Lack of Evidence for Existence of Vitamin D and 25-Hydroxyvitamin D Sulfates in Human Breast and Cow’s Milk

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Summary The presence of water-soluble vitamin D and 25-OH-D sulfates in human breast and cow’s milk was studied. We first confirmed that synthetic vitamin D$_2$ and D$_3$ sulfates could not be hydrolyzed by alkali but by acid. Breast or cow’s milk was separated into milk whey containing water-soluble components and milk curd containing crude proteins and lipophilic components. The separated milk whey and curd were hydrolyzed by acid or alkali and each lipid extract was subjected to HPLC analysis. Neither peak due to vitamin D and 25-OH-D was observed in the chromatograms of acid- and alkali-hydrolyzed milk whey, whereas the peaks due to vitamin D$_3$ and 25-OH-D$_3$ were found in the chromatograms of both acid- and alkali-hydrolyzed milk curd and there was no significant difference between the respective peak heights. The eluates corresponding to the respective peaks observed on the latter’s chromatograms were collected and subjected to UV, HPLC, GC-MS and GLC to identify the existence of vitamin D$_3$ and 25-OH-D$_3$, respectively. We concluded from these results that neither breast nor cow’s milk contained water-soluble vitamin D and 25-OH-D sulfates, whereas they

1 Abbreviations: 25-OH-D, 25-hydroxyvitamin D; $^3$H-D$_3$, tritiated vitamin D$_3$; $^3$H-25-OH-D$_3$, tritiated 25-hydroxyvitamin D$_3$; UV, ultraviolet; HPLC, high-performance liquid chromatography; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; GC-MS, gas chromatography-mass spectrometry; FD-MS, field desorption-mass spectrometry; EI, electron impact; NMR, proton nuclear magnetic resonance; AUFS, absorption unit as full scale; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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contained fat-soluble vitamin D₃ and 25-OH-D₃. The concentrations of vitamin D₃ and 25-OH-D₃ in breast milk were about 125 and 350 ng/liter, while those in cow’s milk were about 420 and 270 ng/liter, respectively. The experiments on the transfer of ³H-D₃ and ³H-25-OH-D₃ perorally dosed to lactating rats into suckling pups through their milk also supported the above conclusion.

**Key Words** vitamin D₃, 25-hydroxyvitamin D₃, vitamin D sulfate, human breast milk, cow’s milk, high-performance liquid chromatography, lactating rat, suckling pups

Sahashi et al. (1) reported that human breast and cow’s milk contained water-soluble vitamin D sulfate and that the concentrations measured by a colorimetric method were 950 and 204 IU/liter, respectively, which were much higher than those of fat-soluble vitamin D (5-30 IU/liter). They also reported that synthetic vitamin D₂ sulfate had similar biological activity to fat-soluble vitamin D₂ itself (2). They deduced from these results that the superiority of breast milk to cow’s milk for preventing rickets might be due to the difference of vitamin D sulfate concentrations. This attractive assumption was supported by Lakdawala and Widdowson (3) using Sahashi’s method and Le Boulch et al. (4) using a GLC method.

Recently, Reeve et al. (5) reported that the biological activity of synthetic vitamin D₃ sulfate was far lower than that of vitamin D₃ itself when intestinal calcium transport, bone calcium resorption, plasma phosphorus levels and antirachitic activity were measured. Similar results showing little activity of vitamin D₃ sulfate were also reported by Nagubandi et al. (6). Moreover, Hollis et al. (7) reported that they could not show the existence of the sulfate in human breast milk whey by an HPLC method. Greer et al. (8) commented that the story of vitamin D sulfate was a myth and that fat-soluble vitamin D and its metabolites should be evaluated for the nutritional status of vitamin D in the milk.

