J. Biochem. 124, 648-653 (1998)

Sexual Differences in Branched Chain Amino Acid Metabolism into Fatty Acids and Cholesterol in Harderian Gland of Golden Hamster[1]

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Received for publication, May 11, 1998

The Harderian gland of golden hamster (*Mesocricetus auratus*) secretes copious lipids, most of which is 1-alkyl-2,3-diacylglycerol (ADG). We previously reported that the composition of ADG shows marked sexual dimorphism [Seyama *et al.* (1995) *J. Biochem.* 117, 661-670]. Male ADG contains only straight chain alkyl and acyl groups, but female ADG contains a lot of branched chain ones too. In this study, we investigated the metabolism of branched chain amino acids (BCAAs) and analyzed the incorporation of the metabolites into lipids in the Harderian gland. Golden hamsters were injected intraperitoneally with [*U*-^{14}C]BCAAs, and Harderian glands were obtained at 3, 6, 9, and 24 h after injection. Lipids were then extracted from the glands and analyzed. Thin layer chromatography revealed that the ADG was labeled in both sexes, but the profile depended on the sex. The cholesterol fraction was labeled only in the male gland. The alkyl and acyl groups of ADG were subjected to radio-gas liquid chromatography. As for the alkyl groups, radioactivity was detected in straight-C16 and -C18 chains in males, while branched-C17 and -C19 chains were labeled in females. As for the acyl groups, straight-C14, -C15, and -C16 chains were labeled in males, while in females, branched-C17 and -C19 chains were labeled as well as a straight-C16 chain. These results suggest that the BCAA metabolism should be regulated as to the sex at the step of branched chain acyl-CoA degradation in the Harderian gland of golden hamster, which causes the sexual dimorphism in the lipid composition in this gland.

Key words: acyl-CoA dehydrogenase, androgen, branched chain amino acid, fatty acid synthesis, golden hamster.

The Harderian gland is an exocrine gland located in the orbit. This gland is prominent in rodents and secretes a large amount of lipids (*1*). The Harderian gland of golden hamster shows marked sexual differences in cell types (*2, 3*), protein composition (*4, 5*), porphyrin accumulation (*6, 7*), and also lipid composition (*8, 9*). We reported that the major exocrinal lipid of the gland was mostly 1-alkyl-2, 3-diacylglycerol (ADG) in golden hamster, and also showed that the composition of alkyl and acyl groups of ADG differed with the gender (*9*). Namely, male ADG is composed of straight chain alkyl and acyl groups, whereas female ADG is also composed of *iso*- and *anteiso*-branched chain ones. We also found that this difference was inversely regulated by the serum testosterone level (*10, 11*).

Since the alkyl and acyl groups of ADG are produced *via* fatty acids, we assumed that the production of fatty acids should be regulated by androgen in this gland, and branched chain fatty acids might be produced when the serum androgen level was low. As *iso*- or *anteiso*-branched chain fatty acids were known to be produced with branched chain acyl-CoAs as primers, we supposed that the androgenic regulation occurred at the step of branched chain amino acid (BCAA) degradation.

In this study, we investigated the incorporation profile of BCAA metabolites, which are the source of branched chain acyl-CoAs in the Harderian gland of golden hamster, and deduced the enzymes which cause the sexual differences in the lipid composition in this gland.

MATERIALS AND METHODS

Animals—Golden hamsters and Chinese hamsters were obtained from Saitama Experimental Animals Supply (Saitama), housed in a vivarium, and allowed access to food and water *ad libitum*. They were kept under a controlled temperature (20±2°C), with a photo period of 12 h light/day (lights on at 8:00 a.m.).

Time-Dependent Incorporation of the Isotope Label into Various Tissues of Golden Hamsters—Golden hamsters

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and a grant from the Asahi Glass Research Foundation.

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Abbreviations: ADG, 1-alkyl-2,3-diacylglycerol; BCAA, branched chain amino acid; BCFA, branched chain fatty acid; CoA, coenzyme A; D-PBS, Dulbecco's phosphate-buffered saline; FID, flame ionization detector; GLC, gas-liquid chromatography; IVD, isovaleryl-CoA dehydrogenase; SBCAD, short/branched chain acyl-CoA dehydrogenase; TLC, thin layer chromatography.

