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Research Article

Abnormal estrogen metabolism has been found in cirrhosis after administration of intravenous tracers of estradiol³H to 6 patients and 23 healthy controls. The major abnormalities observed involved estrogen metabolites other than the 3 "classic" ones, i.e., estrone (E1), estradiol (E2), and estriol (E3). Urinary recovery of radioactivity was regularly elevated in the patients, to an average of 71% of the dose compared to 51% in normals. This is considered to reflect the component of intrahepatic cholestasis in cirrhosis. The per cent dose recovered as urinary glucosiduronates (42%) was normal in cirrhotics in contrast to impaired glucuronidation of cortisol metabolites in this disease. E1 and E2 were present in normal amounts, and E3 was slightly elevated to 21% of the extract compared to 14% in controls. There were strikingly decreased excretion of 2-hydroxyestrone (3% compared with normal 20%) and 2-methoxyestrone (2% compared with 5%) and increased excretion of 16 α -hydroxyestrone (12% compared with normal 6%). Thus cirrhosis, too, is characterized by the reciprocal relationship between decreased 2-hydroxylation and increased 16 α -hydroxylation previously described in hypothyroidism and male breast cancer. However, unlike these latter, the increase of 16 α -hydroxy metabolites was less than the decrease of 2-hydroxy metabolites. The data indicate clearcut impairment of 2-hydroxylation, suggestive impairment of 16 α -hydroxylation, and a definite depression of the reaction 16 α -hydroxyestrone $\rightarrow$ estriol, the latter finding so far unique to cirrhosis. [...]

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Estradiol Metabolism in Cirrhosis

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ABSTRACT Abnormal estrogen metabolism has been found in cirrhosis after administration of intravenous tracers of estradiol- ^3H to 6 patients and 23 healthy controls. The major abnormalities observed involved estrogen metabolites other than the 3 "classic" ones, i.e., estrone (E1), estradiol (E2), and estriol (E3). Urinary recovery of radioactivity was regularly elevated in the patients, to an average of 71% of the dose compared to 51% in normals. This is considered to reflect the component of intrahepatic cholestasis in cirrhosis. The per cent dose recovered as urinary glucosiduronates (42%) was normal in cirrhotics in contrast to impaired glucuronidation of cortisol metabolites in this disease. E1 and E2 were present in normal amounts, and E3 was slightly elevated to 21% of the extract compared to 14% in controls. There were strikingly decreased excretion of 2-hydroxyestrone (3% compared with normal 20%) and 2-methoxyestrone (2% compared with 5%) and increased excretion of 16 α -hydroxyestrone (12% compared with normal 6%). Thus

cirrhosis, too, is characterized by the reciprocal relationship between decreased 2-hydroxylation and increased 16 α -hydroxylation previously described in hypothyroidism and male breast cancer. However, unlike these latter, the increase of 16 α -hydroxy metabolites was less than the decrease of 2-hydroxy metabolites. The data indicate clear-cut impairment of 2-hydroxylation, suggestive impairment of 16 α -hydroxylation, and a definite depression of the reaction 16 α -hydroxyestrone $\rightarrow$ estriol, the latter finding so far unique to cirrhosis. Demonstration of abnormal peripheral metabolism of estrogen in cirrhosis provides a new approach to the origin of the hyperestrogenic syndrome in this disease.

INTRODUCTION

The central role of the liver in the transformation of steroids and the well-recognized occurrence of a hyperestrogenic syndrome in liver disease (2) have stimulated investigation of estrogen metabolism in liver disease. The current status has been summarized in a recent review by Schedl (3). Virtually all the available data have been obtained either by bioassay or by chemical assay methods limited to the 3 "classical" estrogen metabolites,¹ E1 (estrone), E2 (estradiol), and E3 (estriol).

A preliminary report of this work has been published in abstract form (1).

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¹ The following trivial names have been used for metabolites:

estradiol	(E2)	$\Delta^{1,3,5(10)}$ estratriene-3,17 β -diol
estrone	(E1)	3-hydroxy- $\Delta^{1,3,5(10)}$ estratrien-17-one
estriol	(E3)	$\Delta^{1,3,5(10)}$ estratriene-3,16 α ,17 β -triol
16-epiestriol	(EE3)	$\Delta^{1,3,5(10)}$ estratriene-3,16 β ,17 β -triol
16 α -hydroxyestrone	(16 α -OH-E1)	3,16 α -dihydroxy- $\Delta^{1,3,5(10)}$ estratrien-17-one
16-ketoestradiol	(16-O-E1)	3,17 β -dihydroxy- $\Delta^{1,3,5(10)}$ estratrien-16-one
2-hydroxyestrone	(2-OH-E1)	2,3-dihydroxy- $\Delta^{1,3,5(10)}$ estratrien-17-one
2-methoxyestrone	(2-MeO-E1)	2-methoxy-3-hydroxy- $\Delta^{1,3,5(10)}$ estratrien-17-one

