Endocrinol. Japon. 1980, 27 (2), 175-182

The Metabolism of Estriol-3-Glucosiduronate and Estriol in the Rabbit

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Abstract

Urinary metabolites of [6, 7-^{3}H]-estriol-3-glucosiduronate and of [6, 7-^{3}H]-estriol in intact female rabbits were analyzed. The separation of urinary metabolites was performed by countercurrent distribution followed by DEAE-Sephadex A-25 column chromatography. Each conjugate was then hydrolyzed with the enzymes and the aglycone thus liberated was identified. In either case, major urinary metabolites were found to be diconjugates, a considerable part of which was glucosiduronate-N-acetylglucosaminide of 17-epiestriol. In addition, estriol-16-glucosiduronate or monoglucosiduronate of 17-epiestriol was identified as a minor urinary metabolite of [6, 7-^{3}H]-estriol. From these results, it was concluded that the greater part of the estriol-3-glucosiduronate was converted to diconjugates and that estriol-3-glucosiduronate was probably an intermediate metabolite in the conversion pathway from estriol to diconjugates in this species.

The metabolic fate of estriol (E_3)* in the rabbit has been studied by several groups of investigators. Matsuda reported that a part of non-radioactive E_3 administered to the intact rabbit (i.e. without the biliary drainage) was converted to 17-epiestriol (17 epiE_3) which was excreted in the urine as conjugates hydrolyzable with β-glucuronidase (Matsuda, 1964). Collins *et al* investigated the urinary metabolites of [6, 7-^{3}H]-E_3 in the intact rabbit (Collins and Layne, 1968) and identified estriol-diglucosiduronate (E_3-diG) as a major metabolite and 17-epiestriol-3-glucosiduronate-17-N-acetylglucosaminide (17 epiE_3-3G-17 NAG) as a minor one.

Subsequently, Kirdani *et al.* studied the biliary and urinary metabolites of [6, 7-^{3}H]-E_3 in rabbits with biliary fistulas (Kirdani *et al.*, 1973) and the major metabolite both in the bile and in the urine was found to be estriol-3-glucosiduronate (E_3-3G). From

Received July 27, 1979.

*The following trivial names and abbreviations are used: estriol (E_3)=estra-1, 3, 5 (10)-triene-3, 16α, 17β-triol. 17-epiestradiol(17epiE_3)=estra-1,3,5(10)-triene-3, 16α, 17α-triol. 17β-estradiol=estra-1,3,5(10)-triene-3, 17β-diol. 17α-estradiol=estra-1, 3, 5(10)-triene-3, 17α-diol. estrone=3-hydroxyestra-3, 3, 5(10)-trien-17-one. estriol-3-glucosiduronate(E_3-3G)=16α,17β-dihydroxyestra-1,3,5(10)-triene-3-yl-β-D-gluco-pyranosiduronate. (E_3-16G)=3, 17β-dihydroxyestra-1, 3, 5(10)-triene-16α-yl-β-D-glucopyranosiduronate. 17β-estradiol-3-glucosiduronate=17β-hydroxyestra-1, 2, 3(10)-triene-3-yl-β-D-glucopyranosiduronate. estrone-3-glucosiduronate=17-oxoestra-1, 3, 5(10)-triene-3-yl-β-D-glucopyranosiduronate. 17-espiestriol-3-glucosiduronate-17-N-acetylglucosaminide(17epiE_3-3G-17NAG)=16α-hydroxyestra-1,3,5(10)-triene-3-yl-β-D-glucopyranosiduronate-17α-yl-2'-acetamido-2'-deoxy-β-D-glucopy-ranoside. 17α-estradiol-3-glucosiduronate-17-N-acetylglucosaminide=estra-1, 3, 5(10)-triene-3-yl-β-D-glucopyranosiduronate-17α-yl-2'-acetamido-2'-deoxy-β-D-glucopyranoside. Estriol-3-sulfate(E_3-3S)16α, 17β-dihydroxy-estra-1, 3, 5(10)-triene-3-yl-sulfate. Estriol-3-sulfate-16-glucosiduronate=17β-hydroxyestra-1, 3, 5 (10)-triene-3-yl-sulfate-16α-yl-β-D-glucopyranosiduronate.

these results, they speculated that E_3-diG identified by Collins *et al.* must, in fact, have been E_3-3G. Thus, there exists an ambiguity regarding the exact nature of the major urinary metabolites of E_3 and it is not known whether E_3-3G is an end product in this species.