(10 weeks old) were injected intraperitoneally with 2.5 μCi of [*U*-^{14}C]leucine, [*U*-^{14}C]valine, or [*U*-^{14}C]isoleucine (Du Pont, Boston, MA, USA) diluted with Dulbecco's phosphate-buffered saline (D-PBS). Three, 6, 9, and 24 h after injection, Harderian gland, liver, heart, and brain tissues were obtained under anesthesia with sodium pentobarbital. They were kept overnight in 5 ml of chloroform/methanol (2:1, v/v) at room temperature and then lipids were extracted in the liquid phase. Five hundred microliters of this fraction was evaporated under nitrogen gas, and then dissolved in 2 ml of Clear-sol I (Nacalai Tesque, Kyoto). Radioactivity in the solution was measured with a liquid scintillation spectrometer (Tri-Carb 1500; Packard Instruments, Downers Grove, IL, USA).

TLC Analysis of Lipids in Harderian Glands of Golden Hamsters and Chinese Hamsters—Golden hamsters (10 weeks old) were injected with 2.5 μCi of [*U*-^{14}C]BCAAs as described above. Chinese hamsters (10 weeks old) were injected with 1.0 μCi of labeled BCAAs instead. One, 3, and 6 h after injection, Harderian glands were obtained and lipids were extracted as described above. The lipids were then dissolved in 5 ml of chloroform/methanol (2:1), and 50 μl of the resulting solution was applied to a TLC plate (Silica Gel 60; Merck, Darmstadt, Germany). The plates were developed with a solvent system of hexane/diethylether/acetic acid (80:20:1, v/v/v). Autoradiograms of the plates were obtained and measured using a Fujix BAS 2000 system (Fuji Photo Film, Tokyo). The same plates were subsequently sprayed with 20% H_2SO_4 and the spots of total lipids were located by heating at 150°C.

Radio-GLC Analysis of Lipids in Harderian Glands of Golden Hamsters and Chinese Hamsters—Golden hamsters (10 weeks old) and Chinese hamsters (10 weeks old) were injected intraperitoneally with 25 and 10 μCi of [*U*-^{14}C]leucine, respectively. Six hours after injection, when the incorporation of the radioactivity was maximum, the glands were obtained and lipids were extracted as described above. ADG was fractionated from the whole lipids and derivatized as described by Seyama *et al.* (*11*). Briefly, the lipids were applied on a Silica SEP-PAC column (Millipore, Milford, MA, USA), and then the ADG fraction was eluted with hexane/benzene (4:6, v/v). The acyl groups of ADG were then extracted as fatty acid methyl esters by standard techniques. On the other hand, the alkyl groups of ADG were obtained as isopropylidene derivatives of alkylglycerols.

The fatty acid methyl esters and the isopropylidene derivatives of alkylglycerols prepared from ADG were dissolved in hexane and chloroform, respectively. They were subsequently analyzed using a Gas Chromatograph GC-6AM (Shimadzu, Kyoto) equipped with a coated column (3.2 mm × 2.1 m, 2% OV-1; Shinwa Chemical Industries, Kyoto), with nitrogen as a carrier gas. The oven temperatures for the analysis of the fatty acid methyl esters and isopropylidene derivatives of alkylglycerols were 230 and 260°C, respectively, and the temperature of the injector and the detector was 300°C. The column effluent was passed through an Aloka peak analyzer radiogas chromatograph system (Aloka, Tokyo) using methane gas to give radioactivity traces.

RESULTS

Time Dependency of Isotope Incorporation into Lipids in Various Tissues—The radioactivity incorporated into lipids was monitored after the injection of [*U*-^{14}C]leucine into the peritoneum of golden hamsters (Fig. 1). The leucine metabolites were efficiently incorporated into the lipids in the Harderian gland in both sexes as compared with those in other tissues such as liver, heart, and brain. The level of tracer incorporation reached the maximum after 6 to 9 h and then decreased gradually. Similar results were obtained when [*U*-^{14}C]isoleucine or [*U*-^{14}C]valine was injected (data not shown).

