BINDING PROTEINS FOR 1,25-DIHYDROXYCHOLECARCIFEROL AND 25-HYDROXYCHOLECARCIFEROL

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The proteins participating in translocation of vitamin D₃ metabolites inside the cell of the rat intestinal mucosa were identified by sucrose density gradient ultracentrifugation.

Sixteen hours after an intracardial administration of radioactive vitamin D₃ (100 I. U.) to the vitamin D deficient rat, 3.5% of the administered radioactivity was distributed in the intestinal mucosa, and about half of this amount in the crude nuclear fraction. In this case, approximately 60% of the radioactivity extracted from the intestinal mucosa was observed to be 1,25-DHCC and about 20% as 25-HCC, respectively.

In an addition of radioactive 1,25-DHCC or 25-HCC to the intestinal mucosa in vitro, both 1,25-DHCC and 25-HCC were incorporated into the nuclear fraction of rat intestinal mucosa. However, little of the D₃ was found in the nuclei.

Furthermore, for transport of vitamin D₃ metabolites into the nuclei in cell free system of the rat intestinal mucosa, the presence of the cytoplasmic fraction was found to be essential. Subsequently, the individual binding proteins specific to 1,25-DHCC and 25-HCC were identified in the cytoplasmic fraction of rat intestinal mucosa by means of sucrose density gradient ultracentrifugation. The sedimentation constants of these two proteins were approximately 5.3 and 6.3, respectively. However, the binding protein specific to vitamin D₃ was not found in the cytoplasmic fraction by sucrose density gradient ultracentrifugation without prior treatment by Sephadex G-200 column chromatography.

From these results, it was concluded that the cytoplasmic fraction of rat intestinal mucosa contained the specific binding proteins participating in the intracellular translocation of 1,25-DHCC and 25-HCC in rat intestinal mucosa.

Abbreviations used in this paper are: 25-HCC, 25-hydroxycholecarciferol; 1,25-DHCC, 1,25-dihydroxycholecarciferol; G-6-P, glucose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; Vo, void volume; Ve, elution volume.
Evidences have been accumulated that vitamin D regulates calcium absorption from intestines of rats and chickens through the induction of calcium-binding protein (1, 2). Recent studies of vitamin D metabolism have demonstrated that vitamin D₃ is converted to 25-HCC in the liver (3-6) and then to 1,25-DHCC in the kidney (7-11) prior to the elevation of calcium transport. After injection of radioactive vitamin D₃ or 25-HCC into the rachitic chick and rat, both 1,25-DHCC and 25-HCC are transported to the intestinal mucosa (12-14). These hydroxylations (activation of vitamin D₃) are now considered to be essential steps for physiological functions of target tissues such as the intestine and the bone.

On the other hand, it has been shown that several steroid hormones are bound to the specific receptor in the cytoplasmic fraction of each target tissue before the steroids show their physiological function (15-18), and that the steroid-receptor complex has a two-step mechanism in the function of steroid transport from the cytoplasmic fraction to the nuclei (15-17). Intracellular transport of vitamin D₃ metabolites will be considered to be similar to the transport of steroid hormones.

This paper reports that radioactivities corresponding to both 1,25-DHCC and 25-HCC were found in the intestinal mucosa after injection of radioactive vitamin D₃ to the vitamin D deficient rat, and radioactive metabolites of vitamin D₃ were also incorporated into the cytoplasmic fraction, when incubated with the intestinal mucosa in vitro. Subsequently, identification of specific binding proteins for individual metabolites of vitamin D₃ was attempted in the cytoplasmic fraction of the rat intestinal mucosa by means of sucrose density gradient ultracentrifugation. This communication also presents evidence for partial characterization and physiological function of these binding proteins which play a role in the transport of vitamin D₃ metabolites from the cytosol into the nuclei.

METHODS

1. Animals. Female Wister rats weighing 50 to 60 g were kept in a dark room at 22°C and fed rachitogenic diet No. 2 U.S.P. (Nutritional Biochemical Co.) for 4 weeks (vitamin D deficient rat). One-day-old Neochroth cockerels were raised on a vitamin D deficient diet according to OMDAHL et al. (19), and rachitic chickens after 3 to 4 weeks were used for experiment. Animals were starved for 16 hr prior to injection or decapitation.

