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THE METABOLISM OF ESTRIOL IN THE GUINEA-PIG

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SUMMARY

Labeled estriol (E_3) was injected i.v. into intact guinea-pigs and animals with biliary fistulas. It was shown that biliary excretion constitutes a major route for the conjugates of E_3, probably followed by a significant enterohepatic circulation. The conjugation pattern of E_3 revealed the preponderant amount of the steroid to be excreted as a 3-glucosiduronate, both in the urine and bile. In contrast, the 3,16α-disulfate of E_3 was found in significant amount only in the bile. Unconjugated E_3 and its sulfate constituted a minor percentage of the radioactivity found both in bile and urine. The E_3 nucleus remained intact during the conjugation processes in the guinea-pig. The kidney was shown to glucuronidate E_3 *in vitro* to some extent, but to a much lower degree than observed with kidneys of other species; sulfation of E_3 by the guinea-pig kidney was of an even much lower magnitude. Thus, we interpret the results to indicate that in guinea-pigs, the liver plays a paramount role in the conjugation of E_3 followed by biliary excretion of most of the conjugates and subsequent enterohepatic circulation. The role of the kidney in the conjugation of E_3 in the guinea pig appears to be secondary to that of the liver.

INTRODUCTION

Studies with estriol (E_3) in various species have revealed a number of important differences in the metabolism, conjugation and sites of excretion of this steroid. Thus, the metabolism and fate of E_3 in the baboon [1], dog [2], rabbit [3] and human [4] have been reported from our laboratory, the latter species also having been studied by Emerman *et al.* [5], and considerable differences in the quantitative and qualitative pattern of metabolism of E_3 shown to exist among the species. In all of the studies referred to above, it was shown that the E_3 was conjugated without undergoing any significant modification of its steroid nucleus. This latter fact allowed the determination of the conjugation patterns of a phenolic steroid without the necessity of having to deal with complicated metabolic patterns and transformations of the E_3.

In some species the kidney plays an important role in the conjugation (primarily glucuronidation) and excretion of E_3 [6], whereas in others the biliary route is a major pathway for excretion [7]. The factors which control biliary excretion of E_3 are still unknown, though some workers have postulated that it is a major pathway for diconjugates of estrogens [5, 9]. We have preliminarily reported on the biliary and urinary pattern of excretion of E_3 in the guinea-pig, and have compared the percent of radioactivity excreted in the bile and urine after the injection of E_3 in that animal with those in others [8, 10]. E_3-3-glucosiduronate appeared to be the major urinary and biliary conjugate of E_3, though other conjugates were present in guinea-pig urine and bile, but were not identified. Goebelsmann *et al.* [11] reported that E_3-3-glucosiduronate can be biosynthesized by incubation of E_3 and UDPGA with guinea-pig liver homogenate. Recently, Levitz *et al.*[12], identified another product of this incubation as being E_3-3,16α-disulfate.

We wish to present here results of a comprehensive study on the biliary and urinary metabolic patterns of excretion in the guinea-pig following the administration of labeled E_3. The present study points to the fact that E_3-3-glucuronide is readily excreted in the bile and urine of the guinea-pig and that a novel diconjugate (disulfate) of E_3 is excreted in the bile. Apparently, there is an enterohepatic circulation of the conjugated E_3 excreted in the bile of the guinea-pig.

MATERIALS AND METHODS

4-^{14}C-Estriol (60.1 mCi/mM) was purchased from the New England Nuclear Corporation, Boston, Massachusetts; its purity was checked by chromatography on paper in the system: benzene–water–methanol; 100:45:55 by vol., development time being 16 h. Urine was collected from female Hartely guinea-pigs (350–400 g) by suprapubic catheterization, the bladder being ligated around the catheter to insure quantitative recovery of urine. Bile was collected from guinea-pigs by cannulation of the bile duct using tubing that had been drawn to proper size after slight heating in a flame (Tygon Micro-Bore Formulation, Norton Plastics and Synthetics Division, Akron, Ohio). The animals were hydrated during the period of specimen

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collections by an i.v. saline drip delivered either into the tail vein or a vein in the inguinal region. Into this saline delivery tubing was injected 5 μCi of labeled estriol in 2 ml of 10% ethanol in saline. Since excretion rates had been previously reported by us for both urine and bile in the guinea-pig [10], and since the objective of the present work was the determination of the conjugation profile, one collection of urine and bile was made 6 h after injection.