TLC Analysis of Lipids in Harderian Glands of Golden Hamsters—The incorporation of tracers into lipids was then investigated. Lipid extracts were obtained from glands at 1, 3, and 6 h after injection, developed on TLC plates, and then analyzed by autoradiography. Figure 2 shows a representative autoradiogram when [*U*-^{14}C]leu-

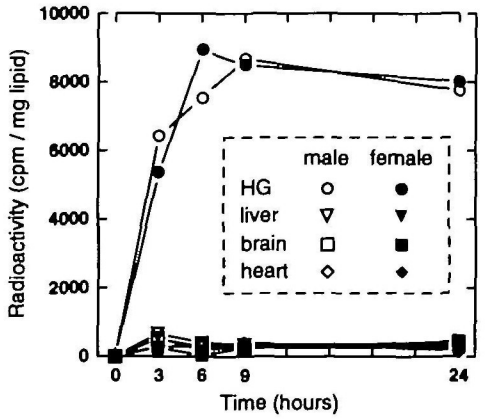

Fig. 1. **The incorporation of radioactivity into lipids in tissues of golden hamsters after [*U*-^{14}C]leucine injection.** Golden hamsters were injected with 2.5 μCi of [*U*-^{14}C]leucine as indicated under "MATERIALS AND METHODS." The incorporation of radioactivity into lipids from the gland was determined with a liquid scintillation spectrometer (Tri-Carb 1500; Packard).

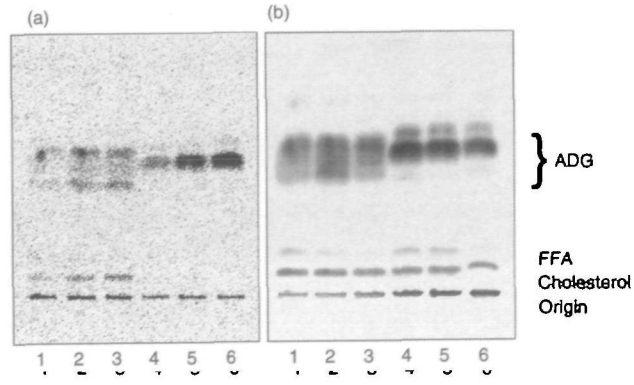

Fig. 2. **TLC analysis of lipids in the Harderian glands of golden hamsters.** Golden hamsters were injected with 2.5 μCi of [*U*-^{14}C]leucine into the peritoneum. Lipids in the gland were obtained and developed on a TLC plate. (a) A representative autoradiogram of a plate. (b) H_2SO_4 detection of lipids on the same plate in panel (a). Lipids obtained at 1 h after injection were spotted on lanes 1 and 4, at 3 h on lanes 2 and 5, and at 6 h on lanes 3 and 6. Lanes 1 to 3 contained male lipids, while lanes 4 to 6 contained female lipids. "ADG" and "FFA" indicate the positions of 1-alkyl-2,3-diacylglycerol and free fatty acids, respectively.

cine was injected. The most strongly labeled fraction was ADG both in males and females, but the radioactivity incorporation profiles differed with the gender, like those observed of the total ADG detected by H_2SO_4. The cholesterol fraction was also labeled in males, but not in females. The incorporation of the isotope into the ADG fraction reached the maximal level at 6 h after injection.

When [U-^{14}C]isoleucine or [U-^{14}C]valine was injected instead of leucine, the radioactivity in the lipid fraction reached the maximum level at 6 h after injection (data not shown). The TLC results were quite the same as those of leucine injection. Namely, sexual differences in the ADG profile were observed both on autoradiography and H_2SO_4 detection, and male ADG gave 3 spots, while female ADG gave 2 spots. Sexual dimorphism was also observed in the cholesterol profile and radioactivity was detected only in males (Fig. 3).

For comparison with the incorporation of BCAA into lipids in the glands of other rodents, we injected [U-^{14}C]-leucine into the peritoneum of Chinese hamsters, and the Harderian gland was obtained after 6 h. Lipids were subsequently extracted from the gland, developed on a TLC plate, and analyzed by autoradiography. The most strongly labeled lipid fraction in the gland was again ADG (lanes 1 and 2 in Fig. 4a), and the cholesterol fraction and the substance at the origin were also labeled in Chinese hamsters of both sexes. ADG of Chinese hamster Harderian gland seemed to give three spots, as observed in lanes 1 and 2 in Fig. 4b, but the substance obtained from the lowest spot (indicated by an arrow) was not ADG according to its IR spectrum (data not shown). Consequently, the incorporation of radioactivity in ADG did not differ with the gender in the Chinese hamster Harderian gland.