The present experiments *in vivo* have been undertaken in order to get more information about these unknown aspects of E_3 metabolism in the rabbit.

Materials and Methods

Compounds

[6, 7-^{3}H]-E_3 (42.2–59 mCi/m mol) was purchased from New England Nuclear Corporation, Boston, Mass. Radiochemical purity was checked by paperly chromatography (PC) in system I (see Table 1). E_3 was obtained from Tokyo-Kasei Chemical Co., Tokyo or Sigma Chemical Co., St. Louis, Mo. 17epiE_3 was purchased from Sigma Chemical Co. E_3-3G and estriol-16-glucosiduronate (E_3-16G) were generous donated by the Dr. Y. Osawa (Medical Foundation of Buffalo, Buffalo, N. Y.). The chemical purity of these standard compounds was checked by PC in appropriate systems. Saccharo-1, 4-lactone was a gift from Chūgai Pharmaceutical Co., Tokyo. Uridine diphosphoglucuronic acid (UDPGA) was purchased from Sigma Chemical Co.

Enzymic hydrolysis and of measurement radioactivity

Methods for these procedures have been previously published (Miyazaki *et al.*, 1977; Miyazaki *et al.*, 1978).

Separation of the urinary metabolites

The separation of the conjugates into the diconjugate fraction and the monoconjugate fraction was performed by means of countercurrent distribution (CCD). The separation of each fraction into subfractions was carried out by means of DEAE-Sephadex A-25 column chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden; a K 9/60 Sephadex column was used) as described by Hobkirk *et al.* (Hobkirk *et al.*, 1969). Aglycones liberated by enzymic hydrolysis of the conjugate were separated by PC. Solvent systems used for CCD and PC ars shown in Table 1.

Table 1. Solvent systems used for analysis.

Countercurrent distribution

System A; n-butanol: 10% NH_4OH (1 : 1)
System B; n-butanol: ethyl acetate: 0.2% NH_4OH (3 : 1 : 4)
System C; n-butanol: ethyl acetate: 0.2% NH_4OH (1 : 1 : 2)
System D; ethyl acetate: water (1 : 1)

Paper chromatography

System I; benzene: methanol: water (100 : 55 : 45)
System II; acetic acid: water: ethylene dichloride: tert-butanol (30 : 70 : 75 : 25)
System III; n-butanol: benzene: methanol: water (1 : 9 : 5 : 5)

Biosynthesis of [6, 7-^{3}H]-estriol-3-glucosiduronate ([6, 7-^{3}H]-E_3-3G)

[6, 7-^{3}H]-E_3-3G was biosynthesized by incubating [6, 7-^{3}H]-E_3 with guinea pig liver homogenate in the presence of UDPGA (Goebelsmann *et al.*, 1965a). The conjugate thus formed was subjected to CCD in system B. A radioactive peak with a K (partition coefficient) of 0.33 was pooled.

Radiochemical purity of this conjugate was checked by thin layer chromatography [chloroform: isopropyl alcohol: formic acid (10 : 6 : 2)]. A single peak, the mobility of which was identical with that of standard E_3-3G (Rf=0.20), was observed. Moreover, upon being chromatographed on paper in system II, this material showed a single peak with an Rf of 0.21.

This conjugate was hydrolyzable with β-glucuronidase (83% hydrolysis) and hydrolysis was significantly inhibited (>95%) in the presence of saccharo-1, 4-dilactone. When the aglycone thus obtained was chromatographed on paper in system I, a single peak which moved at the same rate as standard E_3 (Rf=0.09) was observed.

Thus, identification of this conjugate as E_3-3G was established.

Studies in vivo (A)

Three female, non-pregnant rabbits weighing 2–3 kg were used in the experiment. [6, 7-^{3}H]-E_3-3G was dissolved in 0.9% (w/v) saline. The saline which contained $2.7\times10^6-6.7\times10^6$ dpm of [6, 7-^{3}H]-E_3-3G was injected as a bolus into the marginal ear vein of the rabbits. Following the injection, urine was collected for 72 hr using metabolic cages.