However, more recently, Asano et al. (9) reported that concentrations of vitamin D sulfate in breast and cow’s milk were 720–800 and 1,600–1,700 IU/liter, respectively, when the sulfate was determined by an HPLC method. Moreover, Le Boulch et al. (10) again published the separation and identification of the sulfate in breast milk by a method including gel filtration, HPLC and estimation of a UV spectrum. Thus, the problem has become very confused. In order to resolve this confusion, we studied the problem by our own methods and could not find any evidence for the existence of the sulfates of vitamin D and 25-OH-D in breast and cow’s milk. The methods and results are described in this paper.

**EXPERIMENTAL**

1. **Materials and reagents**

Commercial grades (Duphar Co., The Netherlands) of crystalline vitamin D₂,

D₃ and 25-OH-D₃ were used. [1α,2α-3H]-D₃ (specific activity, 12.3 Ci/mmol) and [26(27)-methyl-3H]-25-OH-D₃ (specific activity, 15.6 Ci/mmol) were purchased from the Radiochemical Centre (Amersham/Buckinghamshire, UK). Organic solvents of analytical grade were distilled before use. Other guaranteed reagents were used.

2. Milk samples

Human breast milk samples were collected from 20 healthy mothers between 5 and 10 days postpartum. The collected milk samples were combined, stirred and stored below −20°C until use. On the other hand, cow's milk samples were collected from 6 healthy Holstein cows fed at the Ikawadani Farm (Kobe). The collected milk samples were similarly combined, stirred and stored until use.

3. Instrumentation

1) High-performance liquid chromatography (HPLC). The following conditions were used for HPLC analysis. Shimadzu LC-3A high-performance liquid chromatographs equipped with UVD-2 detectors (254 nm, AUFS 0.001) were used for all the HPLC analyses and the retention times of authentic vitamin D₂, D₃, 25-OH-D₂ and 25-OH-D₃ were taken by reference to a previous paper (II)

Reversed-phase HPLC

Column: Nucleosil 5C₁₈ (7.5 i.d. × 300 mm, Nagel Co., Federal Republic of Germany).
Mobile phase: 50% methanol in acetonitrile.
Flow rate: 2.0 ml/min (50 kg/cm²).

Straight-phase HPLC I for vitamin D analysis

Column: Zorbax SIL (4.6 i.d. × 250 mm, DuPont Co., USA).
Mobile phase: 0.4% isopropanol in n-hexane.
Flow rate: 1.8 ml/min (60 kg/cm²).

Straight-phase HPLC II for 25-OH-D analysis

Column: Zorbax SIL (4.6 i.d. × 250 mm).
Mobile phase: 2.2% isopropanol in n-hexane.
Flow rate: 1.7 ml/min (45 kg/cm²).

Straight-phase HPLC III for ³H-D₃ and ³H-25-OH-D₃ analysis

Column: Zorbax SIL (4.6 i.d. × 250 mm).
Mobile phase: 5.5% isopropanol in n-hexane.
Flow rate: 0.6 ml/min (20 kg/cm²).

2) Gas chromatography-mass spectrometry (GC-MS). The GC-MS was performed on a Hitachi Super Mass M-80 gas chromatograph double-focusing mass spectrometer equipped with a unit of electron impact (EI) as an energy source. A glass column (0.3 i.d. × 100 cm) packed with 3% OV 17 on Gas Chrom Q (80–100 mesh) was operated at 270°C for vitamin D₃ analysis or 290°C for 25-OH-D₃ analysis with a helium flow rate of 30 ml/min. The separator temperature, ionizing current and voltage were 320°C, 60 μA and 25 eV, respectively. When peaks were
observed on a gas chromatogram, the mass spectra were recorded with a computer (Hitachi M-003).

3) Gas-liquid chromatography (GLC). Reference was made to the results of GLC for the chromatograms monitored by the total ion collection of GC-MS.

4) Field desorption-mass spectrometry (FD-MS). The FD-MS was performed on a Hitachi Super Mass M-80 double-focusing mass spectrometer equipped with a unit of FD using 10 μ tungsten-carbon as an emitter. The emitter current and voltage were kept at 5.0 mA and 3 kV, respectively, while the magnetic field scan rate was controlled at m/z 1–1,500/8 sec.