The inadequacies of both methods have been well understood by workers in the field. In addition, studies utilizing chemical methods have been shown to be subject to two serious limitations. First, the three classical metabolites together have been shown to constitute about 40% of the urinary estrogens (4). Other identified metabolites, e.g. 2-hydroxyestrone, 2-methoxyestrone, 16-epiestriol, 16 α -hydroxyestrone and 16-ketoestradiol, make up most of the remainder. Studies that do not include these compounds are incomplete and may, on occasion, be misleading. It was found in the present study that an analysis restricted to the three classical metabolites would have yielded a quite erroneous picture of estrogen metabolism in cirrhosis. Second, studies of the "urinary production rate" of estradiol have shown discrepancies between values determined from different metabolites (5). This indicates that the pathways of formation of different estrogen metabolites may include varying proportions of multiple precursors, some of which may never have been effective estrogens.

It appears, therefore, that critical evaluation of the fate of biologically active estrogen is best accomplished by studies of estradiol biotransformation, with radioactive tracers. Very few studies of this type have been reported. A preliminary communication by Crowell, Eren, and Preedy (6) reported normal estradiol production in cirrhosis, but gave few details concerning the pathways of estrogen metabolism. A preliminary communication by Stöa and Thorsen (7) reported little deviation from normal in the estradiol metabolism of cirrhotic patients. In a somewhat more detailed preliminary communication, Shaver, Roginsky, and Christy (8) reported evidence that suggested an elevated blood estrogen level and delayed hepatic estrogen inactivation in cirrhosis.

In the present study the metabolism of tracer doses of radioactive estradiol was studied in six patients with cirrhosis and was found to be consistently abnormal, in total excretion, conjugation, and the relative quantities of metabolites. Decreased efficiency of certain pathways of peripheral estradiol metabolism was demonstrated, and there was increased formation of a normally minor metabolite, 16 α -hydroxyestrone. The existence of abnormal peripheral estrogen metabolism in cirrhosis provides a new approach to the understanding of the hyperestrogenic syndrome in this disease.

METHODS

Five men, aged 39–50, and one woman, aged 37, were studied. Each patient had typical clinical and laboratory findings of cirrhosis (Table I), as well as a long history of alcoholism. At the time of study, each patient was hospitalized at the Clinical Research Center and had had a period of rest, adequate diet, and enforced abstinence from alcohol.

Each subject studied received approximately 1 μ c of estradiol-6,7-³H by intravenous injection of a weighed amount of a solution of the steroid in freshly redistilled pyrogen-free propylene glycol. A weighed aliquot of propylene glycol solution from which the individual tracer doses were obtained was used for calculation of the administered dose of radioactivity. Complete urine collections as judged from the consistency of creatinine measurements were obtained for 3 days after each injection. The specimens were refrigerated immediately after voiding and at all times until the collection was complete. If not processed immediately, and this was done in most instances, the entire sample was frozen and kept at –15°C until it was possible to separate the steroid metabolites.

The radioactive steroids used were homogeneous by isotopic dilution analysis. The carrier steroids used were recrystallized to constant melting point before addition to the extract. Purity of carrier steroids was also checked by thin-layer chromatography and by infrared spectrometry. All solvents employed were redistilled and of high quality.

In each study the 3 day urine collections were combined and divided into 10, 50, and 40% aliquots, of which the last was frozen and retained. A known amount (approximately 30 mg) of carrier 2-hydroxyestrone was added to the 10% aliquot; no carrier was added to the 50% aliquot. Each was incubated with 300 U of β -glucuronidase² per ml of urine at pH 5 with acetate buffer at 38°C for 5 days. The urine at pH 5 was extracted continuously with ether for 48 hr. The ether extract was washed with 9% sodium bicarbonate solution saturated with sodium chloride, with saturated sodium chloride solution, and finally with a very small amount of water, and the solvent was removed. The residue is designated "glucosiduronate extract." It should be noted that sulfate conjugates are not hydrolyzed by this procedure although 2-deoxy-2-acetamido-D-glucosides (if present) may be; double conjugates with glucuronic and sulfuric acid would not be cleaved to lipid-soluble compounds. These, therefore, would be included as "nonglucosiduronate."