2. Preparation of subcellular fractions. The rat was killed by decapitation and the small intestine was immediately removed. It was slit open, rinsed 3 times with 0.9% sodium chloride solution and once with 0.25 M sucrose in 0.01 M Tris-HCl buffer (pH 7.4). After blotting by filter paper, the mucosa layer was scraped free from the serosa layer by using a microscopic slide. The intestinal mucosa preparation was then homogenized with 4 volumes of 0.25 M sucrose
in 0.01 M Tris-HCl buffer (pH 7.4) by a Potter-Elvehjem homogenizer with a Teflon pestle. After filtering through double layers of nylon cloth (50 mesh), cellular components were obtained by the modification of HOGEBOOM and SCHNEIDER (20) as follows: Crude nuclei were isolated by centrifugation at 800 × g for 10 min. Mitochondria were isolated from the resulting supernatant at 10,000 × g for 20 min. The supernatant after sedimentation of mitochondria was then recentrifuged in a Hitachi preparative ultracentrifuge 55 PA at 105,000 × g for 60 min to remove the microsomal fraction.

Purified nuclei were isolated according to the slightly modified method of HAUSSLER et al. (21) as follows: The pellet sedimented at 800 × g for 10 min, was resuspended and washed twice with 0.25 M sucrose containing 0.05 M Tris-HCl (pH 7.4), 0.025 M KCl and 0.005 M MgCl₂ (0.25 M sucrose-TKM), and then once with 0.1 % triton X-100 in 0.25 M sucrose-TKM. This nuclear pellet was mixed with 2.4 M sucrose-TKM to yield a final molarity of 1.75 M for sucrose. This suspension was centrifuged at 65,000 × g for 60 min in a Hitachi preparative ultracentrifuge 55 PA, and the sediment was washed with 0.25 M sucrose-TKM. The resulting pellet was used as purified nuclei.

3. Extraction and identification of vitamin D₃ and its metabolites. Vitamin D₃ and its metabolites were extracted by 4 volumes of chloroform, methanol (1:1, v/v) from the intestine, and its subcellular fraction. The chloroform phase was removed, and the residue was reextracted with 2 volumes of chloroform (22). The combined chloroform phase was evaporated and dissolved in 2 to 3 ml of chloroform: n-hexane (65:35, v/v). The generated vitamin D₃ metabolites were analyzed in a Sephadex LH-20 column (2.4 × 34 cm) using the method of HOLICK and DeLUCA (23). The column was eluted successively with the same solvent, and 5 ml of effluents were collected (flow rate; 0.7 ml/min). Fifty µl was used from each fraction to determine radioactivity.

4. Intracardial administration of vitamin D₃-U⁻³H. One hundred I. U. (specific activity 0.05 µCi/100 I.U.) of labeled vitamin D₃-U⁻³H was dissolved in 0.2 ml of propylene glycol and administered intracardially to vitamin D deficient rats. After 4, 8 and 16 hr, the rats were killed by decapitation.

5. In vitro incorporation of vitamin D₃ and its metabolites into subcellular fraction of the intestinal mucosa. The intestinal mucosa of vitamin D deficient rats was suspended in 4 volumes of Krebs-Ringer-Bicarbonate buffer (pH 7.4) (KRB) containing 10 µg/ml glucose, and incubated with 8.5 × 10⁻⁹ M of tritium labeled vitamin D₃ or its metabolites at 37°C for indicated periods. After incubation, the mucosa was washed 3 times with 10 volumes of KRB buffer and then subjected to subcellular fractionation. Vitamin D₃ and its metabolites were extracted according to the method described above.

On the other hand, the mucosal suspension was preincubated at 2°C for 10 min in 4 volumes of KRB buffer containing 10 µg/ml glucose and 8.3 × 10⁻⁹ M of labeled vitamin D₃ or its metabolites. After the preincubation, the mucosa was
washed and transferred to fresh medium, free from labeled materials, for the incubation at 37°C.