Countercurrent distribution (CCD) was performed using three instruments: two Post instruments of 100 and 200 tubes, respectively (Spectrum Medical Instruments, Inc., New York, N.Y.), and a Quickfit Steady State Distribution Apparatus of 125 tubes (Instrutec Corporation, Fairfield, N.J.). All three instruments had 10 ml per phase capacity. Two solvent systems were used throughout: system A, 2% ammonia in water–n-butanol–ethyl acetate; 4:3:1 by vol. and system B, 10% ammonia–n-butanol; 1:1 v/v. Statistical computations of partition coefficients (K) and percentages of compounds in the CCD train were performed by a computer program previously described [4].

DEAE Sephadex was purchased from Pharmacia Fine Chemicals of Piscataway, N.J., swollen in deionized water, and packed in K 9/60 columns (0.9 cm. dia. and 60 cm. long) obtained from the same firm.

Two Packard Instruments (Downers Grove, Illinois), Tri-carb Liquid Scintillation Counter, models 3375 and 2450, as well as a sample oxidizer model 306 were used in the present work. Samples were routinely oxidized if (i) the external standard ratio (A.E.S.) was ≤ 0.5 and (ii) if radioactivity in bile was being determined. A premixed scintillation solution (ACS, Aquous Counting Scintillant, Amersham–Searle Co., Arlington Heights, Illinois) was used after dilution (2:1) with toluene for economy. In all samples enough counts (≥ 500) were accumulated to give a percent standard deviation of ≥ 5.

One solvent system was used throughout for the development of paper chromatograms: benzene–methanol–water; 100:55:45 by vol.

Enzymatic hydrolyses. For β-glucuronidase hydrolysis, triplicate incubations were performed using 15 ml 0.1 M acetate buffer, pH 5.0 at 37°C for 72 h. All incubations were extracted with water-saturated ether. An incubation containing only conjugates was used as the control to ascertain stability of the conjugates in the buffer. A second incubation contained conjugates and enzyme, while the third contained additionally 10^{-3} M saccharo-δ-lactone (Calbiochem., LaJolla, Calif.). Ketodase (Warner–Chilcott, Morris Plains, N.J.) was used in the early part of this study (500 units per ml of incubation medium). Later, bovine liver β-glucuronidase (Sigma, St. Louis, Missouri; Type B-1) was substituted, using an identical number of enzyme units. Phenolsulfates were hydrolyzed in 15 ml of 0.1 M acetate buffer, pH 6.0 at 37°C for 20 h, a duplicate control incubation being included. Either Mylase P (Schwartz–Mann, Orangeburg, N.Y.), 5 mg per ml of buffer, or, alternatively, phenolsulfatase from limpets (Sigma Type II, low β-glucuronidase) was used. In the latter case, 5 mg of enzyme was used in a total of 2 ml of incubation medium.

For solvolysis the conjugates in 15 ml of 1 N HCl were heated at 70°C under reflux for 24 h. The solution was then neutralized with 1 N NaOH and extracted with water-saturated ether.

Table 1. Excretion of radioactivity in 6 h after the injection of 4-^{14}C-estriol into guinea-pigs (Results expressed as % of injected radioactivity)

Biliary fistula			Intact	
Animal no.	Urine	Bile	Animal no.	Urine
1	11.7	36.0	4	30.5
2	13.0	52.6	5	30.0
3	18.8	40.4	6	20.1
Average	16.9	43.0	Average	26.9

RESULTS

In Table 1 is shown the per cent excretion of radioactivity 6 h following the injection of labeled E_3 into 3 intact and 3 biliary fistula animals. The data in Table 1 and those previously reported by us [10] for 24 h excretion show that the excretion of labeled E_3 in the urine of intact animals is greater than that in the urine from biliary fistula animals, indicating an enterohepatic circulation of E_3 in the guinea-pig.