The extract from the 10% aliquot was used only for measurement of 2-hydroxyestrone and estradiol. A known amount of estradiol-17 β approximately equal to the amount of 2-hydroxyestrone was added. The mixture was acetylated by standing overnight in pyridine and acetic anhydride. Excess reagent was removed under a nitrogen stream on a water bath, and the acetates were chromatographed on 8 g of acid-washed alumina. Elution with 3:2

² β -Glucuronidase, known as Ketodase, was obtained from Warner-Chilcott Laboratories, a division of Warner-Lambert Pharmaceutical Company, New York.

TABLE I
Clinical Description of Cirrhotic Patients

Subject	Age	Sex	Degree of illness (0→5)*	Portacaval shunt	Ascites	Serum bilirubin (normal <0.8)	Glutamic-oxaloacetic transaminase (Karman-Ladue units) (normal <40)	Alkaline phosphatase (Bessey-Lowry units) (normal <3.0)	Total cholesterol	Per cent free cholesterol (normal <34)
						mg/100 ml			mg/100 ml	
BO	50	M	3	No	No	0.3	76	4.2	285	38
NS	42	M	3	No	No	1.0	30	4.6	169	39
IN	49	M	1	No	No	0.9	36	2.4	235	30
LY	37	F	3	No	No	0.3	20	4.2	191	34
HS	39	M	4	Yes	No	2.0	59	3.5	230	40
CY	41	M	3	No	Yes	2.0	69	3.9	217	40

* 1 = good health; 5 = critically ill.

(v/v) benzene-petroleum ether (boiling range 58°–62°C) gave estradiol diacetate, which was recrystallized to constant specific activity from petroleum ether-acetone. Elution with 1:9 (v/v) ethyl ether-benzene gave 2-hydroxyestrone diacetate, which was recrystallized to constant specific activity from petroleum ether-acetone. The total radioactivity of each compound was then calculated from the specific activity and the weight of carrier. The values are expressed as the free steroid.

One-half of the glucosiduronate extract from the 50% aliquot was submitted to gradient elution partition chromatography on acid-washed celite column as described by Engel et al. (9). This procedure yields well-defined and homogenous peaks for 2-methoxyestrone, estrone, 16-epiestriol, and estriol, and the values for these compounds were directly determined by combining the pertinent fractions and measurement of radioactivity. The peak containing 16 α -hydroxyestrone also contains 16-ketoestradiol.

TABLE II
Recovery of Radioactivity after i.v. Estradiol-³H

Subject	% Dose in urine			% Dose as glucosiduronate conjugate	
	72 hr	1st day	2nd + 3rd day		
BO	57	48	9	38	19
NS	67	54	13	45	22
IN	66	40	26	37	29
LY	58	44	14	30	28
HS	84	70	14	48	36
CY	89	77	12	52	37
Average of cirrhotic patients	71	56	15	42	28
Average of 11 normal men	51	28	23	40	11
Range	(41–77)	(17–53)	(8–31)	(32–51)	(1–28)
Average of 12 normal women	63	39	24	42	21
Range	(40–79)	(26–59)	(14–39)	(22–54)	(13–31)

Since these two metabolites are not separable by routine chromatographic procedures, the amount of 16 α -hydroxyestrone was determined by reverse isotope dilution. Aliquots of the radioactive peak were diluted with unlabeled carrier 16 α -hydroxyestrone and then acetylated overnight with acetic anhydride and pyridine. Purification was accomplished by preparative thin-layered chromatography on silica gel, employing successive development in the systems 30% ethyl acetate:cyclohexane and 50% ethyl acetate:cyclohexane. The separated 16 α -hydroxyestrone acetate was then crystallized to constant specific activity from acetone:petroleum ether.

In subject CY, carrier 2-hydroxyestrone was added to the urine which was processed in the usual manner. The "glucosiduronate extract" was separated by countercurrent distribution, as described previously (10). Estradiol and 2-hydroxyestrone were separated from the appropriate tubes after carrier addition by acetylation and chromatography, as above. The radioactivity present as estrone, estriol, and 2-methoxyestrone was calculated from the contents of the peak tubes. 16 α -Hydroxyestrone, 16-ketoestradiol, and 16-epiestriol are recovered together as one area in the countercurrent distribution. The amount of 16 α -hydroxyestrone present in this study of CY was determined by reverse isotope dilution, as above.

Radioactivity was measured in a Packard Liquid Scintillation Spectrometer with appropriate corrections for quenching. Specific activities were the mean of at least triplicate measurements and were accurate within the counting error of $\pm 5\%$.