Studies in vivo (B)

Three females, won-pregnent rabbit useghing 2–3 kg were used for experiment. [6, 7-^{3}H]-E_3 was dissolved in 0.2 m*l* of ethanol. The ethanolic solution was diluted to 10 m*l* with 0.9% (w/v) saline. The solution which contained $1.9\times10^7-4.9\times10^7$ dpm of [6, 7-^{3}H]-E_3 was injected as a bolus into the marginal

ear vein and urine was collected for 72 hr using metabolic cages.

Preparation of urinary samples for analysis

The XAD-2 (Rohm and Haas Co., Philadelphia, Pa.) columns were used to recover the radiometabolites from the rabbit urine by the technique described by Bradlow (Bradlow, 1968).

Results

Studies in vivo (A)

The excretion of radioactivity in the urine is shown in Table 2. Usually, the largest amount of radioactivity appeared in the urine during the first 24 hr of collection. In case I, a considerable part of radioactivity was excreted in the urine during the second 24 hr. During 72 hr of collection, 50–71% of the administered does was excreted in the urine.

Table 2. The urinary excretion of radioactivity following an intravenous administration of [6, 7-^{3}H]-estriol-3-glucosiduronate into intact rabbits [Expressed as percentage of the administered dose].

	Rabbit I	Rabbit II	Rabbit III
0–24 hr	33.9	40.0	40.1
24–48	28.1	8.2	11.9
48–72	8.7	1.8	4.3
0–72	70.7	50.0	56.3

Rabbit I, Urine

The urinary samples voided during 0 to 48 hr of collection were pooled. The pooled urine was applied to on XAD-2 column and the radiometabolites were eluted with methanol. This material was then submitted to CCD in system C (n=24, n is number of transfers) and two fractions of radioactivity were present (Fig. 1).

Fraction I: 90%, K=0.14

Fraction II: 10%, K=3.00

Fraction I was further distributed in system A for 59 transfers and two fractions were separated (Fig. 2).

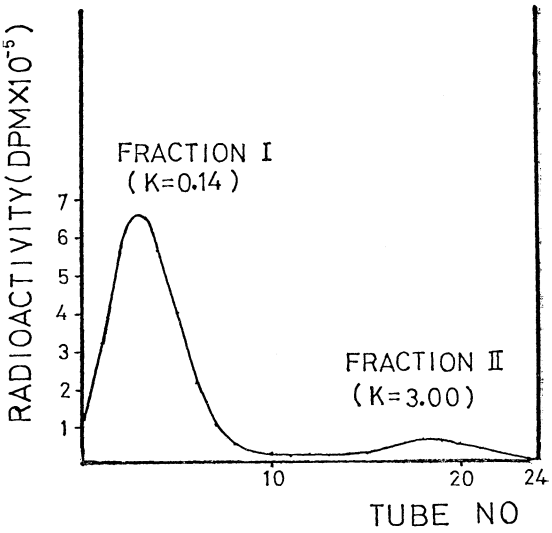

Fig. 1. CCD pattern of urinary metabolites obtained from rabbit-I injected with [6, 7-^{3}H]-estriol-3-glucosiduronate in solvent system C (n=24).

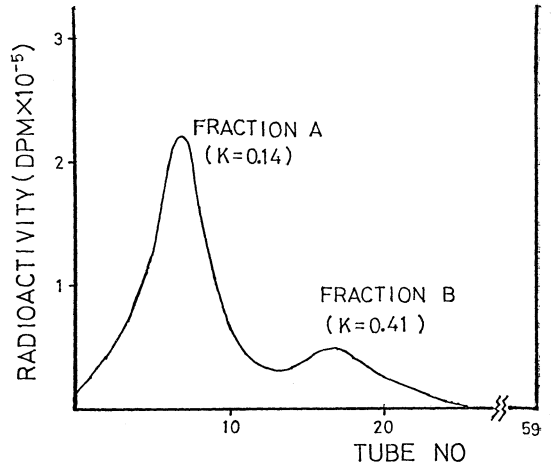

Fig. 2. CCD pattern of fraction I material in the urinary metabolites obtained from rabbit-I injected with [6, 7-^{3}H]-estriol-3-glucosiduronate in solvent system A (n=59).

