IN VIVO AND IN VITRO CONVERSION OF 7-DEHYDROCHOLESTEROL INTO VITAMIN D₃ IN RAT SKIN BY ULTRAVIOLET RAY'S IRRADIATION^{1,2}

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Summary In order to confirm the photochemical conversion of 7-dehydrocholesterol (7-DHC) into vitamin D₃ in rat skin, the following *in vitro* and *in vivo* experiments were carried out. In the first (*in vitro*) experiment, the skin stripped off from a sacrificed normal rat was irradiated with an ultraviolet (UV) lamp for a constant period. In the second (*in vivo*) experiment, the normal rat, irradiated under the same condition mentioned above, was sacrificed and then the skin was stripped off. Lipids were individually extracted with chloroform-methanol (1:1) from the skin obtained in the two experiments and the solvent was evaporated. The resulting residue was saponified and the unsaponifiable matter extracted with benzene was purified by application to hydroxyalkoxypropyl (HAP) Sephadex column chromatography. The resulting purified vitamin D₃ fraction was applied to high-performance liquid chromatography (HPLC) in order to estimate vitamin D₃. No peak, aside from that of α -naphthol as an internal standard, was observed in the HPLC chromatogram on the skin obtained from the non-irradiated rat, whereas the peak corresponding to vitamin D₃ was observed in each HPLC chromatogram on both the irradiated skin (*in vitro* experiment) and the skin obtained from the irradiated rat (*in vivo* experiment). The peaks, confirmed to be due to vitamin D₃ by the results of co-chromatography, were increased according to the increase of irradiation energy and there were little differences between the corresponding estimated values of vitamin D₃ in the two experiments. These results prompted the conclusion that 7-DHC in rat skin was photochemically converted into

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² Following abbreviation are used: 7-DHC, 7-dehydrocholesterol; D₃, cholecalciferol (vitamin D₃); pre-D₃, precholecalciferol (previtamin D₃); HAP, hydroxyalkoxypropyl; UV, ultraviolet; HPLC, high-performance liquid chromatography.

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vitamin D₃ by UV irradiation and that the *in vivo* conversion mechanism might be the same as the *in vitro* one.

It was reported in a previous paper (1) that the existence of 7-DHC in rat skin was physico-chemically confirmed from the results of the UV spectrum, HPLC, GLC and GC-MS and that a HPLC method for the determination of the sterol in the tissue was proposed. When the proposed method was applied to the normal rat skin (20 weeks old), with and without UV irradiation, the estimated average values of 7-DHC were 107.7 and 103.8 $\mu\text{g/g}$ of the fat tissue-removed skin, respectively, which were significantly higher than the corresponding values of vitamin D-deficient rat skin. It is believed that the preventive effect of sunlights on vitamin D deficiency of human beings and animals might be due to the photochemical and thermal formation of vitamin D₃ *via* pre-D₃ in the exposed skin from the 7-DHC by the action of UV rays in sunlight (Dorno rays) (2). Although the photochemical and thermal conversion mechanism of 7-DHC to vitamin D₃ *via* pre-D₃ as a solution state in a test tube has been well clarified (3), the *in vivo* conversion had never been directly confirmed before our previous communication (4) because the vitamin D₃ contents in tissues is usually too small to be detected and determined. We solved this problem by using a HPLC method for the identification and micro-determination of vitamin D₃ and pre-D₃ besides 7-DHC in rat skin. The detailed results are described in this paper. Recently, PETROVA *et al.* (5) and HOLICK *et al.* (6) investigated on the same problem by using ³H-7-DHC and clarified that the sterol in rat skin exposed to UV rays was photochemically converted to pre-D₃ which might be thermally transferred into vitamin D₃. Their conclusions are identical with ours.

EXPERIMENTAL

1. General

1) *Materials and reagents.* Vitamin D₃. Commercial grades, recrystallized from acetone-water (4: 1), Philips-Duphar Co., mp 85–86°C. Pre-D₃. Pre-D₃ was obtained as an oil by purifying a refluxed solution of vitamin D₃ in ethylene dichloride on a preparative TLC. Other materials and reagents were used according to a previous paper (1).

2) *UV absorption spectra.* UV absorption spectra were recorded with a Hitachi 323 automatic spectrophotometer with ethanol as solvent.

3) *HPLC.* HPLC was carried out according to a previous paper (1).

2. Animals

Male rats of Wistar strain weighing 100–120 g fed a normal diet (MF diet from Oriental Yeast Co. containing 200 I.U. of vitamin D₃/100 g) were used. When the content of 7-DHC in the skin was estimated by the method proposed

in a previous paper (1), the average value was 84.5 $\mu\text{g/g}$.