RESULTS

Recovery of radioactivity (Table II). The 3 day total urinary excretion of radioactive metabolites was elevated to 71% of the dose compared with 51% in normal men and 63% in normal women. This increase was confined to the first 24 hr, 56% of the dose compared with 28% in normal men and 39% in normal women. (Women

TABLE III
Estradiol-³H Metabolites in the Glucosiduronate Extract

Subject	Per cent of extract							16 α -OH-E1	2-oxygenated metabolites
	E1	E2	E3	2-Meo-E1	2-OH-E1	16 α -OH-E1	EE3	+ E3	+ 16 α -hydroxy metabolites
BO	8	5	21	1	1	17	4	38	40
NS	15	6	24	3	4	10	5	34	41
IN	15	6	29	1	5	7	5	36	42
LY	5	5	19	1	1	15	2	34	36
HS	26	6	14	4	7	15	4	29	40
CY	23	9	20	1	2	7	*	27	30
Average of cirrhotic patients	15	6	21	2	3	12	4	33	38
Average of normal patients	20	9	14	5	20	6	5	20	45

* Not determined.

normally excrete a larger fraction of the radioactive estradiol metabolites into urine, both 1st day and 72 hr total, then do men (11).) Excretion on the 2nd + 3rd days was somewhat less than normal, 15% compared with 24% in normal subjects of either sex. The 72 hr recovery of radioactivity in the glucosiduronate extract was essentially identical in the normals and cirrhotic patients. Thus, by difference, the cirrhotics excreted an increased proportion of the dose as nonglucosiduronate conjugates. These latter were nearly twice as high in cirrhotics as in the combined normal group, and nearly three times as high as in normal men.

Individual metabolites (Table III). The sum of the seven analyzed metabolites was normal but the pattern was abnormal. 2-Hydroxyestrone and 2-methoxyestrone were formed in $\frac{1}{5}$ the normal amounts; estriol formation was 50% higher than normal, and 16 α -hydroxyestrone was about twice normal. The other three metabolites, estradiol, estrone, and 16-epiestriol, were present in normal amounts.

In subjects IN and NS the "nonglucosiduronate" conjugates after ketodase treatment were solvolyzed (12), and the metabolites were separated by the described procedures. It was found that 2-methoxyestrone and 16 α -hydroxyestrone represented almost exactly the same per cent of this extract as they did of the "glucosiduronate conjugates." Estriol represented a somewhat smaller per cent of the solvolyzed extract but the amount

present would not change the total recovery of this metabolite to any significant degree.

DISCUSSION

Estradiol metabolism of the cirrhotic patients was abnormal in several respects. An increased fraction of the estradiol metabolites was recovered in urine, as a result of greater 1st day excretion, with the 2nd + 3rd day significantly below normal. The normal course of estrogen metabolite disposition offers a possible explanation for these findings. Studies with i.v. estradiol tracers in patients with complete biliary fistulas (13) have shown quantitative recovery of radioactivity within 24 hr, about half in bile and half in urine. This has led to the conclusion that in intact subjects the 1st day's urinary excretion comprises chiefly metabolites that have not undergone biliary excretion, whereas subsequent collections contain metabolites that have undergone prior enterohepatic circulation. It would be anticipated that a decrease in hepatobiliary excretory capacity, a regular pathophysiological feature of cirrhosis corresponding to the anatomically demonstrable intrahepatic cholestasis (14), would cause increased 1st day urinary elimination of estrogen metabolites. This is analogous to the increase of urinary bilirubin when hepatobiliary excretory capacity is reduced in disease. In agreement with this explanation, the highest 1st day recovery of estrogen metabolites occurred in the two patients with the greatest elevation of serum bilirubin concentration. The decreased 2nd +

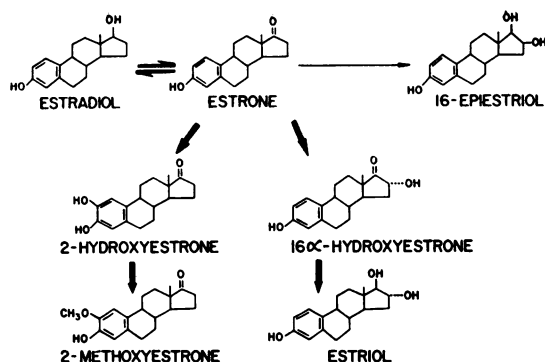


FIGURE 1 Normal pathways of estradiol metabolism in man.

