. J. Physiol.* **211:** 217.

22. Fordtran, J. S., F. C. Rector, Jr., M. F. Ewton. N. Soter, and J. Kinney. 1965. Permeability characteristic of the human small intestine. *J. Clin. Invest.* **44:** 1935.

23. Schedl, H. P., C. E. Pierce, A. Rider, and J. A. Clifton. 1968. Absorption of L-methionine from the human small intestine. *J. Clin. Invest.* **47:** 417.

24. Levitz, M., J. Katz, and G. H. Twombly. 1965. The biosynthesis of labeled estriol-3-sulfate-16-glucosiduronate. *Steroids.* **6:** 553.