A polar fraction (K=0.14, designated as fraction A) represented the major one and comprised 72% of the urinary metabolites, whereas a lesser polar fraction (K=0.41, designated as fraction B) comprised 18% of the urinary tritium.

Fraction II was not analyzed further because of the small amount of radioactivity.

Analysis of fraction A

This fraction was considered as representing diconjugates, judging from the K value. This material was, then, chromatographed on a DEAE-Sephadex A-25 column using a linear gradient of 0–0.5 M NaCl and at least four subfractions were present (Fig. 3). A major subfraction (A-II) eluted at an NaCl concentration of 0.10–0.13 M, which comprised 60% of fraction A, was analyzed further.

An aliquot of subfraction A-II was incubated with hyaluronidase. After the incubation, the medium was subjected to CCD (system A, n=24). It was found that the original conjugate was almost completely converted to a less polar compound (K=1.67). The latter substance could be readily hydrolyzed with β-glucuronidase (97% hydrolysis) and hydrolysis was inhibited (>65%) by saccharo-1, 4-lactone. When aglycones thus obtained were chromatographed on paper in system I, two radioactive peaks were found. The less polar peak, which comprised 88% of total aglycones and moved at the same rate as standard 17 epiE_3 (Rf=0.65), was identified as 17 epiE_3 by reverse isotope dilution (RID).

When another aliquot of subfraction A-II was incubated with β-glucuronidase, 88% of the radioactivity became ethyl acetate extractable. The ethyl acetate extract was then submitted to CCD (system D, n=24). A radioactive peak with a K of 1.18 was pooled. The latter substance was hydrolyzable with hyaluronidase (80% hydrolysis) and this hydrolysis was inhibited by N-acetylglucosamine (>75%). The aglycone thus obtained had an Rf identical with that of standard 17 epiE_3 on paper (system I).

Thus, 88% of subfraction A-II was indentified as glucosiduronate-N-acetylglucosaminide of 17-epiestriol (17 epiE_3-G-NAG). In the present studies, however, the positions of hydroxyls in 17 epiE_3 nucleus, to which glucuronic acid and N-acetylglucosamine were attached, could not be determined.

The other minor subfractions obtained by submitting fraction A to DEAE-Sephadex column chromatography were not analyzed further because of the small amount of radioactivity available. Even if one of these subfractions might represent E_3-diG, this comprised at most about 12% of the urinary metabolites.

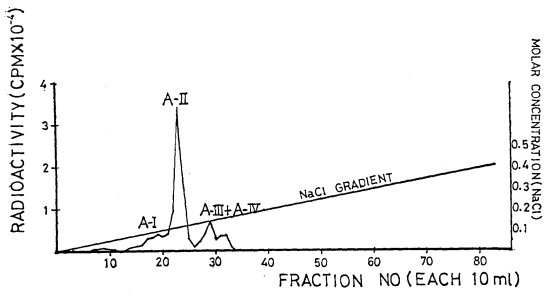

Fig. 3. Chromatographic profile of fraction A in the urinary metabolites obtained from rabbit-I injected with [6, 7-^{3}H]-estriol-3-glucosiduronate on a DEAE-Sephadex column in a linear gradient of 0–0.5 M NaCl.

Analysis of fraction B

An aliquot of this material was chromatographed on paper in system II and a single radioactive peak which moved at the same rate as standard [6, 7-^{3}H]-E_3-3G (Rf=0.26) was observed.

Another aliquot of this material was chromatographed on DEAE-Sephadex A-25 column using a linear gradient of 0.05–0.15 M NaCl and a single peak eluted at an NaCl concentration of 0.09 to 0.10 M was obtained (Standard [6, 7-^{3}H]-E_3-3G was also eluted at an NaCl concentration of 0.09 to 0.10 M)

When the third aliquot was incubated

with β-glucuronidase, 88% of hydrolysis was attained. This hydrolysis was significantly inhibited by saccharo-1, 4-lactone (>95%). The aglycone thus obtained was chromatographed on paper in system I and in system III. In either case, the mobility of the aglycone was identical with that of standard E_3 [Rf's in system I and in system III were 0.09 and 0.68, respectively]. Finally, this aglycone was identified as E_3 by RID.