5) Proton nuclear magnetic resonance (NMR) spectrum. The NMR spectra were obtained on a Varian XL-200 spectrometer (200 MHz, USA) with d6-DMSO as a solvent.

6) Ultraviolet (UV) spectrum. The UV spectra were obtained on a Hitachi 323 spectrometer with ethanol as a solvent.

4. Syntheses of vitamin D2 and D3 sulfates

Vitamin D2 and D3 sulfates were synthesized as ammonium salts according to Higaki et al. (13). Each crystal thus obtained was further purified by application twice to a preparative TLC using Kieselgel GF254 as an adsorbent and methanol–chloroform (1:4) as a developing solvent. Both sulfates gave the same Rf value (0.38).

Vitamin D2 sulfate: mp 113–115°C (white crystal as ammonium salt); UV (nm), λmax 265, λmin 230; NMR (δ), 0.55 (3H, s, 18-CH3), 4.23 (1H, s, 3-CH), 4.70 (m) and 5.05 (m) (2H, 19=CH2), 5.21 (2H, m, 22,23-CH=CH–), 6.10 (2H, AB quartet, 6,7-CH=CH–, J=12), 7.16 (1H, s, 3-SO4H, disappeared by addition of D2O); FD-MS (m/z), 476 (M+), 378 (M+–H2SO4).

Vitamin D3 sulfate: mp 115–117°C (white crystal as ammonium salt); UV (nm), λmax 265, λmin 230; NMR (δ), 0.55 (3H, s, 18-CH3), 4.26 (1H, m, 3-CH), 4.70 (m) and 5.06 (m) (2H, 19=CH2), 6.12 (2H, AB quartet, 6,7-CH=CH–, J=12), 7.20 (1H, 3-SO4H, disappeared by addition of D2O); FD-MS (m/z), 464 (M+), 366 (M+–H2SO4).

Since vitamin D2 and D3 sulfates are difficult to vaporize, the respective molecular ions are unable to be detected on the spectra of usual MS with a unit of EI as reported by Reeve et al. (5) and Nagubandi et al. (6). Therefore, we used the FD-MS and succeeded in finding the respective molecular ions on the spectra as shown above.

5. Procedure for identification of vitamin D, 25-OH-D and their sulfates in breast and cow’s milk

1) Separation of milk curd and whey. Two hundred ml of breast or cow’s milk was taken in a 500 ml Erlenmeyer flask and 8 ml of 20% acetic acid solution was added to it. The solution was heated without stirring to 40°C and then mixed vigorously. The mixed solution was immediately cooled and the resultant white

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precipitate was separated by filtration through a Whatman 5A filter paper. The separated precipitate and filtrate were denoted as milk curd and whey, respectively. Milk curd usually contains milk casein and lipophilic components, while milk whey contains lactoglobulin, lactoalbumin, minerals and other water-soluble components. The milk curd and whey thus obtained were lyophilized and powdered until the following procedures were performed.

2) Hydrolysis of milk curd and whey with acid (Acid solvolysis). The powdered milk curd or whey obtained from 200 ml of milk was taken in a 300 ml round-bottom flask. Twenty ml of water and 200 ml of ethyl acetate previously saturated overnight with sulfuric acid were added to it and mixed vigorously. After allowing the mixture to stand for 1 h at 39°C, the solution was neutralized with sodium bicarbonate solution, the lipid then being repeatedly extracted with ethyl acetate. The extracts were combined, washed with water and evaporated under reduced pressure. The resulting residue was used for the following HPLC.

3) Hydrolysis of milk curd and whey with alkali (Alkali saponification). The powdered milk curd or whey obtained from 200 ml of milk was taken in a 300 ml round-bottom flask and dissolved in 100 ml of 10% pyrogallol-ethanol solution. After adding 25 ml of 90% (w/v) KOH solution, the solution was refluxed for 1 h. It was immediately cooled and the unsaponifiable matter was extracted with benzene according to a previous paper (11). After evaporating the solvent under reduced pressure, the resulting residue was used for the following HPLC.

