PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 48–52 (1974)

Liver Microsomal Hydroxylation of Steroid Hormones After Establishing an Indigenous Microflora in Germfree Rats (37745)

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In a recent investigation from our laboratories it was demonstrated that germfree rats are characterized by a significantly more efficient liver microsomal hydroxylation of steroid hormones and certain sterols than the conventional counterparts (1). The hypothesis was formulated that this difference could be secondary to the differences in bile acid metabolism in germfree as compared to conventional rats and explained by induction of the liver microsomal hydroxylase systems in germfree rats by some specific bile acid, possibly chenodeoxycholic acid. Because of the important biological implications of a relationship between the intestinal microflora and the liver microsomal hydroxylating capacity it was considered of interest to investigate further the principles characterizing this relationship. As a first step of this analysis we studied the effects of conventionalization of germfree rats upon their hepatic steroid hormone-metabolizing capacity.

Materials and Methods. Steroids. The sources of unlabeled steroid substrates and steroid reference compounds have been given in a previous paper (1). 4-[4-^{14}C]Androstene-3,17-dione (sp radioact, 1.2 μCi/mg) was obtained from the Radiochemical Centre (Amersham, England). 5α-[4-^{14}C]Androstane-3α,17β-diol was prepared from 4-[4-^{14}C]androstene-3,17-dione (sp radioact, 210 μCi/mg) as described before (2). The labeled steroids were assayed for purity by radio-gas chromatography prior to use and were found to be more than 98% pure. Prior to incubation the synthesized 5α-[4-^{14}C]androstane-3α,17β-diol was diluted with unlabeled 5α-androstane-3α,17β-diol (the purity of which was assayed by gas–liquid chromatography) to yield a specific activity of 3.0 μCi/mg.

Animals and preparation of homogenates. Germfree animals of the Long–Evans strain were reared according to the technique of Gustafsson (3, 4) and fed a standard diet *ad libitum* (4). This diet is semisynthetic with 10% arachis oil as source of fat. Control animals of the same strain were reared outside the germfree isolators on the same sterilized diet. Male rats weighing about 300 g (age: 3–5 mo) were used. Five germfree rats were removed from the isolators and were given a rectal infusion of a suspension of feces from conventional rats to secure a quick and complete establishment of an intestinal microflora. The exgermfree rats were then reared outside the germfree isolators in the same way as conventional rats. Twenty-nine days after conventionalization five germfree rats were taken out of their isolator and killed immediately by a blow on the head. The five exgermfree rats and five conventional rats were also killed in the same way. The livers were excised immediately and chilled on ice. Liver homogenates, 20% (w/v), were prepared in a modified Bucher medium (5) (pH 7.4), with a Potter-Elvehjem homogenizer equipped with a loosely fitting Teflon pestle. The homogenate was centrifuged at 20,000g for 15 min. The microsomal fraction was obtained by centrifuging the 20,000g supernatant fluid at 105,000g for 70 min. The microsomal fraction was suspended in the homogenizing medium in a volume corresponding to that of the 20,000g supernatant fluid, from which it had been isolated, and

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was homogenized with a loosely fitting pestle. The protein concentration of the 20,000g supernatant fluid and of the microsomal fraction was determined according to Lowry *et al.* (6).

Incubations with 4-[4-^{14}C]androstene-3,17-dione and 5α-[4-^{14}C]androstane-3α,17β-diol. Incubations of the labeled steroid substrates were carried out with the microsomal fraction with the addition of NADPH or NADP and an NADPH-regenerating system as described before (1). The conditions during which the incubations were performed had been tested and, using the conditions described, the obtained conversions were linear with respect to time and enzyme concentration. The incubations were terminated, the incubation mixtures were extracted, and the extracts were applied on precoated silica gel plates and chromatographed as described previously (1). The chromatoplates were subjected to autoradiography with an exposure time of 10 days. The radioactive zones on the thin-layer chromatographic plates were determined exactly from the X-ray film and were scraped off, eluted with methanol and measured for radioactivity in a Packard liquid scintillation spectrometer, Model 3003. Student's t test was used for the statistical analysis and the significance level was set at 0.05.

