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METABOLITES OF ESTRADIOL-17β IN BOVINE LIVER: IDENTIFICATION OF THE 17-β-D-GLUCOPYRANOSIDE OF ESTRADIOL-17α

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SUMMARY

An investigation of the unidentified polar metabolites from the livers of steers treated with [4-^{14}C]-estradiol-17β or its 3-benzoate revealed them to consist principally of glycosidic derivates of estradiol-17α. The major polar metabolite was the 17-β-D-glucopyranoside of estradiol-17α. The 3-β-D-glucosiduronate of estradiol-17α, and other 17-glycosides of estradiol-17α and estradiol-17β were also characterized. This study demonstrates for the first time the presence of estrogen glycosides in bovine liver tissue.

INTRODUCTION

In a recent study Dunn *et al.*[1] observed the presence of a polar estrogen metabolite in extracts of the livers of steers and heifers treated with [4-^{14}C]-estradiol-17β and its 3-benzoate. This unknown metabolite constituted about 40% of the total radioactivity in the liver; it was chromatographically more polar than estriol, and was found to be the major metabolite recovered after β-glucuronidase hydrolysis of the glucosiduronate fraction. The purpose of the present investigation was to characterize and identify this and other unknown metabolites of estradiol-17β ($17\beta E_2$) in bovine liver tissue. During the course of the work it became apparent that these estrogen metabolites cannot be adequately characterized on the basis of their partition from ether and their recovery after enzymatic hydrolysis. We have confirmed the presence of the major estrogen metabolite in bovine liver in about 40% yield, and identified it as the 17-β-D-glucopyranoside of estradiol-17α ($17\alpha E_2$).

EXPERIMENTAL

Veterinary protocol[*1, 2*]. Four steers weighing 450–500 kg each were procured from a commercial source and given a maintenance diet of feed and water *ad libitum*. The steers were run into a squeeze chute daily for restraint and injection. They were primed for 11 days with a daily injection into the parotidoauricularis muscle of either $17\beta E_2$ (2 mg/day) or $17\beta E_2$ 3-benzoate (3 mg/day) dissolved in sesame oil (1 cc). The injection site was alternated daily between the right and left muscle. On completion of the priming, the animals were injected for three additional days with the same amount of $17\beta E_2$ or $17\beta E_2$ 3-benzoate to which 400 μCi [4-^{14}C]-$17\beta E_2$ or 600 μCi[4-^{14}C]-$17E_2$ 3-benzoate, respectively, had been added. The radioactive compounds were obtained from New England Nuclear Corp. Three hours after the final dose of radiolabeled estrogen, the animals were slaughtered, the livers excised *in toto*, representative areas sliced and homogenized in a Waring blender, and two 1.5 kg lots from each liver frozen immediately.

Extraction and partial purification of metabolites. The flow chart for the extraction and partial purification of radioactive metabolites from liver tissue is shown in Fig. 1. Each 200 g sample was extracted with 800 ml of Delsal's reagent[3] (methylal–methanol, 4:1, v/v). Ascorbic acid (200 mg) was added to the extract to serve as an anti-oxidant. The extract was filtered through a coarse, sintered glass filter, and the residue washed four times with 800 ml of Delsal's reagent. The combined extracts were evaporated to dryness under reduced pressure at 37°C and defatted and further purified according to procedures described by Kaltenbach *et al.*[2]. The conjugated estrogen fractions were chromatographed on Sephadex LH-20 columns (1.9 × 55 cm) and the radioactive eluates hydrolyzed with β-glucuronidase[2]. After hydrolysis, the aqueous phase was extracted with diethyl ether[2].

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Trivial names: The following trivial names and abbreviations are used: estrone (E_1) = 3-hydroxy-1,3,5(10)-estratrien-17-one; 17α-estradiol ($17\alpha E_2$) = 1,3,5(10)-estratriene-3,17α-diol; 17β-estradiol ($17\beta E_2$) = 1,3,5(10)-estratriene-3,17β-diol; estriol (E_3) = 1,3,5(10)-estratriene-3,16α,17β-triol; $17\alpha E_2$-17-glucopyranoside = 3-hydroxy-1,3,5(10)-estratrien-17α-yl-β-D-glucopyranoside; $17\beta E_2$-17-glucopyranoside = 3-hydroxy-1,3,5(10)-estratrien-17β-yl-β-D-glucopyranoside; $17\alpha E_2$-3-glucosiduronate = 17α-hydroxy-1,3,5(10)-estratrien-3-yl-β-D-glucosiduronate; $17\beta E_2$-3-glucosiduronate = 17β-hydroxy-1,3,5(10)-estratrien-3-yl-β-D-glucosiduronate.