4) HPLC. The residue obtained in section 2) or 3) was used for the reversed-phase HPLC mentioned above for purification and the fractions corresponding to vitamin D and 25-OH-D were individually collected. After evaporating the solvent under reduced pressure, each residue was dissolved in the respective mobile phases and then subjected to the straight-phase HPLC I or II mentioned above. When the peaks corresponding to the authentic vitamin D₃ or 25-OH-D₃ were observed on the chromatograms, the respective eluates were collected and used for the analysis of UV, GC-MS or HPLC.

6. Animal experiments

1) Animals. Pregnant rats of the Wistar strain weighing approximately 250 g each were purchased from the Shizuoka Experimental Animals and Agricultural Co. (Hamamatsu, Japan). The rats were fed on a normal diet (Nosanko Co., Osaka, Japan) and water ad libitum before and after delivery. The newborn pups were fed their mother’s milk ad libitum.

2) Administration of ³H-D₃ or ³H-25-OH-D₃ to lactating rats. After a week postpartum, lactating rats were each perorally given 1 μCi of ³H-D₃ or ³H-25-OH-D₃ by force.

3) Collection of the mother’s milk and the blood of the suckling pups. Fifteen h after the administration of the labeled compounds, the mother’s milk was collected from the mammary glands by suckling with a mini-aspirator tube. On the other hand, immediately after the administration of the labeled compounds to the
mother rats, the suckling pups were allowed to suck the mother’s milk ad libitum. After 15 h lactation, the pups were sacrificed by decapitation and their blood was collected. The plasma was obtained by centrifugation.

4) Radioactive determination of milk and plasma. According to a previous paper (12), each 0.5 ml of the milk or plasma thus obtained was extracted with a mixture of methanol and methylene dichloride (2:1) with subsequent separation into fat-soluble and water-soluble layers.

Eight ml of the dioxane counting solution containing 120 g of naphthalene, 5 g of PPO and 0.3 g of POPOP per liter of dioxane was added to the water-soluble layer and the radioactivity was measured by a liquid scintillation spectrophotometer (Packard model 3375, Downner, USA). On the other hand, the solvent of the fat-soluble layer was evaporated under reduced pressure. The resulting residue was dissolved in 8 ml of the Triton-toluene counting solution containing 5 g of PPO, 100 mg of POPOP, 210 ml of Triton X-100 and 790 ml of toluene, and the radioactivity was similarly measured.

5) HPLC. The residue obtained from the fat-soluble layer of each 0.5 ml of the milk or plasma was dissolved in 0.5 ml of 5.5% isopropanol in n-hexane. Fifty μl of the solution was subjected to the straight-phase HPLC III. Each 40-drop (0.5 ml) fraction of the eluates was collected in small test tubes using a mini-fraction collector incorporating a drop counter (Gilson Co., USA) and the solvent was evaporated under reduced pressure. The resulting residue was dissolved in the Triton-toluene counting solution and the radioactivity was similarly measured.

RESULTS

1. Hydrolysis of synthetic vitamin D$_2$ and D$_3$ sulfates with acid or alkali

According to section 5-2) of EXPERIMENTAL, hydrolysis of synthetic vitamin D$_2$ and D$_3$ sulfates with acid (acid solvolysis) was performed. Exactly 1 μg of

![Fig. 1. Acid solvolysis or alkali-saponification of synthetic vitamin D$_3$ sulfate. (a) acid solvolysis; (b) alkali-saponification.](image-url)
vitamin D$_2$ or D$_3$ sulfate was dissolved in 5 ml of ethyl acetate previously saturated with sulfuric acid and incubated at 39°C for the desired time. The extracted lipid was subjected to the straight-phase HPLC I described in EXPERIMENTAL to measure the rate of production of hydrolyzed vitamin D. As shown in Fig. 1a, vitamin D$_3$ sulfate was completely hydrolyzed after 1 h solvolysis. Similar results were also obtained on vitamin D$_2$ sulfate. Therefore, we concluded that acid solvolysis for 1 h was sufficient to hydrolyze vitamin D$_2$ and D$_3$ sulfates in a sample.