Figure 1 is a composite of two representative curves of excretion patterns obtained upon CCD of urine and bile. The urine distribution in Fig. 1 is from a

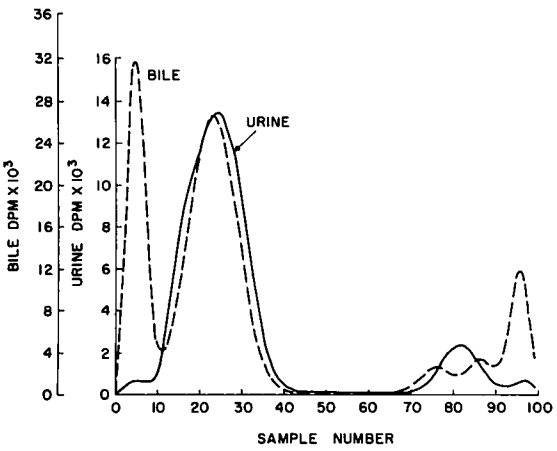

Fig. 1. CCD patterns of a representative guinea-pig urine and bile following the injection of ^{14}C-E_3. Peak I, which is very prominent in the bile (present in very small amounts in the urine) consists of E_3-3,16α-disulfate. Peak II (tubes 10–40) is the major peak in both the urine and bile and consists of the 3-glucosiduronate of E_3. Peak III (tubes 70–90) in the urine was shown to be E_3-3-sulfate; its counterpart in the bile consisted of a more complicated pattern, which was difficult to identify. Peak IV is unconjugated E_3.

biliary fistula animal; distribution of urines from intact guinea-pigs gave identical patterns. The CCD curves (Fig. 1) have been divided into four general areas: Peak I, tubes 0–9; Peak II, tubes 10–40; Peak III, tubes 68–90 (in the bile curve, this area includes Peaks IIIa and IIIb) and Peak IV, tubes 91–100. Computation of the CCD data gave the following estimates:

Peak no.	Per cent in CCD train	K value	Calculated peak tube no.
	Urine		
I	1.1	0.05	4.6
II	89.0	0.3	23.6
III	9.0	4.7	81.8
IV	1.0	30.0	96.3
	Bile		
I	26.8	0.05	4.6
II	56.5	0.3	23.0
IIIa	3.4	3.3	76.1
IIIb	7.1	7.1	87.1
IV	9.1	26.6	95.9

It is apparent that both urine and bile contain Peak II, ultimately shown to be of similar origin in both fluids, that accounts for the preponderant amount of the radioactivity in bile and urine. However, in the urine another significant Peak (III) was also present, without a counterpart in the bile. In the latter fluid a sharp and prominent Peak I was present, Peak III consisted of 2 components and a prominent Peak IV was observed.

Analysis of urine. Peak I was not analyzed, since it contained very small amounts of radioactivity. Peak II was hydrolyzed with β-glucuronidase. Almost no radioactivity could be extracted from the control, whereas 43.8–84.2% of the incubated ^{14}C was extractable from the enzyme incubation and 12.5–15.4% from the incubation with inhibitor. Chromatography on paper of the extract gave one peak which had a mobility identical with that of co-chromatographed E_3 standard. From the position of Peak II in the CCD train ($K \simeq 0.3$) and the above results, it was concluded that Peak II consisted of E_3-3-glucosiduronate. Peak III was hydrolyzed with phenolsulfatase and less than 1% and 84% or more of the radioactivity could be extracted from the control and enzyme incubations, respectively. One peak was observed on chromatography of the extract, which corresponded to the E_3 standard. From the K value of Peak III ($\simeq 4.5$), it was concluded that it consisted of E_3-3-sulfate. Peak IV was chromatographed directly on paper and had the same mobility as that of the E_3 standard.