3. UV irradiation experiments

1) *First experiment (in vitro experiment)*. Normal rats whose hair was shaved with an electric shaver were sacrificed and then about 10 cm² area of the skin was immediately stripped off from the back of each. After removing the fat tissue from the skin, it was fixed onto filter paper placed on a table at 4°C. The epidermis of the fixed skin was irradiated from the right upper side (distance, 25 cm) with an UV lamp (National Erythema lamp, Matsushita Electric Co.; main wavelength, 280–310 nm; quantum of irradiated energy, 85 $\mu\text{W/cm}^2/\text{min}$) for 30, 60 and 120 min. The irradiated skin was immediately cut into small pieces and then treated according to the proposed method described in the following section to determine vitamin D₃.

2) *Second experiment (in vivo experiment)*. Normal rats whose hair was shaved with an electric shaver were put in small cages (a rat was put in a cage) and irradiated from the right upper side (distance, 25 cm) with the same UV lamp mentioned above under the same conditions as described in the first experiment. The rats were immediately sacrificed and then about 10 cm² area of the skin was stripped off from the back of each. After removing the fat tissue from the skin, it was immediately cut into small pieces and then treated according to the proposed method in the following section to determine vitamin D₃.

4. Determination of vitamin D₃ in rat skin

1) *Extraction of lipids*. The small pieces of rat skin obtained by either the first or the second experiment were shaken under argon gas with about 300 ml of chloroform–methanol (1: 1) for 48 hr at 4°C in order to extract the lipids. The solvent was evaporated under reduced pressure below 40°C (evaporation of solvent in this work was always carried out under this condition) and a milk-yellow oily residue was obtained.

2) *Saponification and isolation of the unsaponifiable matter*. The residue obtained above was saponified and then the unsaponifiable matter was isolated according to the directions described in the previous paper (1). One hundred ml of benzene was used to isolate the unsaponifiable matter and the separated benzene layer was filtered through a Whatman 1PS filter paper.

3) *HAP Sephadex column chromatography*. Exactly 90 ml of the filtrate obtained above was placed in a round bottom flask and evaporated to dryness. The resulting residue was dissolved with adequate amounts of β -carotene as a marker in small quantities of a mixture of *n*-hexane and chloroform (65: 35) and then applied to a HAP Sephadex column (45 $\times$ 2.1 cm *i.d.*) prepared according to ELLINGBOE *et al.* (7) and JONES *et al.* (8). The column was eluted with the same solvent mixture with flow rate of 1.5 ml/min and each 3 ml was fractionated. The vitamin D₃, including pre-D₃ fractions (fraction no. 48–60), were joined and evaporated to dryness.

4) *HPLC*. The residue obtained above was dissolved in 1.0 ml of an internal standard solution (α -naphthol was dissolved in *n*-pentane to obtain a concentration of 10 $\mu\text{g/ml}$). Five μl of the sample solution obtained thus was taken with a microsyringe and applied to the HPLC described in the previous paper (1).

Five μl of a vitamin D_3 standard solution prepared by dissolving vitamin D_3 in the internal standard solution to obtain a concentration of $S \mu\text{g/ml}$ (usually 5 $\mu\text{g/ml}$) was similarly applied to the HPLC.

5) *Calculation*. The peak height ratios of vitamin D_3 to α -naphthol were estimated on the HPLC chromatograms obtained from the sample and vitamin D_3 solutions, respectively. The contents of vitamin D_3 in fat tissue-removed rat skin was calculated by the following formula:

$$\text{Content of vitamin D}_3 (\mu\text{g/g}) = \frac{P_{\text{sa}}}{P_{\text{st}}} \times \frac{V \times S}{W} \times \frac{100}{90}$$

P_{sa} : Peak height ratio of vitamin D_3 to α -naphthol on the HPLC Chromatogram of sample solution

P_{st} : Peak height ratio of vitamin D_3 to α -naphthol on the HPLC chromatogram of vitamin D_3 standard solution

V : Volume of *n*-pentane in the final sample solution for the HPLC (This is 1.0 in the above case)

S : Concentration of vitamin D_3 in the vitamin D_3 standard solution ($\mu\text{g/ml}$)

W : Weight of the pieces of fat tissue-removed rat skin taken for the determination (g)

The ratio, 100/90: See the methods for determination of vitamin D_3 in rat skin.