On the other hand, hydrolysis of synthetic vitamin D$_2$ and D$_3$ sulfates with alkali (alkali saponification) was performed according to section 5-3) of EXPERIMENTAL. Exactly 1 µg of vitamin D$_2$ or D$_3$ sulfate was dissolved in 10 ml of 1–5 N ethanolic KOH solution and refluxed for 2 h to perform alkali saponification. The extracted unsaponifiable matter was subjected to the straight-phase

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**Fig. 2.** HPLC profiles on the vitamin D fractions isolated from human breast milk whey and curd after acid solvolysis or alkali-saponification. According to EXPERIMENTAL, the separated human breast milk whey and curd was hydrolyzed with acid or alkali. Each fraction of lipid extract and authentic vitamin D$_3$ was subjected to the reversed-phase HPLC and the collected vitamin D fractions were then subjected to the straight-phase HPLC I.

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Fig. 3. HPLC profiles of the 25-OH-D fractions isolated from human breast milk whey and curd after acid-solvolysis or alkali-saponification. According to EXPERIMENTAL, the separated human breast milk whey or curd was hydrolyzed with acid or alkali. Each of the lipid and authentic 25-OH-D$_3$ extracts were subjected to the reversed-phase HPLC and the 25-OH-D fractions collected were then subjected to the straight-phase HPLC II.

HPLC I to measure the rate of production of hydrolyzed vitamin D. Vitamin D$_3$ sulfate could not be hydrolyzed in 1–5 N ethanolic KOH solutions as shown in Fig. 1b, though Higaki et al. (13) have reported that vitamin D$_2$ sulfate could be completely hydrolyzed by dissolving the sulfate in ethanolic KOH solutions of a concentration higher than 2 N and refluxing for 30 min. Since similar results were also obtained on vitamin D$_2$ sulfate, we concluded that vitamin D$_2$ and D$_3$ sulfates could not be hydrolyzed by alkali-saponification.

2. Hydrolysis of human breast milk curd and whey with acid or alkali

According to EXPERIMENTAL, 200 ml of human breast milk was separated into milk curd and whey which were lyophilized and powdered. The powdered milk curd or whey was hydrolyzed with acid or alkali. Each lipid extract was applied to
the reversed-phase HPLC and the vitamin D and 25-OH-D fractions collected were then subjected to the straight-phase HPLC I and II, respectively. The profiles are shown in Figs. 2 and 3. As shown in these figures, the peaks corresponding to vitamin D₃ and 25-OH-D₃ were observed in the profiles of milk curd in both chromatograms of acid solvolysis and alkali saponification, whereas no peak due to the two compounds was found in those of milk whey. No significant difference between the respective peak heights on the acid and alkali hydrolyzed milk curd was obtained. The concentrations of vitamin D₃ and 25-OH-D₃ calculated by the peak heights were about 125 and 350 ng/liter, respectively.

3. Identification of vitamin D₃ and 25-OH-D₃ in human breast milk curd

The eluates corresponding to vitamin D₃ and 25-OH-D₃ observed in the chromatograms of milk curd (Figs. 2 and 3) were collected and used for the analysis of UV, HPLC, GC-MS and GLC. As shown in Table 1, the analytical data agreed with those of the respective authentic compounds.

4. Identification of vitamin D₃ and 25-OH-D₃ in cow’s milk curd

According to EXPERIMENTAL, 200 ml of cow’s milk was separated into milk curd and whey. Each was treated similarly as with human breast milk. The chromatograms of the straight-phase HPLC I and II on the milk curd and whey were quite similar to those shown in Figs. 2 and 3, respectively. The concentrations of vitamin D₃ and 25-OH-D₃ calculated by the peak heights were about 420 and 370 ng/liter, respectively.