Gas chromatography–mass spectrometry analysis. The methanol extracts left after radioactivity measurements were (trimethyl) silylated and analyzed by gas chromatography–mass spectrometry (LKB 9000 instrument) using a 1.5% SE-30 column. Mass spectra were recorded on magnetic tape using the incremental mode of operation and were then treated in an IBM 1800 computer (7). A compound was considered identified only if it had the same mass spectrum and gas–liquid chromatographic behavior as the reference compound.

Results. Measurements of microsomal protein. The protein concentrations of the microsomal suspensions were found to be the following: germfree rats: 5.0 ± 0.5 mg protein/ml microsomal suspension, conventional rats: 4.1 ± 0.7 mg/ml; and exgermfree rats: 3.8 ± 0.4 mg/ml. No significant difference was obtained between germfree and conventional rats ($P < 0.10$) but exgermfree rats showed significantly lower protein concentration than germfree rats ($P < 0.005$).

Incubations of 4-[4-^{14}C]androstene-3,17-dione with the microsomal fraction fortified with NADPH. After incubation of 4-[4-^{14}C] androstene-3,17-dione with the microsomal fraction of livers from germfree, exgermfree and conventional rats there were total conversions of the substrate of 10–20%. The following compounds chromatographed in distinct zones on the thin-layer plates and could be quantitated separately: (the compounds are listed in order of decreasing polarity) 7α-hydroxy-4-androstene-3,17-dione (8); 6β-hydroxy-4-androstene-3,17-dione (8); 16β-hydroxy-4-androstene-3,17-dione (tentative identification); 16α-hydroxy-4-androstene-3,17-dione (8); 17β-hydroxy-4-androsten-3-one; 3α- and 3β-hydroxy-5α-androstan-17-one (not separable in the solvent system used) (9); 3β-hydroxy-4-androsten-17-one (10); 4-androstene-3,17-dione (= the substrate) (1); and 5α-androstane-3,17-dione (1). The silyl ether of the metabolite tentatively identified as 16β-hydroxy-4-androstene-3,17-dione gave a molecular ion at m/e 374 and a base peak at m/e 117; the mass spectrum resembled closely that of 16α-hydroxy-4-androstene-3,17-dione silyl ether (8). The ratio between the relative retention times (t_R:s) on SE-30 of the silyl ethers of the unknown steroid ($t_R = 0.97$) and of 16α-hydroxy-4-androstene-3,17-dione ($t_R = 0.88$) was 1.10 which is similar to the ratio between the t_R values of 3β,16β- and 3β,16α-dihydroxy-5-androsten-17-one silyl ethers (1, 13).

Table I summarizes the quantitative data on microsomal hydroxylation of 4-androstene-3,17-dione. It can be seen that microsomal preparations from germfree rats were significantly more active in hydroxylating 4-androstene-3,17-dione in position 6β than corresponding preparations from conventional rats ($P < 0.02$). Conventionalization of germfree rats led to decreased activity of the 6β-hydroxylase system. Also 7α-, 16β- and 16α-hydroxylation of 4-androstene-3,17-dione tended to be more efficient in germfree than

TABLE I. Hydroxylation of 4-[4-^{14}C]Androstene-3,17-dione in Liver Microsomes from Germfree, Conventional and Exgermfree Rats.[a]

Compound	Metabolite formed (nmoles/mg protein • 10 min)		
	GF	C	Ex-GF
7α-Hydroxy-4-androstene-3,17-dione	3.25 ± 0.83	3.15 ± 0.57	3.10 ± 0.72
6β-Hydroxy-4-androstene-3,17-dione	14.42 ± 1.82	11.36 ± 1.44[b]	12.82 ± 1.75
16β-Hydroxy-4-androstene-3,17-dione	2.92 ± 0.36	2.47 ± 0.59	2.34 ± 0.26[b]
16α-Hydroxy-4-androstene-3,17-dione	41.71 ± 4.63	37.25 ± 4.51	39.81 ± 6.63