Specificity of the Incorporation of the Isotope Labels into the Aliphatic Chains of ADG—We further investigated the specificity of the incorporation of BCAA metabolites into ADG in the gland. Hamsters were injected with [U-^{14}C]-leucine and thereafter ADG in the gland was eluted. The derivatives of the alkyl and acyl groups of ADG were obtained and analyzed by radio-GLC. As for the alkyl groups, straight-C16 and -C18 chains were labeled in males (Fig. 5a), and branched-C17 and -C19 chains were labeled in females (Fig. 5b). While as for the acyl groups, straight-C14, -C15, and -C16 chains were labeled in males (Fig. 6a), and branched-C17 and -C19 chains were labeled in females (Fig. 6b). Additionally, a straight-C16 chain was also labeled in female glands but the radioactivity was quite low in spite of the large amount detected in the total fraction on GLC. These data clearly show that the BCAA metabolites were efficiently incorporated into the branched aliphatic chains in female ADG.

The same analysis was performed on ADG in the Harderian gland of Chinese hamster, which showed no sexual difference in its TLC profile. The alkyl groups of ADG in male (Fig. 7a) and female (Fig. 7b) glands contained both straight chains (C14, C15, C16, C17, and C18) and branched chains (C15 and C17), and showed quite similar profiles. Among these chains, branched-C15 chains were strongly labeled. A straight-C16 chain was also labeled, but the efficiency of the incorporation was quite low. As for the acyl groups of ADG, straight-C13 and branched-C13 chains were also present in both sexes, and radioactivity was additionally detected in a branched-C13 chain (Fig. 8). These results showed that a sexual difference was not detected in the alkyl nor acyl groups of ADG in Chinese hamster. Therefore, tissue- and species-specific regulation of the catabolism of BCAA into fatty acids was observed only in the Harderian gland of golden hamster.

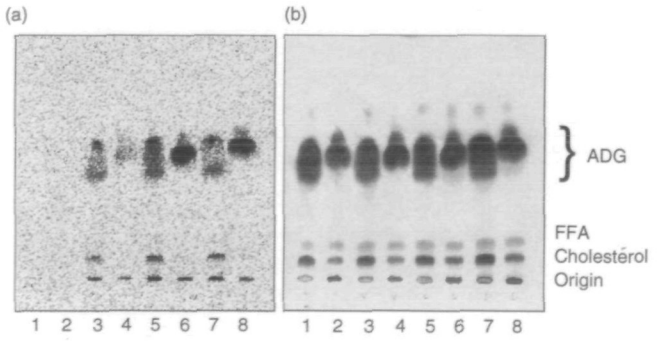

Fig. 3. **TLC analysis of lipids in the Harderian glands of golden hamsters after injection of [U-^{14}C]BCAA.** Golden hamsters were injected with 2.5 μCi of [U-^{14}C]BCAA or D-PBS into the peritoneum. Six hours after injection, glands were obtained and lipids were extracted. They were then analyzed as described in Fig. 2. (a) A typical autoradiogram of a plate. (b) The profile of lipids in panel (a) detected with H_2SO_4. Male lipids were spotted on lanes 1, 3, 5, and 7, while female ones were spotted on lanes 2, 4, 6, and 8. Samples obtained from hamsters injected with D-PBS were spotted on lanes 1 and 2; [U-^{14}C]leucine on lanes 3 and 4; [U-^{14}C]valine on lanes 5 and 6; and [U-^{14}C]isoleucine on lanes 7 and 8.