Analysis of bile. The polar Peak I (Fig. 1) had a K value of 0.05 in CCD system A. When this peak was subjected to β-glucuronidase hydrolysis and the incubation medium redistributed, 96.8% of the radioactivity in the CCD train had a K value of 15.3, whereas the remaining ^{14}C had a K of 0.05, i.e., identical with the starting material. When this experiment was repeated with the addition of the inhibitor, 89.3% of the radioactivity in the CCD train was less polar, indicating little or no inhibition. Upon repetition of this experiment with purified phenolsulfatase, 19.8% of the radioactivity became less polar. This indicated that the hydrolysis with β-glucuronidase was probably due to the presence of phenolsulfatase in the enzyme preparation and, hence, hydrolysis was uninhibitable with saccharo-δ-lactone. A figure of about 20% hydrolysis after a single incubation with purified phenosulfatase is acceptable, in our experience, since this enzyme is very susceptible to inhibition by impurities in the bile, which upon CCD are usually found in the polar region of Peak I. The peak resulting from the hydrolysis (with phenosulfatase either from the purified enzyme or present in the β-glucuronidase) will be henceforth designated as the "hydrolysis peak" to avoid confusion.

The remainder of the work described in this section was performed with the "hydrolysis peak" obtained from successive phenolsulfatase hydrolyses of Peak I. Because the "hydrolysis peak" was located in the nonpolar area of the CCD train in system A, the possibility that the compound represented by the radioactivity was an aglycone was further explored by chromatography on paper. The peak proved to be much more polar than E_3 and, hence, it was concluded that the "hydrolysis peak" was not an aglycone. Redistribution in solvent system B showed that the peak had become much more polar with a $K = 2.19$. We concluded that the "hydrolysis peak" had a free phenolic hydroxyl group by analogy with the chromatographic behavior of E_3-3-glucosiduronate and E_3-16α-glucosiduronate in systems A and B [13]. Upon chromatography on E_3-DEAE Sephadex (Fig. 2) a single peak was obtained, indicating that the compound in question was a charged conjugate. When an aliquot of the "hydrolysis peak" was solvolyzed overnight at 70°C in 1 N HCl, 41.4–62.2% of the radioactivity could be extracted with water-saturated ether after neutralization. Another aliquot of the "hydrolysis peak" was mixed with 10 mg of E_3-16α-esulfate* and the mixture redistributed in system B. The radioactivity and O.D. (280 nm) curves coincided, with a peak in tube 68 ($n = 99$, $K = 2.1$). Another aliquot of the "hydrolysis peak" was mixed with 1.5 mg of E_3-16α-sulfate, and the mixture was solvolyzed. The extract was then chromatographed on paper, whereupon the radioactive peak and the color spot (after spraying with phenol reagent and exposing to ammonia) coincided and had identical mobilities when co-chromatographed with an E_3 standard. We concluded from the above experiments that the "hydrolysis peak" was E_3-16α-sulfate and, therefore, Peak I must be E_3-3,16α-disulfate.

When ^3H-E_3-3,16α-disulfate* was added to the bile, the ^3H and ^{14}C invariably traveled together in the

*We are grateful to Dr. Mortimer Levitz for supplying us with E_3-16α-sulfate and ^3H-E_3-3,16α-disulfate standards.

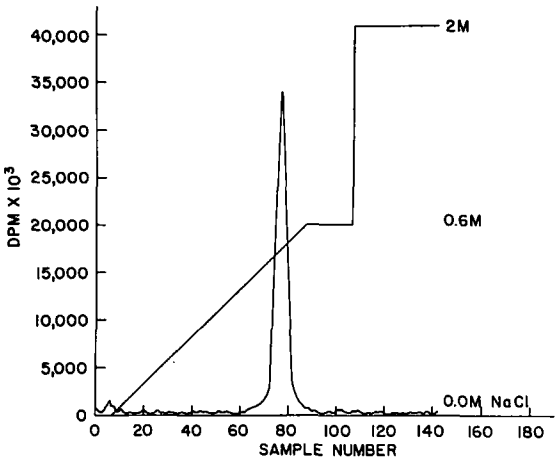

Fig. 2. Chromatography pattern of the "hydrolytic peak" on DEAE-Sephadex, showing a simple major peak, compatible with the behavior of a charged conjugate.

ratio present originally. Thus, when Peak I was subjected to CCD, the two labels had identical distribution curves. Solvolysis of Peak I led to the formation of E_3, which contained ^3H and ^{14}C in a ratio very similar to that before solvolysis. Because of the small amounts of ^3H-disulfate standard available to us, no further studies could be performed.