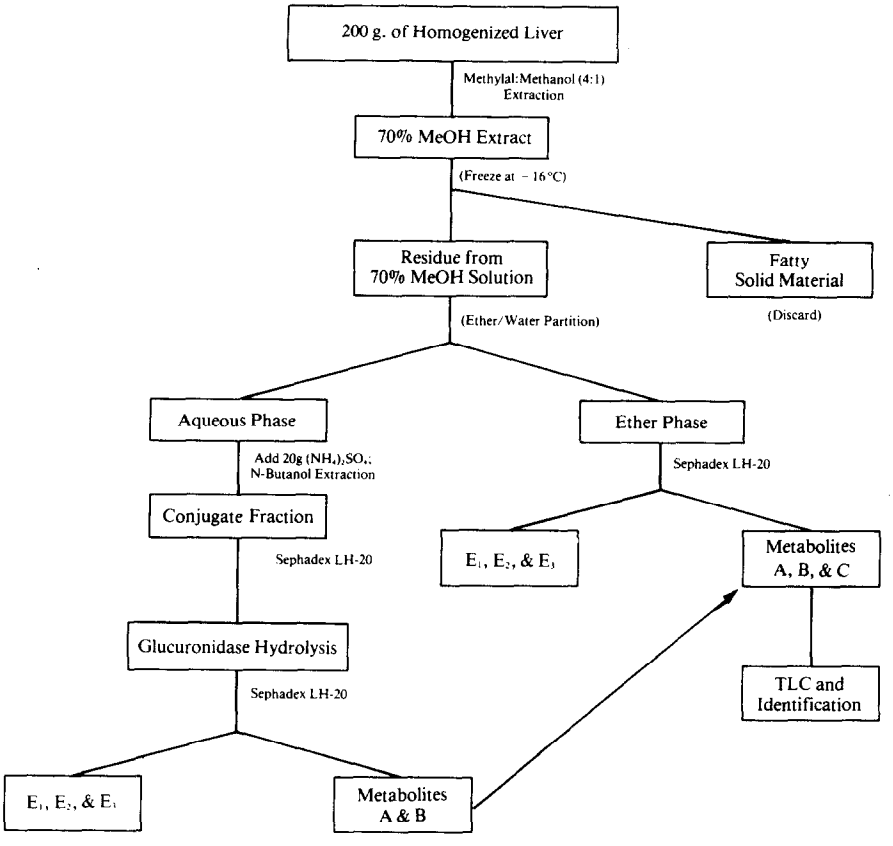

Fig. 1. Flowchart for the separation of estrogen metabolites from bovine liver.

[^{3}H]-Labeled internal standards of E_1, 17βE_2 and E_3 were added to the ether extracts obtained before and after hydrolysis with β-glucuronidase[2]. These extracts were then dried under reduced pressure and dissolved in 5 ml of benzene–methanol (85:15). They were then quantitatively applied to Sephadex LH-20 columns (1.9 × 55 cm) and eluted with the same solvent mixture. After chromatography, the columns were eluted with methanol to remove residual radioactivity.

Radioactivity determinations. Radioactivity was measured in a Packard Model 3320 Tri-Carb liquid scintillation spectrometer using a scintillation fluid consisting of 42 ml Liquifluor (New England Nuclear Corp.) per liter of toluene. For the conjugate samples, 0.2 ml of water was added to each vial and Bio-Solv solubilizer BBS-3 (66 ml/l, Beckman Instrument Co.) was also included in the counting mixture to insure complete solubilization of the samples. An on-line computer interfaced with the spectrometer provided quench corrected values of d.p.m. Efficiencies were obtained from a curve generated by counting a series of quenched standards with and without external irradiation before each experiment. Combustion analysis of liver tissue, using about 600 mg, was performed in duplicate by Dr. Lee Beck of the University of Alabama Medical Center, Birmingham, Alabama.