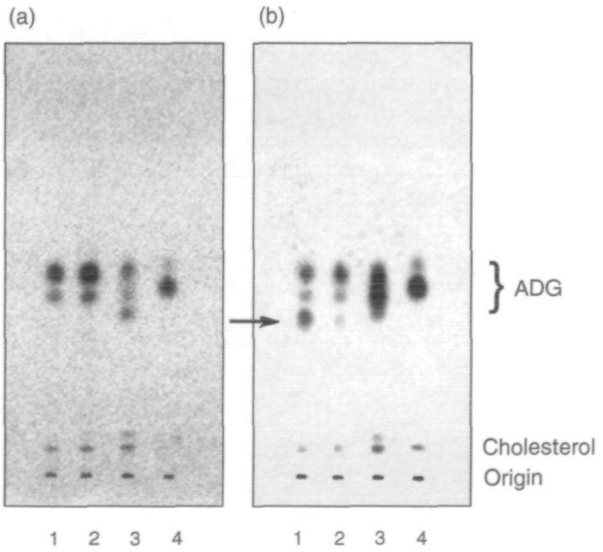

Fig. 4. **TLC analysis of lipids extracted from the Harderian glands of Chinese hamsters after injection of [U-^{14}C]BCAA.** Chinese hamsters were injected with 10 μCi of [U-^{14}C]BCAA into the peritoneum. Six hours after injection, lipids in the glands were obtained and analyzed as described in Fig. 2. (a) A representative autoradiogram of a plate. (b) The lipid profile of the plate in panel (a). Lane 1 contained the lipids from male glands and lane 2 contained those obtained from female glands. Also analyzed on the same plate were the lipids from the male (lane 3) and female (lane 4) golden hamster glands which were prepared and used in Fig. 3. The arrow indicates a substance other than ADG, according to its IR spectrum.

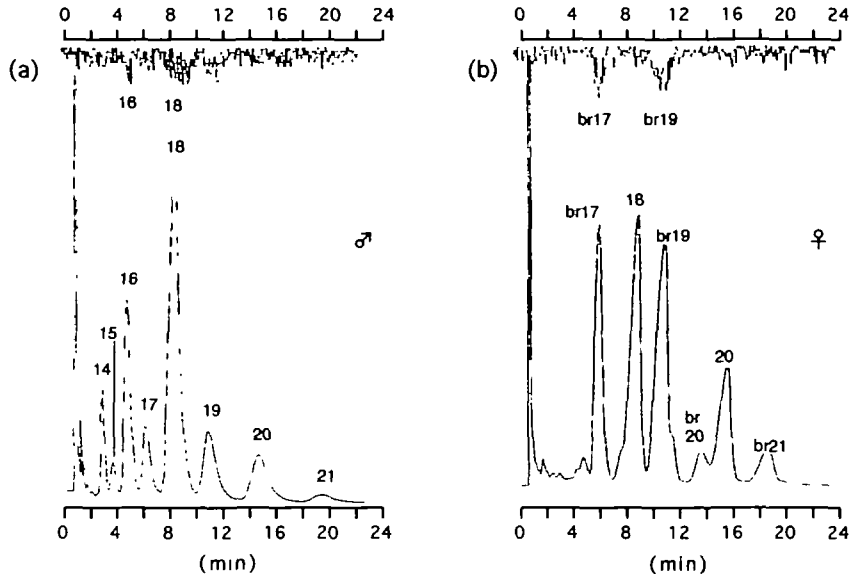

Fig. 5. **Radio-GLC analysis of isopropylidene derivatives of alkylglycerols prepared from ADG of golden hamster Harderian glands.** Golden hamsters were injected with 25 μCi of [U-^{14}C]leucine and the glands were obtained at 6 h after injection. Lipids in the glands were obtained and ADG was eluted. The acyl groups of ADG were extracted as fatty acid methyl esters, and the alkyl groups of ADG were subsequently obtained as isopropylidene derivatives of alkylglycerols. The latter were dissolved in chloroform and analyzed with a Gas Chromatograph GC-6AM (Shimadzu). The column effluent was passed through an Aloka peak analyzer radio-gas chromatograph system (Aloka). The voltage of the flame ionization detector (FID) was plotted upward and radioactivity was plotted downward. (a) Male. (b) Female. Each number indicates the chain length of each peak. "br" means branched chain.