Peak II was hydrolyzed with β-glucuronidase. Extraction of the control, enzyme and enzyme plus inhibitor incubations yielded on the average 0.26%, 89% and 42% of the incubated radioactivity, respectively. From these data and the position of the peak in the CCD train ($K = 0.31$), it was concluded that this Peak II represented E_3-3-glucosiduronate.

Peaks IIIa, IIIb and IV were combined and chromatographed on DEAE-Sephadex, whereupon two peaks appeared, one in the non-charged eluent vol. (early collections) and the other in the charged, conjugate area of the elution pattern from this type of column. The first non-charged peak was chromatographed on t.l.c. and the plate exposed for eight weeks to NS54T Kodak X-ray film. Upon development, a spot appeared which had the same mobility as co-chromatographed standard E_3. The charged peak was not investigated because of the small amount of radioactivity associated with it.

In vitro *conjugation.* The ability of guinea-pig liver preparations to synthesize the 3-glucuronide, 3-sulfate and 3,16α-disulfate has been demonstrated by other workers [5, 11, 12]. Hence, the *in vitro* capacity of the guinea-pig kidney to conjugate labeled E_3 was investigated utilizing kidney homogenates and UDPGA for glucuronidation and the cytosol fraction of the guinea-pig kidney with ATP plus $MgSO_4$ for sulfation, according to previously described methods [5, 11, 12]. It was shown that the kidney homogenate readily conjugated E_3 with glucuronic acid, though to a much lesser degree than shown by the kidney of other species [6]. Conjugation of the E_3 with sulfate was of a much lower percentage than that exhibited for glucuronidation (6% vs. 25%). The glucuronide formed by the kidney preparation was shown to the 16α-glucosiduronate of E_3 and the sulfate in the 3-position of the steroid by CCD and hydrolytic procedures.

DISCUSSION

The factors which determine the excretion of E_3 in the bile and urine in various species are not well understood, particularly since there is such remarkable variability in the quantitative and qualitative patterns among the species [7, 13]. In some it is probable that the kidney plays an important role in the conjugation and excretion of i.v. administered labeled E_3, e.g., in the human and baboon. On the other hand, in some species the liver is the site of the paramount conjugating and excretory systems of E_3, usually leading to significant biliary excretion of the products of such conjugation. In the guinea-pig the excretion in the bile of the disulfate and 3-glucuronide of E_3, conjugates which have been shown to be synthesized *in vitro* by guinea pig liver preparations, indicates that both mono- and diconjugates are readily excreted into the bile; and the preponderant excretion of the 3-glucuronide in the bile points to the fact that double conjugation is not a prerequisite for biliary excretion, at least in the guinea-pig.

Even though the kidney may play some role in the conjugation of E_3 in the guinea-pig, particularly of glucuronidation and to a much lesser extent sulfation, as demonstrated by the *in vitro* studies, we believe that in this animal, in contrast to others (human, baboon), the preponderant site of conjugation is the liver; this may also account for the large biliary excretion of the radioactivity associated with E_3. In other words, phenolic steroids which are readily and extensively conjugated by the liver are preponderantly excreted in the bile, whereas those which can also be readily conjugated by the kidney, are excreted very significantly in the urine.

The identification of the disulfate of E_3 in guinea-pig bile is, as far as we know, the first such demonstration *in vivo* of this conjugate in any species, though it has been synthesized *in vitro* by guinea-pig liver. It is interesting to note that very little of the disulfate of E_3 was found in guinea-pig urine, whereas it was present in substantial amounts in the bile. The failure to find significant amounts of the disulfate in the urine, in spite of a considerable enterohepatic circulation in the guinea-pig of E_3 and/or its conjugates, indicates that (1) either the disulfate is not hydrolyzed and/or reabsorbed in the intestinal tract, (2) that it is not formed in the kidney in any significant amounts, or (3) that probably very little of the disulfate enters the circulation. This was not true of the major conjugate of E_3 in the guinea pig, i.e., the 3-glucosiduronate, which appeared in large amounts both in the urine and bile.

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