Thin-layer chromatography. Brinkmann E-M (silica gel 60F-254, 5 × 20 cm) plates were used in the thin layer chromatography (t.l.c.) of the polar metabolites. The solvent systems were those employed by Lisboa[4]: S-1, ethyl acetate–hexane–ethanol (16:3:1, by vol.); S-2, chloroform–ethanol (3:2, v/v); S-3, chloroform–ethanol (4:1, v/v); S-4, ethyl acetate–ethanol–acetic acid (9:10:1, by vol.). All plates were placed in a desiccator and vacuum dried for 5 min after development with the various solvent systems. Radioactive metabolites were located on the plates with an Atomic Radiochromatograph Scanner, Model RSS. Recovery of the radioactive metabolites from t.l.c. plates was accomplished by elution with methanol. Nonradioactive estrogen standards were located on the plates by spraying with 15% perchloric acid and heating on a hot plate.

Enzymatic hydrolysis. Enzymatic hydrolysis with β-glucosidase of metabolites purified by t.l.c. was carried out by dissolving the metabolite in 5 ml 0.1 M sodium citrate buffer, pH 4.3, and incubating with 5 mg β-glucosidase (Calbiochem) for 24 or 48 h at 37°C. The reaction mixture was then extracted three times with 10 ml ethyl acetate and the combined organic extracts dried over Na_2SO_4 and evaporated *in vacuo*. The inhibitor glucono-1,5-lactone (6 mg) was used in some incubations to distinguish between the hydrolysis of 3- and 17-glycosides[5].

Derivative formation. The methyl esters of the 3- and 17-glucosiduronates of 17βE_2 (Sigma Chemical Co.) and metabolite C were prepared by dissolving

the compounds in methanol, cooling in an ice bath, and adding 0.5 to 1 ml of a cold dry ether solution of diazomethane. After 5 s, a few drops of acetic acid were added to destroy the excess diazomethane, and the solutions were evaporated to dryness at 37°C under nitrogen. The 3-methyl ethers of $17\alpha E_2$-17-glucopyranoside[6] and metabolites A_2, A_1 and B were similarly prepared except that the reaction with diazomethane was allowed to proceed for 20 h at 0°C. Under these conditions, t.l.c. showed that methyl ester formation was complete in 5 s and methyl ether formation was not detectable until after 20 min but was complete after 20 h.

RESULTS

Column chromatography

The chromatographic analyses of the ether phases[2] (Fig. 1) of the liver extracts are illustrated in Fig. 2. In addition to the expected radioactivity coincident with the reference areas of E_1 and $17\beta E_2$, an unknown metabolite, termed "A", slightly more polar than E_3 was found. A much larger amount of an unknown polar metabolite, termed "B", was found in all four of the liver extracts. In the case of the liver extract from steer 2, a substantial amount of an even more polar unknown metabolite, designated "C", was found.

The column chromatographic analysis of the fraction containing the steroid conjugates (obtained from the aqueous phase of the partitions with ether[2] (Fig. 1) from liver tissue revealed a single major peak of radioactivity. This material was subjected to hydrolysis by β-glucuronidase[2] and then chromatographed on Sephadex LH-20 columns. Small amounts of the unknown polar metabolites A and B were found in the extracts from steers 1, 3 and 4. These were pooled with the respective fractions described above from the ether phases of the liver extracts.