RESULTS AND DISCUSSIONS

1. Chromatographic behavior of pre- D_3 , vitamin D_3 and 7-DHC on the HPLC

A mixed solution of α -naphthol, pre- D_3 , vitamin D_3 and 7-DHC in *n*-pentane having the concentrations of 10, 2, 2 and 20 $\mu\text{g/ml}$, respectively, was prepared and then applied to the HPLC according to EXPERIMENTAL. The chromatogram is shown in Fig. 1 and the relative retention times of pre- D_3 , vitamin D_3 and 7-DHC

Fig. 1. HPLC chromatogram of authentic samples of α -naphthol, pre- D_3 , vitamin D_3 and 7-DHC (α -naphthol: internal standard).

to α -naphthol (retention time: 8.04 min) were 1.43, 2.27 and 2.88, respectively.

2. Calibration curve of vitamin D₃

Vitamin D₃ was dissolved with 10 μ g of α -naphthol in 1 ml of *n*-pentane to make the solutions whose weight ratios of vitamin D₃ to α -naphthol were 0.1, 0.2, 0.3, 0.4 and 0.5, respectively. The solutions were applied to the HPLC in order to obtain a calibration curve. The peak height ratios showed linear correlations with the weight ratios (vitamin D₃/ α -naphthol) as shown in Fig. 2.

Fig. 2. Calibration curve of vitamin D₃ by the HPLC method. (α -naphthol: internal standard)

3. Chromatographic behavior of vitamin D₃, pre-D₃ and 7-DHC on the HAP Sephadex column

Suitable amounts of vitamin D₃, pre-D₃, 7-DHC and β -carotene (marker) were dissolved in small quantities of *n*-hexane-chloroform (65:35) and then applied to the HAP Sephadex column chromatography described in EXPERIMENTAL. The chromatogram obtained by estimating the UV absorption of each fraction at 280 nm is shown in Fig. 3. β -Carotene used as a marker was eluted in the fraction no. 20, while vitamin D₃ and pre-D₃ which were not separated to one another in the chromatographic condition were eluted in the fractions between no. 48 and 60

Fig. 3. HAP Sephadex column chromatogram of authentic samples of pre-D₃, vitamin D₃ and 7-DHC (* marker: β -carotene).

(maximum: fraction no. 54–55). 7-DHC which was separated from vitamin D₃ and pre-D₃ was eluted in the fractions between no. 70 and 82 (maximum: fraction no. 75–76). The corresponding fractions were individually joined and then applied to the HPLC. The results showed that each joined fraction contained the corresponding compounds. The joined fractions of vitamin D₃ including pre-D₃ and 7-DHC are denoted as vitamin D₃ and 7-DHC fractions, respectively.

4. Chromatographic behavior of the unsaponifiable matter of lipids obtained from normal rat skin on the HPLC

Lipids were extracted from the normal rat skin without UV irradiation and the unsaponifiable matter was isolated according to EXPERIMENTAL. The HPLC chromatogram of the unsaponifiable matter is shown in no. 1 of Fig. 4. The

Fig. 4. HPLC chromatograms of the unsaponifiable matter of lipid extracts of non-irradiated rat skin and the purified vitamin D₃ fraction obtained by applying them to the HAP Sephadex column (α -naphthol: internal standard). no. 1: Chromatogram of the unsaponifiable matter. no. 2: Chromatogram of the purified vitamin D₃ fraction. no. 3: Chromatogram of the purified vitamin D₃ fraction obtained from the mixture of rat skin lipid extracts and standard vitamin D₃. no. 4: Chromatogram of standard vitamin D₃.

peak, whose retention time agreed with that of 7-DHC, was confirmed to be due to 7-DHC as reported in the previous paper (1), whereas the peaks corresponding to vitamin D₃ and pre-D₃ could not be detected because the positions were disturbed by many other peaks. When the unsaponifiable matters were purified by

Table 1. Yield of vitamin D₃ in rat skin in the first (*in vitro*) and second (*in vivo*) experiments.

Irradiation time (min)	Yield of vitamin D ₃ (ng/g of skin)	
	First experiment (<i>in vitro</i>)	Second experiment (<i>in vivo</i>)
0	n.d.	n.d.
30	3.7	4.1
60	7.4	8.2
120	12.5	14.7

Note 1) Values are shown as the mean of duplicate estimations. 2) n.d., not detected (<2 ng/g of skin).

applying to the HAP Sephadex column chromatography and then the vitamin D₃ fraction was applied to the HPLC, the interfered peaks were eliminated but no peak besides the internal standard was observed as shown in no. 2 of Fig. 4. Therefore, we concluded that the normal rat skin without UV irradiation contained neither vitamin D₃ nor pre-D₃. Then, 10 µg of authentic vitamin D₃ was added to the lipid extracts obtained from the normal rat skin and then treated according to EXPERIMENTAL. As shown in no. 3 of Fig. 4, the peak corresponding to vitamin D₃ was observed in the HPLC chromatogram and the recovery test gave satisfactory data. The results showed that the proposed method was useful for the determination of vitamin D₃ in rat skin.