3rd day recovery in the cirrhotic patients is a result of the large urinary loss on the 1st day, since less of the estrogen metabolites reach the intestine to be available for reabsorption.

In contrast to the elevated total urinary recovery of estrogen metabolites, the proportion excreted as glucosiduronate conjugates was normal. This differs from cortisol metabolism in cirrhosis, where decreased glucosiduronate conjugation of metabolites was observed (15). The glucosiduronate fraction showed a normal total amount of the seven analyzed metabolites, with an abnormal pattern. There was a decrease of the 2-oxygenated metabolites, 2-hydroxyestrone and 2-methoxyestrone, to $\frac{1}{5}$ of normal, with an increase of the 16 α -hydroxy metabolites. Estriol was 50% higher than normal, and 16 α -hydroxyestrone was present in amounts more than twice normal. These changes in estrogen metabolites do not appear to be a consequence of the altered conjugation mechanisms in cirrhosis since analysis of the "sulfate" fraction in two patients disclosed quantities of 2-methoxyestrone and 16 α -hydroxyestrone relatively similar to that present in the "glucosiduronate" extract.

In order to interpret these changes, it is helpful to consider the over-all pattern of estradiol metabolism (Fig. 1) (16). Estradiol forms a reversible redox system with estrone, and all the other products are derived from estrone. The latter is metabolized along two major pathways, the 2-oxygenation pathway (estrone $\rightarrow$ 2-hydroxyestrone $\rightarrow$ 2-methoxyestrone) and the 16 α -hydroxylation pathway (estrone $\rightarrow$ 16 α -hydroxyestrone $\rightarrow$ estriol). Formation of 16-epiestriol represents a separate minor pathway. The relative prominence of the two

major pathways varies in disease but the sum of the two appears to be constant in all diseases studied so far. Depression of one leads to equivalent accentuation of the other.

In cirrhosis, the decrease of 2-oxygenated metabolite formation was profound but there was only a small increase in estriol formation. In this sense the formation of estriol was relatively depressed, since the increase was not consistent with the decrease in 2-oxygenated metabolites. Even when the 16 α -hydroxyestrone excretion was considered, the increase in total 16 α -hydroxy metabolite formation was only about $\frac{2}{3}$ as great as the decrease in 2-oxygenated metabolite formation. This contrasts with hypothyroidism (17) and male breast cancer (10) where the increase in 16 α -hydroxy metabolites is equal to or slightly greater than the decrease in 2-oxygenated metabolites. These data suggest some impairment of the 16 α -hydroxylation reaction in cirrhosis. The ratio of 16 α -hydroxyestrone to estriol in the cirrhotic patients was approximately twice normal and approximately five times as high as in male breast cancer. These results indicate inhibition or partial loss of the dehydrogenase responsible for the reduction of 16 α -hydroxyestrone to estriol.

It is tempting to relate the observed abnormalities of peripheral estrogen metabolism to the genesis of the hyperestrogenic syndrome in cirrhosis. An increased hormonal effect is presumably due to elevated blood (and/or tissue) concentrations of active hormone. This in turn could be due to increased hormone production, decreased inactivation, or both. Studies of radioactive estradiol metabolism by Shaver, Roginsky, and Christy (8) led these authors to the conclusion that blood estrogen levels were indeed elevated in cirrhosis. They concluded also that the elevated blood levels occurred without elevated estrogen production, citing unpublished data of their own and a report by Crowell, Eren, and Preedy (6) which indicated normal estradiol production in cirrhosis, and they speculated that the initiating cause of the hyperestrogenic syndrome in cirrhosis is impaired hepatic removal of estrogen.

As Shaver et al. pointed out, their studies provided no details concerning the possible step(s) of hepatic estrogen metabolism which might be impaired. The present study elucidates but does

not solve this problem. One of the two major metabolic pathways that inactivate estrone, i.e. 2-oxygenation, is clearly impaired. The status of the other pathway, 16 α -hydroxylation, is uncertain. Of its two sequential steps, the second is definitely impaired. The evidence concerning inhibition of the first step is suggestive but not conclusive.

The role of the increased amounts of 16 α -hydroxyestrone requires evaluation. If this metabolite were estrogenic, an explanation for the hyperestrogenic syndrome would be at hand. From animal assays (18) the estrogenicity of 16 α -hydroxyestrone, like that of estriol, is weak. This conclusion is not necessarily transferrable to man. Direct study of the human biological effects of 16 α -hydroxyestrone, its estrogenicity as well as the possibility that it may inhibit 2-hydroxylation and/or 16 α -hydroxylation of estrone, seems indicated.

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