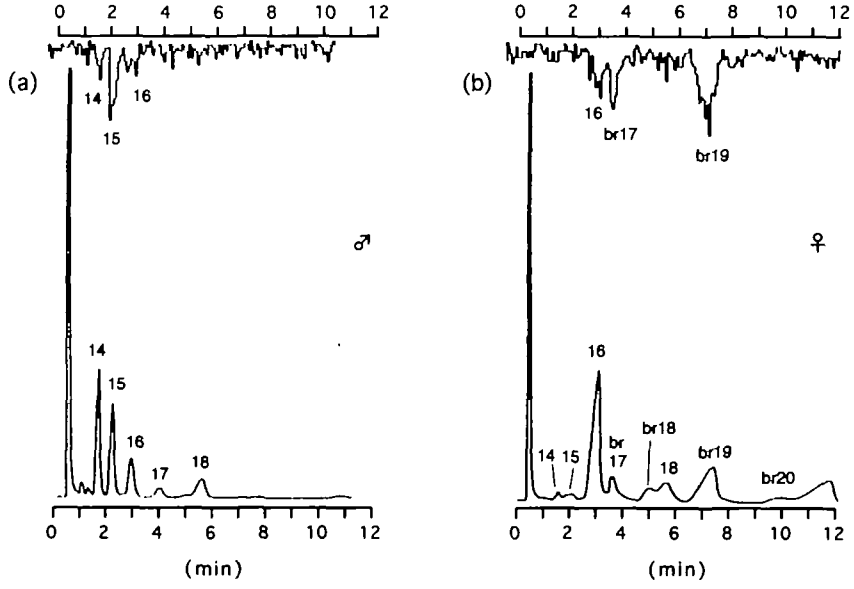

Fig. 6. **Radio-GLC analysis of fatty acid methyl esters prepared from ADG of golden hamster Harderian glands.** Golden hamsters were injected with 25 μCi of [U-^{14}C]leucine and the acyl groups of ADG were prepared as isopropylidene derivatives of alkylglycerols as indicated in Fig. 5. They were dissolved in hexane and then analyzed as indicated in Fig. 5. The FID voltage was plotted upward and radioactivity was plotted downward. (a) Male. (b) Female. Each number indicates the chain length of each peak. "br" means branched chain.

DISCUSSION

We previously reported that the major secretory lipid from the Harderian gland of golden hamster was ADG, and showed the marked sexual dimorphism in the composition of ADG in this gland (*9*). In the present study, we investigated the synthesis of lipids in this gland using [U-^{14}C]-valine, leucine, and isoleucine, and found distinct sexual differences in the incorporation of the isotope into ADG and cholesterol.

BCAA metabolites were incorporated into the lipids of the Harderian gland in both sexes. The high efficiency of isotope incorporation observed in the gland may reflect the high activity of lipid synthesis in this gland, as reported previously (*12, 13*).

Labeled ADG was separated into 3 spots in males and into 2 spots in females on TLC analysis, but each profile was the same as the total ADG profile detected with H_2SO_4. Thus, the BCAA metabolites were uniformly incorporated into ADG. As ADG is known to be produced from fatty acids and glycerol phosphate, the production of these two compounds might be regulated in this gland.

Sexual differences were also observed in the labeling profiles of the cholesterol fraction; the male fraction was strongly labeled but the female one was not, although a total cholesterol spot was detected in both sexes. These results showed that cholesterol was not produced from BCAA in female glands, and thus some enzymes for the production of cholesterol were thought to be regulated in the Harderian gland of golden hamster.

The incorporation of radioactivity did not differ with the gender in other tissues, such as liver, heart, nor brain, in golden hamster (data not shown). Furthermore, in the Chinese hamster Harderian gland, sexual differences were not observed in the TLC profiles of ADG and cholesterol (Fig. 4). Therefore, tissue- and species-specific regulation of BCAA catabolism occurred only in the golden hamster