Similarly to human breast milk, the eluates corresponding to vitamin D₃ and 25-OH-D₃ were used for instrumental analysis and the two compounds was also identified in the milk curd.

Table 1. Analytical data of the vitamin D and 25-OH-D fractions obtained from the second analytical HPLC I or II carried out on human breast milk curd.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Authentic D₃</th>
<th>Vitamin D fraction</th>
<th>Authentic 25-OH-D₃</th>
<th>25-OH-D fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>λ_max (nm)</td>
<td>264</td>
<td>264</td>
<td>264</td>
<td>264</td>
</tr>
<tr>
<td>λ_min (nm)</td>
<td>228</td>
<td>228</td>
<td>228</td>
<td>228</td>
</tr>
<tr>
<td>HPLC⁵</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reversed-phase</td>
<td>22.1</td>
<td>22.0</td>
<td>23.8</td>
<td>23.8</td>
</tr>
<tr>
<td>Straight-phase</td>
<td>21.6</td>
<td>21.6</td>
<td>21.6</td>
<td>21.6</td>
</tr>
<tr>
<td></td>
<td>118, 136, 253</td>
<td>118, 136, 253</td>
<td>253, 271</td>
<td>253, 271</td>
</tr>
<tr>
<td>GC-MS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(m/z)</td>
<td>271, 351,</td>
<td>271, 351</td>
<td>367, 382</td>
<td>367, 382</td>
</tr>
<tr>
<td>384 (M⁺)</td>
<td>400 (M⁺)</td>
<td>400 (M⁺)</td>
<td>400 (M⁺)</td>
<td>400 (M⁺)</td>
</tr>
<tr>
<td>GLC ⁶</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyro-compound</td>
<td>8.2</td>
<td>8.2</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Isopyro-compound</td>
<td>9.8</td>
<td>9.8</td>
<td>6.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

⁵ For reversed-phase HPLC and straight-phase HPLC I for vitamin D and II for 25-OH-D refer to EXPERIMENTAL. The data are shown as the retention time (min).
⁶ The data are shown as the retention time of GLC monitored by the total ion collection of GC-MS.
5. Results of the animal experiments

Each 5 nCi of $^3$H-D$_3$ and $^3$H-25-OH-D$_3$ was mixed and used for straight-phase HPLC III. When each 40-drop (0.5 ml) fraction of the eluates had been collected, radioactivity due to $^3$H-D$_3$ and $^3$H-25-OH-D$_3$ was observed in fractions no. 10–14 and no. 25–28, respectively.

According to EXPERIMENTAL, 1 µCi of $^3$H-D$_3$ or $^3$H-25-OH-D$_3$ was perorally administered to lactating mother rats, and their milk was collected. Figure 4a shows the HPLC profile for the lipid extract obtained from the rats given $^3$H-D$_3$. The two radioactive peaks corresponding to vitamin D$_3$ and 25-OH-D$_3$ were observed in it. On the other hand, the HPLC profile for the lipid extract of milk obtained from the rats dosed $^3$H-25-OH-D$_3$ gave only one radioactive peak corresponding to 25-OH-D$_3$, as shown in Fig. 4b.

The suckling pups were allowed to suck the milk of lactating rats previously given $^3$H-D$_3$ or $^3$H-25-OH-D$_3$ and their blood was collected. Figures 5a and 5b show the HPLC profiles for the lipid extracts of the plasma obtained from the pups having sucked $^3$H-D$_3$ and $^3$H-25-OH-D$_3$ dosed mother’s milk, respectively. The two radioactive peaks corresponding to vitamin D$_3$ and 25-OH-D$_3$ were observed in the former (Fig. 5a), while the one radioactive peak corresponding to 25-OH-D$_3$ was in the latter (Fig. 5b).

When the radioactivity of fat-soluble and water-soluble layers of the milk and plasma was measured according to EXPERIMENTAL, almost all the count was observed in the layers of the former, while no significant radioactivity was found in those of the latter as shown in Table 2.