[a] The conversions are calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms. The values listed are the means ± SD of experiments with five male rats. Abbrev.: GF = germfree rat; C = conventional rat; Ex-GF = exgermfree rat.
[b] $P < 0.02$ compared to GF.

in conventional rats but these differences were not significant statistically. The activities of the same hydroxylase systems tended to decrease upon conventionalization and in the case of 16β-hydroxylation the activity found in exgermfree rats was significantly lower than that found in germfree rats ($P < 0.02$).

Incubation of 5α-[4-^{14}C]androstane-3α,17β-diol with the microsomal fraction fortified with NADPH. After incubation of 5α-[4-^{14}C] androstane-3α,17β-diol with the hepatic microsomal fraction, germfree, exgermfree and conventional male rats hydroxylated 22–43% of the substrate. The following compounds chromatographed in distinct zones on the thin-layer plates: 5α-androstane-3α,7α,17β-triol (11); 5α-androstane-3α,7β,17β-triol (2); 5α-androstane-2β,3α,17β-triol (12); 5α-androstane-2α,3α,17β-triol (2); 7α,17β-dihydroxy-5α-androstan-3-one (13); 5α-androstane-3β,17β,18-triol (14); 5α-androstane-3α,17β,18-triol (14); 5α-androstane-3α,17β-diol (substrate) and 5α-androstane-3β,17β-diol (not separable in the solvent system used) (9); 3α-hydroxy-5α-androstan-17-one and 17β-hydroxy-5α-androstan-3-one (not separable in the solvent system used) (9); and 5α-androstane-3,17-dione (1).

As can be seen from Table II, 2β-, 7α- and 18-hydroxylation of 5α-androstane-3α, 17β-diol were about 30–40% more efficient in germfree as compared to conventional rats. These differences were statistically significant ($P < 0.02$–0.05). After conventionalization of the germfree rats the activities of their 2β- and 7α-hydroxylase systems diminished to the level characteristics of conventional rats. Also the activity of the 18-hydroxylase system of the conventionalized rats tended to ap-

TABLE II. Hydroxylation of 5α-[4-^{14}C]Androstane-3α,17β-diol in Liver Microsomes from Germfree, Conventional and Exgermfree Rats.[a]

Compound	Metabolite formed (nmoles/mg protein • 10 min)		
	GF	C	Ex-GF
5α-Androstane-3α,7α,17β-triol	3.63 ± 0.40	2.74 ± 0.47[b]	2.86 ± 0.24[c]
5α-Androstane-3α,7β,17β-triol	1.05 ± 0.05	0.99 ± 0.10	0.91 ± 0.17
5α-Androstane-2β,3α,17β-triol	2.75 ± 0.58	2.07 ± 0.21[c]	1.95 ± 0.21[b]
5α-Androstane-2α,3α,17β-triol	18.48 ± 1.73	17.03 ± 2.56	17.67 ± 3.36
5α-Androstane-3β,17β,18-triol + 5α-androstane-3α,17β,18-triol	2.99 ± 0.42	2.14 ± 0.40[b]	2.45 ± 0.47

[a] The conversions are calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms. The values listed are the means ± SD of experiments with five male rats. Abbrev.: GF = germfree rat; C = conventional rat; Ex-GF = exgermfree rat.
[b] $P < 0.02$; [c] $P < 0.05$ compared to GF.

proach a value similar to that found for conventional rats but no complete "conventionalization" was observed for this enzyme activity. No significant differences were found between germfree, conventional and exgermfree rats with respect to the activities of the 7β- and the 2α-hydroxylase system.