Thin-layer chromatography

The polar metabolites obtained above were sub-

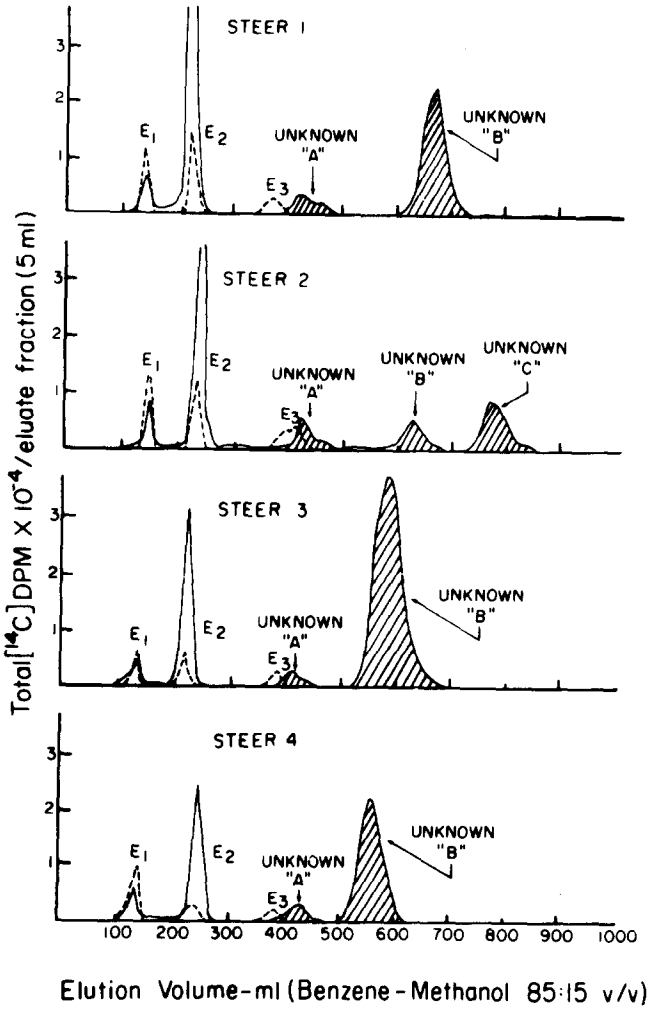

Fig. 2. Column chromatography of the ether phases from extracts of bovine liver on Sephadex LH-20. The 1.9 × 55 cm columns were equilibrated and eluted with benzene–methanol (85:15, v/v). The total [^{14}C] d.p.m. in the 5 ml eluate fractions is shown for each column. The elution of the [^{3}H]-labeled internal standards of estrone (E_1), 17β-estradiol (E_2), and estriol (E_3) is shown by the dashed lines.

Table 1. Recovery of individual polar metabolites from liver

Steer	Metabolite	Actual d.p.m. extracted per 200 g*	d.p.m. Estimated from combustion results†	Overall procedural losses in %‡	% of Total tissue radioactivity as metabolites§
1	A_2	7,890	9,600	19	1.2
	A_1	7,150	8,710	18	1.2
	B	247,000	301,000	18	38.0
2	B	66,000	97,200	32	9.3
	C	65,100	96,200	32	9.2
3	A_2	6,090	7,730	21	1.1
	A_1	2,560	3,400	25	0.5
	B	234,000	298,000	22	42.0
4	A_2	7,010	10,600	34	1.4
	A_1	2,950	4,400	33	0.6
	B	162,000	244,000	34	31.0

* Total DPM extracted for all metabolites: steer 1, 642,000; steer 2, 710,000; steer 3, 550,000; steer 4, 520,000.

† Calculated from [total d.p.m. from tissue combustion analysis/Total d.p.m. extracted for all metabolites] × [Actual DPM extracted per 200 g (column 3)]. The total DPM from tissue combustion analysis were: steer 1, 782,000; steer 2, 1,050,000; steer 3, 701,000; steer 4, 784,000.

‡ General loss during initial extraction procedure and hydrolysis.

§ Calculated from [d.p.m. estimated from combustion results (column 4)/total d.p.m. from tissue combustion analysis] × 100%.

jected to t.l.c. using S-1 developed four times. All of these metabolites were more polar than estriol. The fraction containing polar metabolite A was resolved into two components, A_2 with an R_f of .0.53, and A_1 with an R_f of 0.26. By t.l.c. the polar metabolites B and C behaved as single radioactive components in S-1, with R_f values of 0.20 and 0 respectively.

The recovery of the four polar metabolites from liver tissue after t.l.c. is shown in Table 1. Metabolite B was the major polar radioactive compound isolated from the livers of both steers administered [4-^{14}C]-17βE$_2$ 3-benzoate and one of the two steers given [4-^{14}C]-17βE$_2$, accounting for about 38% of the total radioactivity in the liver tissue. Metabolite C, the most polar of these metabolites, was found in 9% yield in only one of the two animals given [4-^{14}C]-17βE$_2$. The amounts of A_2 and A_1 from the liver of this animal were not determined.