![Fig. 4. HPLC profiles of the lipid extract of milk obtained from lactating rats previously given $^3$H-D$_3$ or $^3$H-25-OH-D$_3$. According to EXPERIMENTAL, $^3$H-D$_3$ (a) or $^3$H-25-OH-D$_3$ (b) was perorally administered to lactating rats. The lipid extract of their milk was subjected to the straight-phase HPLC III.](image-url)
Fig. 5. HPLC profiles of the lipid extract of plasma of pups suckling the milk of lactating rats previously given $^3$H-D$_3$ or $^3$H-25-OH-D$_3$. According to EXPERIMENTAL, pups sucked the milk of lactating rats previously given $^3$H-D$_3$ (a) or $^3$H-25-OH-D$_3$ (b). The lipid extract of the pups plasma was subjected to the straight-phase HPLC III.

Table 2. Distribution of radioactivity in the milk obtained from lactating rats previously given $^3$H-D$_3$ or $^3$H-25-OH-D$_3$ and from the plasma of pups suckling the milk.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Radioactivity (dpm/0.5 ml)</th>
<th>Radioactivity (dpm/0.5 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In fat-soluble layer</td>
<td>In water-soluble layer</td>
</tr>
<tr>
<td>Mother's milk I</td>
<td>50,132</td>
<td>N.D.</td>
</tr>
<tr>
<td>Pup's plasma I</td>
<td>8,915</td>
<td>N.D.</td>
</tr>
<tr>
<td>Mother's milk II</td>
<td>35,091</td>
<td>N.D.</td>
</tr>
<tr>
<td>Pup's plasma II</td>
<td>12,269</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Note: 1) Mother's milk I and II were obtained from the lactating rats previously given $^3$H-D$_3$ and $^3$H-25-OH-D$_3$, respectively. 2) Pup’s plasma I and II were obtained from the pups suckling mother’s milk I and II, respectively. 3) N.D., not detected.

DISCUSSION

The story of vitamin D sulfate in human breast and cow’s milk was started by Sahashi et al. (1) about 20 years ago. They added ethanol and acetic acid to milk for separation into oil and water-soluble layers and precipitate (crude protein). After removing oil and precipitate, the water-soluble layer was mixed with barium hydroxide solution and the precipitate formed involving vitamin D sulfate barium salt was separated. By refluxing the precipitate in 3N ethanolic KOH solution,
vitamin D sulfate was saponified and the free vitamin D liberated was extracted with diethyl ether for subjection to SbCl₃ and Liebermann color reactions, TLC, and measurement of UV spectrum for identification. Although we re-examined the Sahashi procedure on each 500 ml of breast or cow’s milk very exactly, we were unable to find any evidence for the existence of vitamin D sulfate in the milk. Sahashi’s group (13) also reported that vitamin D₂ sulfate could be easily hydrolyzed by alkali saponification in ethanolic solutions of concentrations higher than 2 N. However, when we re-examined this, we found that the sulfates of vitamin D₂ and D₃ could not be hydrolyzed by alkali, as shown in Fig. 1a. Although we repeatedly studied the saponification by changing the concentrations of ethanolic KOH solutions and refluxing times, the sulfates could not be hydrolyzed at all. On the other hand, we found that vitamin D₂ and D₃ sulfates could be easily hydrolyzed by acid solvolysis, as shown in Fig. 1b. Therefore, we concluded that the sulfates could be hydrolyzed not by alkali but by acid. We cannot understand the reason why Sahashi’s group (1, 13) were able to hydrolyze vitamin D₂ sulfate with alkali and isolate the free form of vitamin D from the water-soluble layers of breast and cow’s milk after alkali saponification. Sulfates of some sterols, e.g., cholesterol (14), dehydroisoandrosterone (15) and corticosteroids (14, 16), were found in biological materials. It was reported that these sulfates were generally stable in alkali solutions and could be hydrolyzed either by acid or by enzymatic methods to obtain their free forms (14–16). Therefore, we do not think that vitamin D sulfate is one of the exceptions.