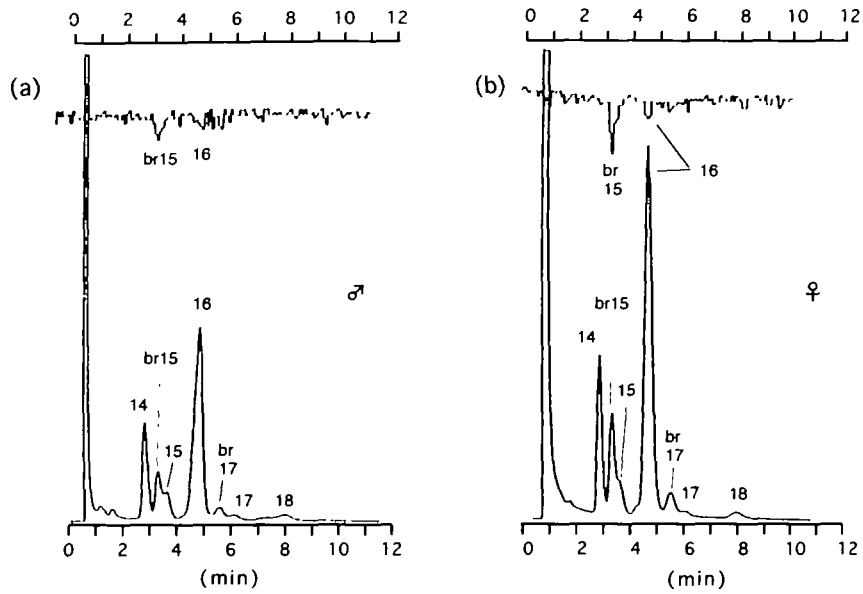

Fig. 7. **Radio-GLC analysis of isopropylidene derivatives of alkylglycerols prepared from ADG of Chinese hamster Harderian glands.** Chinese hamsters were injected with 10 μCi of [*U*-^{14}C]leucine. After 6 h, the Harderian glands were obtained, and the alkyl groups of ADG were prepared and analyzed as indicated in Fig. 5. The FID voltage was plotted upward and radioactivity was plotted downward. (a) Male. (b) Female. Each number indicates the chain length of each peak. "br" means branched chain.

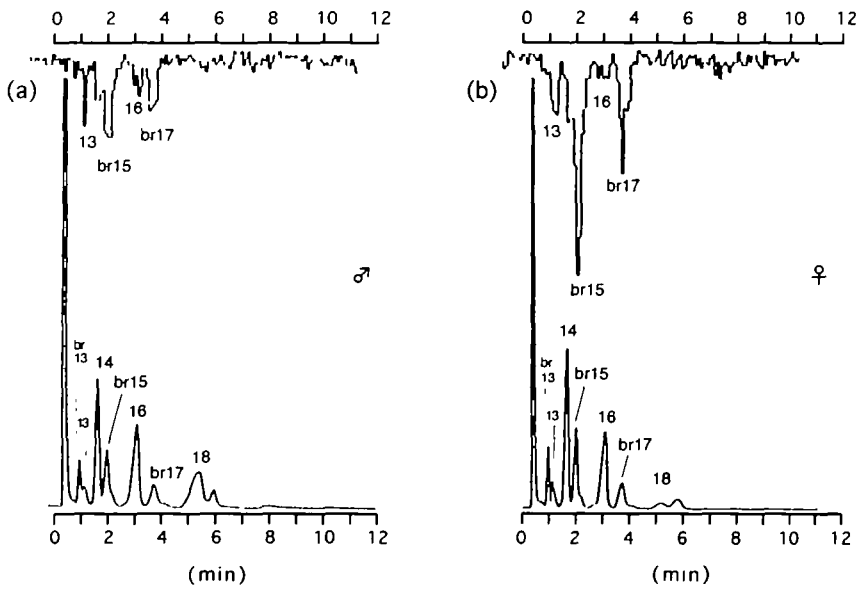

Fig. 8. **Radio-GLC analysis of fatty acid methyl esters prepared from ADG of Chinese hamster Harderian glands.** Chinese hamsters were injected with 10 μCi of [*U*-^{14}C]-leucine and the acyl groups of ADG were obtained as isopropylidene derivatives of alkylglycerols as indicated in Fig. 5. They were dissolved in hexane and then analyzed as indicated in Fig. 5. The FID voltage was plotted upward and radioactivity was plotted downward. (a) Male. (b) Female. Each number indicates the chain length of each peak. "br" means branched chain.

Harderian gland.