Thus, fraction B material was identified as E_3-$_3$G.

Rabbits II and III, urine

In these cases, the diconjugate fraction comprised 75% and 64% of the urinary tritium, respectively (Table 3, Table 4); G-NAG constituted 31% of the diconjugate fraction in rabbit II. It is noted that E_3-3G was not detected in either case.

Table 3. The distribution pattern in solvent system B or C of the urinary metabolites obtained from intact rabbits injected with [6, 7-^{3}H]-estriol-3-glucosiduronate.

	Rabbit I	Rabbit II	Rabbit III
Fraction I	90% *(K=0.09)	75 (K=0.04)	100 (K=0.09)
Fraction II	10 (K=3.00)	—	—
** Fraction III	—	22 (K=23.00)	—

* K represents the value of partition coefficient in solvent system C (Rabbit I and Rabbit II) or in solvent system B (Rabbit III).
** This fraction probably represents free estrogen.

Table 4. The distribution pattern in solvent system A of fraction I material obtained from intact rabbits injected with [6, 7-^{3}H]-estriol-3-glucosiduronate.

	Rabbit I	Rabbit II	Rabbit III
Fraction A	80% *(72) **(K=0.14)	100 (75) (K=0.10)	64 (64) (K=0.10)
Fraction B	20 (10) (K=0.41)	— —	36 (36) (K=0.31)

* The number in this parenthesis shows the percentage of fraction A and fraction B material contained in urinary tritium.
** K represents the value of partition coefficient in solvent system A.

Studies in vivo (B)

The excretion of radioactivity in the urine is shown in Table 5. Usually, the largest amount of radioactivity appeared in the urine during the first 24 hr of collection. In case V, a considerable part of radioactivity was excreted in the urine during the second 24 hr. During 72 hr of collection, 34–52% of the administered dose was excreted in the urine.

Table 5. The urinary excretion of radioactivity following an intravenous administration of [6, 7-^{3}H]-estriol into intact rabbits [Expressed as percentage of the administered dose].

	Rabbit IV	Rabbit V	Rabbit VI
0–24 hr	27.9	24.8	38.8
24–48	4.5	19.9	9.9
48–72	1.4	6.0	3.1
0–72	33.8	50.7	51.8

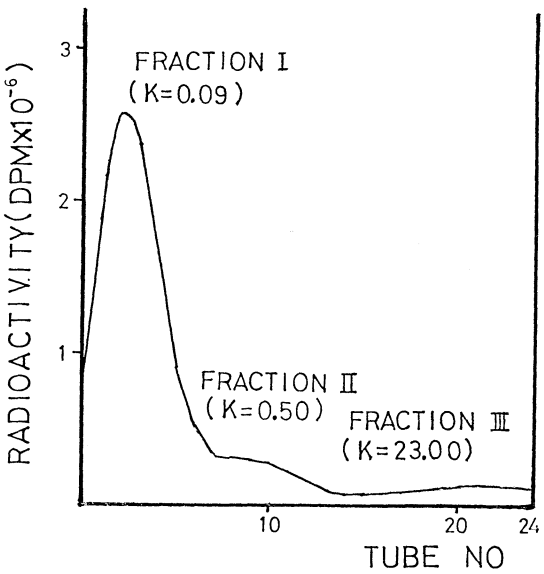

Fig. 4. CCD pattern of urinary metabolites obtained from rabbit-IV injected with [6, 7-^{3}H]-estriol in solvent system C (n=24).

Rabbit-IV, urine

The urinary metabolites recovered through XAD-2 column were subjected to CCD in system C (n=24) and three fractions of radioactivity were present (Fig. 4).