5. First UV irradiation experiment (*in vitro* experiment)

The skin stripped off from the normal rats was irradiated according to the procedure described in the first experiment (*in vitro* experiment) of EXPERIMENTAL. The irradiated skin was treated according to the proposed method for the determination of vitamin D₃. The estimated values of vitamin D₃ and the HPLC chromatograms are shown in Table 1 and nos. 1–3 of Fig. 5, respectively. The mono peak, whose retention time agreed with that of vitamin D₃, was observed besides the peak of the internal standard in each chromatogram and the peak heights were increased according to the increase of irradiation energy as shown in nos. 1–3 of Fig. 5. Co-chromatography performed by mixing the sample solution (120 min irradiated) with authentic vitamin D₃ also gave a mono peak as shown in no. 4 of Fig. 5. It was concluded from these results that the observed mono peaks in nos. 1–3 of Fig. 5 were due to vitamin D₃ which was photochemically formed from 7-DHC in the rat skin.

6. Second UV irradiation experiment (*in vivo* experiment)

The normal rats were irradiated and then treated according to the procedure described in the second experiment (*in vivo* experiment). The skin obtained thus was treated according to the proposed method for the determination of vitamin D₃. The estimated values of vitamin D₃ and the HPLC chromatograms are shown in

Fig. 5.

Fig. 6.

Fig. 5. HPLC chromatograms in the first (*in vitro*) experiment. Procedure is referred to EXPERIMENTAL. (α -naphthol: internal standard) no. 1: Chromatogram on the 30 min irradiated skin. no. 2: Chromatogram on the 60 min irradiated skin. no. 3: Chromatogram on the 120 min irradiated skin. no. 4: Co-chromatogram on the mixture of no. 3 and standard vitamin D_3 . no. 5: Chromatogram on standard vitamin D_3 .

Fig. 6. HPLC chromatograms in the second (*in vivo*) experiment. Procedure is referred to EXPERIMENTAL. (α -naphthol: internal standard). no. 1: Chromatogram on the 30 min irradiated rat. no. 2: Chromatogram on the 60 min irradiated rat. no. 3: Chromatogram on the 120 min irradiated rat. no. 4: Co-chromatogram on the mixture of no. 3 and standard vitamin D_3 . no. 5: Chromatogram on standard vitamin D_3 .

Table 1 and nos. 1–3 of Fig. 6, respectively. The co-chromatogram performed on the mixture of the sample solution (120 min irradiated) and authentic vitamin D_3 is shown in no. 4 of Fig. 6. The results on the second experiment were almost identical with the corresponding results on the first experiment and each mono peak observed in nos. 1–3 of Fig. 6 was also confirmed as that of vitamin D_3 .

The results of the two experiments (*in vitro* and *in vivo*) induced the conclusion that the 7-DHC existed in rat skin was converted to vitamin D_3 (probably *via* pre- D_3) by UV irradiation. It was also deduced that the *in vivo* conversion mechanism might be similar to the *in vitro* conversion because there were little differences between the corresponding estimated values of vitamin D_3 in the two

experiments and the values increased according to the increase of irradiation energy.

The peak due to pre-D₃ was not observed in the HPLC chromatograms of the two experiments. It is well-known that the UV irradiation of 7-DHC first gives pre-D₃ which is thermally converted to vitamin D₃ until the equilibrium of pre-D₃ and vitamin D₃ is reached (3). KEVERLING BUISMAN *et al.* (9) determined the first order rates of the reversible isomerization between pre-D₃ and vitamin D₃ and calculated the rate constants. From these rate constants, they calculated the times to reach $0.99 \times$ the equilibrium at various temperature and the ratios of pre-D₃ and vitamin D₃ in the equilibrated solutions. Since the presently proposed method for the determination of vitamin D₃ in rat skin included the saponification procedure by refluxing in ethanolic KOH solution in order to eliminate interfering substances, most of the pre-D₃ first produced in the irradiated rat skin might be thermally isomerized into vitamin D₃ during saponification. When the ratio of the compounds in the saponified solution mentioned above was calculated according to KEVERLING BUISMAN *et al.* (9), the solution should contain about 20% of pre-D₃ and about 80% of vitamin D₃. Since the peak due to vitamin D₃ itself was observed as small one as shown in nos. 1–3 of Figs. 5 and 6, the peak due to pre-D₃ remained in the solution might be too small to be detected. Recently, PETROVA *et al.* (5) and HOLICK *et al.* (6) showed by using ³H-7-DHC that the sterol in rat skin was first converted to pre-D₃ by UV irradiation, which might be gradually thermal-isomerized into vitamin D₃ at body temperature before showing the biological activity. Their results supported our conclusion and assumption.

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