Table 2. Chromatographic mobilities of metabolites and derivatives in t.l.c. systems

Compound	R_f in solvent system* S-1†	S-2	S-3	S-4
Estriol	0.59	0.74	0.58	0.77
Metabolite A_2 methyl ether	0.58		0.47	
Metabolite A_2	0.53		0.38	
Methyl 17βE$_2$-17-glucosiduronate 3-methyl ether		0.67		0.75
Metabolite C methyl ester	0.45	0.58		0.72
Methyl 17βE$_2$-17-glucosiduronate		0.65		
Methyl 17βE$_2$-3-glucosiduronate	0.40	0.58		0.72
Metabolite B methyl ether	0.27	0.48		
17αE$_2$-17-glucopyranoside 3-methyl ether	0.27	0.49		
Metabolite A_1 methyl ether			0.38	
Metabolite A_1	0.26		0.25	
Metabolite B	0.20	0.46		
17αE$_2$-17-glucopyranoside	0.19	0.45		
17βE$_2$-17-glucopyranoside	0.18	0.44		
17αE$_2$-17-galactopyranoside	0.12	0.39		
17αE$_2$-17-N-acetylglucosaminide	0.07			
Metabolite C	0			0.13
17βE$_2$-3-glucosiduronate	0	0	0	0.11

*S-1, ethyl acetate–hexane–ethanol (16:3:1, by vol.);
*S-2, choloroform–ethanol (3:2, v/v);
S-3, choloroform–ethanol (4:1, v/v);
S-4, ethyl acetate–ethanol–acetic acid (9:10:1, by vol.).

† Developed four times.

Identification of metabolite B as the 17-β-D-glucopyranoside of estradiol-17α

The chromatographic mobility of metabolite B in S-1 (developed four times) was indistinguishable from that of $17\alpha E_2$-17-glucopyranoside[6]. (R_f = 0.19, Table 2). An aliquot of metabolite B (20,000 d.p.m.) was reacted for 20 h with diazomethane and rechromatographed by t.l.c. in S-1. A single radioactive peak was observed on scanning the chromatogram with a mobility similar to that of authentic $17\alpha E_2$-17-glucopyranoside 3-methyl ether (R_f = 0.27). This methylated derivative was rechromatographed in S-2 where its R_f of 0.48 was also the same as that of the authentic 3-methyl ether of $17\alpha E_2$-17-glucopyranoside (Table 2).

In order to establish that the aglycone of B was indeed $17\alpha E_2$ instead of the administered $17\beta E_2$, an aliquot of B (15,000 d.p.m.) was hydrolyzed in 2 N HCl for 1 h at 100°C. After extraction with ethyl acetate, 88% (13,500 d.p.m.) was recovered; its mobility in S-1 (developed once) was similar to that of $17\alpha E_2$ (R_f = 0.56). Another aliquot of B (138,000 d.p.m.) was hydrolyzed with β-glucosidase with similar results. The aglycone obtained from enzymatic hydrolysis was divided into equal parts and each part recrystallized five times with either $17\alpha E_2$ or $17\beta E_2$. In the latter case the specific activity of the crystals continually decreased, whereas with $17\alpha E_2$ the specific activity was within the 5% acceptance range[7] in the second through fifth crystallizations.

In order to provide unequivocal evidence of structure, metabolite B itself was crystallized with authentic $17\alpha E_1$-17-glucopyranoside[6]. Recrystallization to constant specific activity was obtained using the criteria of Axelrod *et al.*[7]. Furthermore, metabolite B was acetylated to form the pentaacetate. An aliquot of B (20,000 d.p.m.) was dissolved in pyridine (0.2 ml) and reacted with acetic anhydride (0.2 ml) overnight at room temperature. The solution was then evaporated under nitrogen and purified by t.l.c. in the solvent system methylene chloride–ethanol (24:1, v/v). A single radioactive peak was observed with an R_f of 0.68. This material was eluted, and it crystallized to constant specific activity with authentic 3-acetoxyestra-1,3,5(10)-trien-17α-yl-2',3',4',6'-tetra-O-acetyl-β-D-glucopyranoside[6].