Fraction I: 76%, K= 0.09
Fraction II: 17%, K= 0.50
Fraction III: 7%, K=23.00

Analysis of fraction I

When this material was further distributed in system A (n=46), K was found to be 0.11. This fraction was considered as representing diconjugates, judging from the K value. An aliquot of this material was, then, chromatographed on a DEAE-Sephadex A-25 column using a linear gradient of 0–0.5 M NaCl and at least five subfractions were present (Fig. 5). Since the separation of subfraction I-c, I-d and I-e was unsatisfactory, another aliquot of fraction I was chromatographed on a DEAE-Sephadex A-25 column using a linear gradient of 0–0.2 M NaCl. Again, the separation of these subfractions was poor. A major subfraction I-b, which was eluted at an NaCl concentration of 0.07–0.08 M and comprised 46% of fraction I, was analyzed further in the manner as described in studies *in vivo* (A) and identified as G-NAG. (17 epiE_3-G-NAG constituted 76% of this subfraction.)

The other, minor subfractions of fraction I were not analyzed further. Even though one of these subfractions might represent E_3-diG, E_3-diG comprised at most 16% of the urinary metabolites.

Analysis of fraction II

When this fraction was redistributed in system B for 24 transfers, K was found to be 1.09. This material was then chromatographed on a DEAE-Sephadex column using a linear gradient 0.05–0.15 M NaCl and a single peak, eluted at an NaCl concentration of 0.11–0.13 M, was observed. The radiochemical purity of this material was checked by PC in system II. A single peak was present, the mobility of which was identical with that of standard E_3-16G (Rf=0.43).

This substance was hydrolyzable with β-glucuronidase (79% hydrolysis) and hydrolysis was inhibited by saccharo-1, 4-lactone (>95%). When the aglycone was submitted to PC in system I, a single peak which moved at the same rate as standard [6, 7-^{3}H]-E_3 (Rf=0.10) was obtained. Finally, the aglycone was identified as E_3 by RID. Thus, identification of fraction II as E_3-16G was established.

Cases V and VI, urine

In these cases the diconjugate fraction comprised 68% and 79% of the urinary tritium (Table 6) and G-NAG constituted 44% and 48% of the diconjugate fraction, respectively. (17 epiE_3-G-NAG comprised 71% of the G-NAG conjugate in case V.)

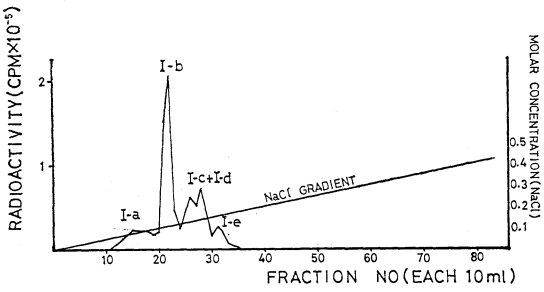

Fig. 5. Chromatographic profile of fraction I in the urinary metabolites obtained from rabbit-IV injected with [6, 7-^{3}H]-estriol on a DEAE-Sephadex column in a linear gradient of 0–0.5 M NaCl.

Table 6. The distribution pattern in solvent system C of the urinary metabolites of intact rabbits injected with [6, 7-^{3}H]-estriol.

	Rabbit IV	Rabbit V	Rabbit VI
Fraction I	76 *(K=0.09)	68 (K<0.04)	79 (K<0.04)
Fraction II	17 (K=0.50)	25 (K=0.50)	15 (K=0.33)
Fraction III	7 (K=23.0)	7 (K=5.0)	6 (K=8.6)

* K represents the value of partition coefficient in solvent system C.

Moreover, E_3-16G (Case V; 25% of the urinary tritium) and 17 epiestriol-monoglucosiduronate (Case VI; 15% of the urinary tritium) were found in the urine. No E_3-3G could be detected in the urine in either case.

Discussion

The biliary and urinary excretion of the metabolites of [16-^{14}C]-E_3 in rabbits with biliary fistulas was studied by Sandberg and coworkers (Sandberg *et al.*, 1967) and it was found that 45% of an administered dose appeared in the bile and 11% in the urine. Moreover, the intestinal reabsorption of the biliary metabolites was estimated as 92% by these authors (Sandberg *et al.*, 1967). Subsequently, the nature of the biliary metabolites of [6, 7-^{3}H]-E_3 in this species was investigated by Kirdani *et al* and E_3-3G was found to be a major metabolite which comprised 85% of the biliary tritium (Kirdani *et al.*, 1973). Thus, following the injection of E_3 into intact rabbits, the fraction of the urinary metabolites which derived from biliary E_3-3G was calculated to be 67%. Therefore, if E_3-3G delivered intraintestinally via the biliary tract undergoes no conversion during the subsequent transport processes, the major urinary metabolites of E_3 in the intact animals must be E_3-3G.