From these results and considerations, we separated human breast or cow’s milk into milk curd and whey and then hydrolyzed with acid or alkali according to EXPERIMENTAL. If the milk contains vitamin D sulfate in very high concentrations as reported by Sahashi et al. (1), a big peak due to the hydrolyzed free form of vitamin D should be observed in the chromatogram of acid solvolyzed milk whey while no peak due to vitamin D should be observed in the chromatogram of alkali-saponified milk whey. As shown in Figs. 2 and 3, the results showed that no peak due to the free forms of vitamin D and 25-OH-D could be observed in the chromatograms of both acid-solvolyzed and alkali-saponified breast milk whey. On the other hand, the peaks due to vitamin D₃ and 25-OH-D₃ were clearly observed in the chromatograms of both acid-solvolyzed and alkali-saponified breast milk whey and there was no significant difference between the respective two peak heights, as shown in Figs. 2 and 3. The observed peaks were respectively identified by the results of UV, HPLC, GC-MS and GLC as shown in Table 1. Similar results were also obtained on cow’s milk curd and whey. We concluded from the results that neither human breast nor cow’s milk contained water-soluble vitamin D and 25-OH-D sulfates, whereas both milk types contained fat-soluble vitamin D₃ and 25-OH-D₃.

The concentrations of vitamin D₃ in breast and cow’s milk calculated from the respective peak heights of the milk curd were 125 and 420 ng/liter (5 and 16.8 IU/liter) while those of 25-OH-D₃ were 350 and 270 ng/liter, respectively. The data were
close to those recently appearing in the literature (17–21) from the use of HPLC methods.

The existence of vitamin D sulfate in breast and cow's milk was reported not only by Sahashi et al. (1) but also by Lakdawala and Widdowson (3), Le Boulch et al. (4, 10) and Asano et al. (9). However, no conclusive evidence for the existence of the sulfate was found in their reports. Asano et al. (9) and Le Boulch et al. (10) used HPLC for the isolation of vitamin D sulfate in breast milk, but we thought that the degree of separation of the peak mentioned as the sulfate from other concomitants was insufficient. We do not know if there was a difference between their samples and ours.

As mentioned above, we could find no evidence for the existence of vitamin D sulfate in breast and cow's milk from our various examinations. Greer et al. (8) commented that the story of vitamin D sulfate was a myth and that fat-soluble vitamin D and metabolites should be evaluated for the nutritional status of vitamin D in breast and cow's milk. We agree with Greer's comments from our own experimental results described in this paper.

In order to confirm the above conclusion more certainly, we performed an animal experiment. When $^{3}$H-D$_{3}$ or $^{3}$H-25-OH-D$_{3}$ was perorally administered to lactating mother rats, the radioactivity in their milk was not observed in the water-soluble layer but in the fat-soluble layer as shown in Table 2. The radioactivity in the plasma of the pup suckling the milk was also mainly found in the fat-soluble layer while no significant count was observed in the water-soluble layer. These results also strongly supported our conclusion mentioned above.

As shown in Figs. 4a and 5a, the two radioactive peaks due to vitamin D$_{3}$ and 25-OH-D$_{3}$ were observed in the HPLC profiles of the lipid extracts of the milk obtained from $^{3}$H-D$_{3}$ dosed rats and the suckling pup’s plasma. The results strongly suggested that the dosed vitamin D$_{3}$ itself and the 25-OH-D$_{3}$ formed in the mother rat's liver were secreted in the milk through the mammary gland and transferred into the suckling pups through the milk. On the other hand, one radioactive peak corresponding to 25-OH-D$_{3}$ was observed in the HPLC profiles of the milk obtained from the $^{3}$H-25-OH-D$_{3}$ dosed rats and the suckling pup's plasma as shown in Figs. 4b and 5b. The results also suggested that the dosed 25-OH-D$_{3}$ itself was directly secreted in the milk through the mammary gland and transferred into the suckling pups.

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