In the following study, we investigated the incorporation of BCAA metabolites into the substituents of ADG by radio-GLC analysis, and showed that BCAA metabolites were incorporated into the straight chains in male glands, but were efficiently incorporated into the branched chains in female glands. On the contrary, as for the ADG substituents of Chinese hamsters, branched chains as well as straight chains were labeled, but no sexual differences were observed (Figs. 7 and 8). These data suggested sex-dependent and species-specific regulation of branched chain fatty acid synthesis in the Harderian gland of golden hamster.

The branched aliphatic chains in female glands were limited to the "even and odd numbered *iso*-form" and "odd numbered *anteiso*-form," and so they should be produced with branched chain acyl-CoAs as the primers. The possible primers are isobutyryl-CoA for "even-numbered *iso*-branched chains," 2-methylbutyryl-CoA for "odd-numbered *anteiso*-branched chains," and isovaleryl-CoA for "odd-numbered *iso*-branched chains." Seyama *et al.* previously reported the existence of isobutyryl, 2-methylbutyryl, and isovaleryl groups among the acyl groups of ADG in female Harderian glands, but these groups were not found in male ADG on NMR analysis (*9*). Therefore, the amounts of the above mentioned acyl-CoAs differed with the gender in this gland, and the production or degradation of these acyl-CoAs was thought to be regulated in the Harderian gland of golden hamster.

Since BCAAs should be metabolized at least to branched chain acyl-CoAs when they are catabolized into the lipid fraction, and since the level of incorporation of radioactivity into the lipid of the gland was almost the same in males and females (Fig. 1), the production of the above mentioned acyl-CoAs probably did not differ so much with the gender.

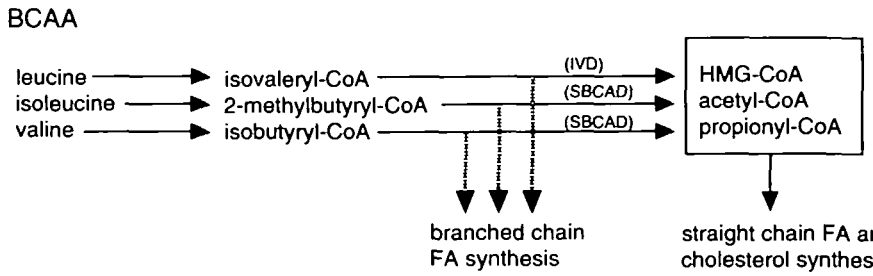

Fig. 9. **The metabolism of branched chain amino acids, and the synthesis of fatty acids and cholesterol.** (IVD) Catalyzed initially by isovaleryl-CoA dehydrogenase. (SBCAD) Catalyzed initially by short/branched chain acyl-CoA dehydrogenase.

Thus, the degradation of the branched chain acyl-CoAs should be regulated in the gland. A candidate for the regulatory endocrine factor is androgen because we previously showed that the composition of ADG in the Harderian gland was inversely changed by the serum testosterone level in castrated male and intact female golden hamsters (*10*). As indicated in Fig. 9, branched chain acyl-CoAs are metabolized to acetyl-CoA or propionyl-CoA in male glands where the androgen level is high, and they are consequently incorporated into straight chain fatty acids. While in the female gland where the androgen level is low, BCAAs are metabolized to branched chain acyl-CoAs, but they would not be efficiently metabolized further, and they were thought to be used as the primers of the fatty acid synthesis (as indicated by broken lines in Fig. 9). This hypothesis is consistent with the sexual difference observed in the isotope incorporation into the cholesterol fraction (Fig. 2), because 3-hydroxy-3-methylglutaryl-CoA, known as the source of cholesterol, would not be produced from BCAAs when branched chain acyl-CoAs were not catabolized (Fig. 9).

In summary, our data clearly show that the BCAA metabolism in the Harderian gland of golden hamster differs with the gender, and this difference is probably caused by the androgenic regulation of branched chain acyl-CoA degradation in tissue- and species-specific manners. Analysis of the enzymes involved in the metabolism of branched chain acyl-CoAs will provide important information on the specific regulation by androgen in this gland.

The authors wish to thank Professor Akihiko Kawaguchi (Graduate School of Arts and Sciences, The University of Tokyo, Tokyo) and Mr. Kazuo Watanabe (Sagami Chemical Research Center, Kanagawa) for their help in the use of the radio-GLC apparatus.

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