Discussion. The turnover of bile acids has been found to be influenced by the intestinal microflora as there is a higher excretion of fecal bile acids in conventional than in germfree rats (15, 16). This difference has mainly been ascribed to the much longer transit time of intestinal contents in germfree as compared to conventional rats (16). Another consequence of the absence of intestinal microorganisms is a considerably larger pool of cholic acid and its metabolites in germfree than in conventional rats (15, 17). In view of the findings of Shefer *et al.* (18) that the *de novo* bile acid synthesis decreased in intact rats after infusion of cholic acid concomitantly with an increase in the relative amount of β-muricholic acid (18), one would expect that the larger pool size of cholic acid in germfree rats should influence their qualitative pattern of bile acids. Indeed, β-muricholic acid has been reported to constitute a much larger fraction of the fecal bile acids in germfree than in conventional rats (19). Our recent finding that liver microsomal 6β-hydroxylation of lithocholic acid is about two times more efficient in germfree than in conventional rats (1) is in agreement with this finding. Furthermore, we found that the liver microsomal 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one was significantly lower in germfree animals than in their conventional counterparts (1), possibly as a result of the greater recirculation of bile acids reported in germfree rats (19). It may be that the relative increase in chenodeoxycholic acid versus cholic acid production that should result from the less efficient 12α-hydroxylation is the direct cause of the "induced" character of the liver microsomal hydroxylase systems of germfree rats. Numerous reports exist on steroids capable of stimulating their own metabolism by increasing liver microsomal hydroxylation (20–22) and in the same way chenodeoxycholic acid could induce the liver microsomal 6β-hydroxylase enzyme system resulting in more extensive formation of β-muricholic acid. Since the 6β-hydroxylase system active on chenodeoxycholic and lithocholic acid has been described as a less specific enzyme system related to the so called common drug- and steroid hormone-metabolizing enzyme system (23), the differences in microsomal hydroxylation of 4-androstene-3,17-dione, 4-pregnene-3,20-dione and 5α-androstane-3α,17β-diol between germfree and conventional rats could also be explained by induction by chenodeoxycholic acid.

The present investigation has demonstrated that conventionalization of germfree rats results in an almost complete change back to normal liver microsomal enzyme activities with respect to hydroxylation of 4-androstene-3,17-dione and 5α-androstane-3α,17β-diol. Furthermore, the microsomal protein concentration, which tended to be higher in germfree rats than in conventional rats as another indication of induction of microsomal enzymes in the former group of animals, also decreased to a normal value upon conventionalization of germfree rats. These results show that the induced state of certain microsomal hydroxylase systems in germfree rats is reversible and dependent on the metabolic changes in the germfree rat consecutive to the absence of the normal indigenous microflora.

Summary. The liver microsomal metabolism of 4-[4-^{14}C]androstene-3,17-dione and 5α-[4-^{14}C]androstane-3α,17β-diol was studied in germfree, conventional and exgermfree rats with a normal indigenous microflora. Germfree rats were significantly more active than conventional rats in hydroxylating 4-androstene-3,17-dione in position 6β ($P < 0.02$) and 5α-androstane-3α,17β-diol in positions 2β ($P < 0.05$), 7α($P < 0.02$) and 18 ($P < 0.02$). In addition 7α-, 16α- and 16β-hydroxylation of 4-androstene-3,17-dione and 2α- and 7β-hydroxylation of 5α-androstane-3α,17β-diol tended to be greater in germfree rats than in conventional rats. In most cases the exgermfree rats, which had been reared outside the germfree isolators for 4 wk, showed similar activities of the microsomal

hydroxylase systems as their conventional counterparts. Thus, exgermfree rats showed significantly less efficient 16β-hydroxylation of 4-androstene-3,17-dione ($P < 0.02$) and 2β- ($P < 0.02$) and 7α- ($P < 0.05$) hydroxylation of 5α-androstane-3α,17β-diol than germfree rats. The results show that the induced state of certain microsomal hydroxylase systems in germfree rats is reversible and dependent on the metabolic changes in the germfree rat consecutive to the absence of the normal indigenous microflora.

This work was supported by grants from Ekhagastiftelsen and from the Swedish Medical Research Council (project 16X-206).

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Received July 30, 1973. P.S.E.B.M., 1974, Vol. 145.