However, the present studies showed that the major urinary metabolites of E_3 in the intact rabbits were diconjugates and that no E_3-3G was detected in the urine. These results clearly indicated that E_3-3G excreted in the intestinal lumen was converted to diconjugates before it was finally excreted in the urine. This statement was also substantiated by other evidence of ours that E_3-3G injected into the intact rabbits was mostly converted to diconjugates. The efficiency of the conversion of E_3-3G to diconjugates probably depends on the rate at which E_3-3G is delivered to the systemic circulation. In fact, in rabbit-I in our studies, a small amount of E_3-3G was detected in the urine after the injection of E_3-3G. In the present studies, however, the sites which participated in the conversion of E_3-3G to diconjugates in this species could not be determined,

Since the nature of urinary matabolites of E_3 in the intact rabbit has already been reported by Collins *et al.* (Collins and Layne, 1968), our present results regarding [6, 7-^{3}H] -E_3 metabolism should be compared with those reported by these authors. First, fraction I in our results will correspond to peak I in those of Collins and coworker. These authors indentified E_3-diG as a major component of peak I and 17 epiE_3-G-NAG as a minor one, whereas 17 epiE_3-G-NAG was a major component of fraction I and E_3-diG, if any, comprised at most 16% of urinary metabolites in our studies. This discrepancy is probably explained by the individual difference among animals studied. Secondly, fraction II material in our analysis will correspond to peak II which was characterized as monoglucosiduronate of E_3 by Collins *et al.* and this material was identified as E_3-16G or monoglucosiduronate of 17 epiE_3.

Now, the nature of urinary metabolites of E_3 in the other species (non-pregnant, intact cases) should be mentioned. In man (Goebelsmann *et al.*, 1965b; Inoue *et al.*, 1969) and baboon (Musey *et al.*, 1973), the major urinary metabolite of labeled E_3 was E_3-16G, whereas E_3-3G and estriol-3-sulfate-16-glucosiduronate were detected in the urine as the minor metabolites. In the dog, the major urinary metabolites of [6, 7-^{3}H]-E_3 were found to be "polyglucuronides" and E_3-3G; small amounts of E_3-16G and estriol-3-sulfate (E_3-3S) were also detected in the urine (Kirdani and Sandberg, 1974). Analysis of the urinary metabolites of [6, 7-^{3}H]-E_3 was carried out in the sheep and it was found that the urinary metabolites consisted predominantly of E_3-3S and E_3-3G (Miya-

zaki *et al*, 1972). The nature of the urinary metabolites of [4-^{14}C]-E_3 was investigated in the guinea pig and E_3-3G was a major urinary metabolite (Kirdani *et al.*, 1976). Thus, an outstanding feature indicated by the above results is the diversity of the nature of the urinary metabolites in the various species.

With regard to the metabolism of E_3-3G, an analysis of the urinary metabolites of doubly labeled E_3-3G was carried out in the human and a major metabolite was found to be E_3-3G (Kirdani *et al.*, 1970), but there have been no reports on the analysis of urinary metabolites of E_3-3G in other species.

In the previous publication from our laboratory, it was shown that [6, 7-^{3}H]-17 β-estradiol-3-glucosiduronate and [6, 7-H]-estrone-3-glucosiduronate administered to the rabbit were mostly converted to a diconjugate, namely 17α-estradiol-3-glucosiduronate-17-N-acetylglucosaminide (Miyazaki *et al.*, 1977). Therefore, we can conclude that the conversions of estrogen-3-glucosiduronate to diconjugate and of the estrogen nucleus to 17α-estrogen are universal phenomena in the rabbit. It is noted that exceptions so far reported were 15α-hydroxyestrogens in which cases monoglucosiduronates were end products and no diconjugates were found in the urine (Polakova *et al.*, 1971).

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