Characterization of polar metabolite C as the 3-β-D-glucosiduronate of estradiol-17α

From its extremely polar chromatographic behavior in S-1 (Table 2) metabolite C resembled a glucosiduronate rather than a glycoside. After reaction for 5 s with diazomethane its mobility in S-1 (developed four times) changed from an R_f of 0 to 0.45. This rapid reaction with diazomethane is characteristic of carboxylic acids such as $17\beta E_2$-3-β-D-glucosiduronate, whose methyl ester has an R_f of 0.40 in S-1 (developed four times). The chromatographic properties of the methyl ester of metabolite C were further compared to those of the 3- and 17-glucosiduronates of $17\beta E_2$ in S-2. The results are shown in Table 2, where it is seen that the methyl ester of metabolite C (R_f = 0.58) more closely resembled that of the 3-glucosiduronate (R_f = 0.58) than of the 17-glucosiduronate of $17\beta E_2$ (R_f = 0.65). This was further confirmed by t.l.c. using S-4 where the methyl esters of metabolite C (R_f = 0.72) and 17β-3-β-D-glucosiduronate are indistinguishable. A further reaction of the methyl ester of metabolite C with diazomethane for 20 h failed to alter its chromatographic behavior in any of the above three solvent systems. This is to be contrasted with the methyl ester of $17\beta E_2$-17-β-D-glucosiduronate which further reacts to form the 3-methyl ether under these conditions.

An aliquot of metabolite C (71,000 d.p.m.) was treated with β-glucuronidase for 5 days at 37°C. The ether extract was chromatographed in S-1 (developed once). The scan of the chromatogram showed that complete hydrolysis occurred, yielding a product with the mobility of $17\alpha E_2$ (R_f = 0.56). This aglycone of C was recrystallized with authentic $17\alpha E_2$ and constant specific activity was obtained from the first through the fifth crystallizations.

Characterization of polar metabolite A_2 as a 17-glycoside of 17α-estradiol

The chromatographic mobility of metabolite A_2 (R_f = 0.53 in S-1 developed four times) clearly distinguished it from the more polar 17-glucopyranoside or 17-galactopyranoside of $17\alpha E_2$ (Table 2). After reaction for 20 h with diazomethane the chromatographic mobility of metabolite A_2 increased to 0.58 in S-1 (Table 2). In the solvent system benzene–ethanol (80:20, v/v) this methylated derivative of metabolite A_2 had an R_f of 0.41, compared to the unreacted A_2 with an R_f of 0.30. This presumed 3-methyl ether of A_2 was mixed with unreacted A_2 and separated by t.l.c. in S-3. There were two distinct radioative peaks (Table 2), confirming that metabolite A_2 is a 17-glycoside rather than a 3-glycoside.

After enzymatic hydrolysis of metabolite A_2 with β-glucosidase, a single radioactive peak was obtained in S-1 (developed once) with a mobility corresponding to that of $17\alpha E_2$. Glucono-1,5-lactone caused a 35% inhibition of β-glucosidase hydrolysis, which is consistent with the conclusion that metabolite A_2 is a 17-glucoside[5].

The aglycone of A_2 obtained after β-glucosidase hydrolysis was recrystallized with authentic $17\alpha E_2$ to constant specific activity in the last three crystallizations.

Characterization of polar metabolite A_1 as a 17-glycoside of estradiol-17β

The chromatographic mobility of metabolite A_1 in S-1 (developed four times) clearly separated it from the more polar 17-glucopyranoside and 17-galactopyranoside of $17\alpha E_2$ (Table 2). An aliquot of A_1 was reacted for 20 h with diazomethane. This material was

added to an unreacted aliquot of A_1 and the mixture separated by t.l.c. in S-3. The methylated derivative of A_1 had an R_f of 0.38 compared to unreacted A_1 with an R_f of 0.25 (Table 2). This suggests that A_1 is also a 17-glycoside, forming a 3-methyl ether on reaction with diazomethane.

An aliquot of metabolite A_1 was incubated with β-glucosidase for 48 h at 37°C. The product was chromatographed in S-1 (developed once) which demonstrated that 45% of the sample had been hydrolyzed to material with a chromatographic mobility (R_f = 0.53) similar to that of 17βE_2 (R_f = 0.54), while 55% remained unchanged. Another aliquot of A_1 was hydrolyzed in 2 N HCl for 1 h at 100°C. The resulting aglycone was divided into equal parts and recrystallized with either 17αE_2 or 17βE_2. When crystallized with 17αE_2 the specific activity continually decreased, whereas with 17βE_2 it remained constant in the last three crystallizations, demonstrating that the aglycone of metabolite A_1 is 17βE_2.

DISCUSSION

The major polar metabolite (B), found in about 40% yield in bovine liver tissue obtained 3 h after the final *in vivo* administration of [4-^{14}C]-17βE_2 or its 3-benzoate has been conclusively identified as the 17-glucopyranoside of 17αE_2. This glycoside was readily hydrolyzed by β-glucosidase and the aglycone so obtained was shown to be 17αE_2. The identification was then established by crystallization to constant specific activity of the glycoside and its pentaacetate. This metabolite reacted slowly with diazomethane to form the 3-methyl ether, providing additional confirmation of its structure as a 17-glycoside.

The liver of one of the steers treated with [4-^{14}C]-17βE_2 contained about 9% of a metabolite (C) characterized as the 3-glucosiduronate of 17αE_2. This glucosiduronate reacted rapidly with diazomethane to form the expected methyl ester but did not react further to yield a 3-methyl ether. This metabolite was hydrolyzed with β-glucuronidase to yield an aglycone which was identified as 17αE_2.

Two minor polar metabolites (A_2 and A_1) isolated from the livers of the other three steers were characterized as 17-glycosides of 17αE_2 and 17βE_2. These 17-glycosides reacted slowly with diazomethane to form 3-methyl ethers, and were hydrolyzed by β-glucosidase to yield their identified aglycones. The nature of their glycosidic moiety was not established since these two metabolites were different in chromatographic mobility from any known glycosides of 17αE_2. It is possible that they are 17-xylosides[8].

None of the above metabolites were found in detectable amounts in the blood of these animals 3 h after administration of [4-^{14}C]-17βE_2 or its 3-benzoate[9].

In this and a previous study[1], the partition with ether of the partially defatted liver extracts was performed using an aqueous phase containing 0.6 M ammonium sulfate and an equal volume of ether, followed by three additional partitions of this aqueous phase with equal volumes of fresh ether. The combined ether phases were found to contain most of the polar metabolites, including the 17-glycosides and the 3-glucosiduronate of 17αE_2. When pure 17-glucopyranoside of 17αE_2 is partitioned with ether in this manner, 81% is found in the ether phase, whereas 94% of the 3-glucosiduronate of 17αE_2 is found in the aqueous phase. Therefore, this partition with ether is not satisfactory for the separation of 17-glycosides from unconjugated estrogens and does not partition glucosiduronates as expected from extracts of fatty tissues like liver. Direct chromatography of the partially defatted liver extracts on Sephadex LH-20 is recommended, taking care that the columns are not overloaded with tissue extract. Overloading of these columns with similar extracts from blood resulted in the apparent occurrence of nonpolar radioactive material[1]; this was eventually shown to be an artifact, probably due to alteration of the elution pattern of these columns by the nonradioactive lipids[9].

The fact that major amounts of the polar metabolites isolated from liver are conjugates of 17αE_2, rather than of the administered hormone 17βE_2, is consistent with the findings of Dunn *et al.*[1], who observed a 12:1 ratio of 17αE_2 : 17βE_2 in the unconjugated estrogen fraction from the liver tissue of animals similarly treated with [4-^{14}C]-17βE_2. It is known that E_1 is converted to 17αE_2 rather than 17βE_2 in bovine blood[10]. The predominance of 17αE_2 and its conjugates in steer liver is most probably due to an initial rapid oxidation of 17βE_2 to E_1, followed by 17α-hydroxysteroid oxidoreductase catalysis to 17αE_2 and subsequent formation of the 17-glycosides. This study provides the first identification of estrogen 17-glycosides in bovine liver tissue.

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