6. Translocation of 1,25-DHCC and 25-HCC from cytosol into nuclei in cell free system of rat intestinal mucosa. The purified nuclei were suspended in 9 volumes of 0.25 M sucrose in a Tris-HCl buffer (pH 7.4). One ml of this suspension was incubated with 2 pmoles of labeled steroids in the presence of 1 ml of cytoplasmic fraction (about 5.5 mg/ml protein) at 37°C or 2°C. After incubation, the reaction mixture was immediately chilled and centrifuged at 2,000×g for 20 min. The pellet was washed with 2 ml of 0.25 M sucrose-TKM and suspended in 2 ml of distilled water. Extraction of vitamin D₃ and its metabolites from the nuclear pellet was carried out by the same method as described above. Each chloroform phase was combined and evaporated to dryness in a counting vial. In some experiments, either an albumin solution, which was adjusted to the same protein concentration as the cytoplasmic fraction, or a 0.25 M sucrose solution was added to the incubation medium instead of the cytoplasmic fraction, and then it was incubated for 30 min at 37°C.

7. Analyses of binding proteins for vitamin D₃ metabolites.

1) Binding assay in cytoplasmic fraction with labeled vitamin D₃ and its metabolites. Binding activities of the cytoplasmic fraction were assayed using tritium labeled vitamin D₃ and its metabolites. Two ml of the cytoplasmic fraction containing approximately 5.5 mg/ml protein were incubated with 3.1×10⁻¹¹ moles (3.72×10⁻⁸ Ci) of radioactive materials for 20 min at 2°C (total volume 2 ml) and subjected to both sucrose density gradient ultracentrifugation and gel filtration of Sephadex G-200 column.

2) Sucrose density gradient ultracentrifugation. 0.2 ml of sample were layered on 4.8 ml of gradient solution ranging from 5 to 20% in sucrose in a 0.01 M Tris-HCl buffer (pH 7.4), and centrifuged for 12 hr at 216,000×g at 2°C by a Hitachi preparative ultracentrifuge 65 P with an RPS-65-TA rotor. The centrifuged tubes were punctured at the bottom and 12 drops each were collected directly in a counting vial to determine its radioactivity, or in a test tube to determine protein concentration. The sedimentation constant was estimated by the method of MARTIN and AMES (25) using bovine serum albumin (4.3 S, MW. = 67,000) as a standard.

3) Gel filtration. The Sephadex G-200 column (1.4×55 cm) was equilibrated with 0.01 M Tris-HCl buffer (pH 7.4). Three ml of protein solution were applied to the column and eluted with the same buffer. Flow rates were 3 to 4 drops per minute, and each 3 ml were collected.

8. Other determinations. Radioactivity was measured by a Packard Tri-Carb scintillation spectrometer, Model 3380. Chloroform extract was evaporated and then dissolved in 10 ml of scintillation solution containing 5 g of 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyloxazoyl)-binzene (dimethyl POPOP) 0.1 g per liter of toluene. The counting efficiency under these conditions was 53
to 54%. The samples obtained from sucrose density gradient ultracentrifugation and gel filtration (0.5 ml) were mixed with 10 ml of dioxane-based scintillation solution containing 5 g of PPO, 0.1 g of dimethyl POPOP and 100 g of naphthalene per liter of dioxane. The counting efficiency of these aqueous samples was 34 to 36%.

Protein was determined by the method of LOWRY et al. (26) using bovine serum albumin as a standard. DNA was extracted from the nuclear pellet by the method of MIZUNO et al. (27), a modification of SCHMIDT-THANNHAUSER-SCHNEIDER (28), and measured by diphenylamine reaction with calf thymus DNA as a standard (29).

9. Radioactive materials and chemicals. Vitamin D$_3$-U-3H (specific activity: 540 mCi/mmmole) and 25-HCC-26,27-3H (specific activity: 1.2 Ci/mmmole) were obtained from New England Nuclear Co., U.S.A., and uniformly labeled tritium vitamin D$_3$ (specific activity 0.05 µCi/100 I.U.) from Daiichi Chemical Co.

1,25-DHCC was prepared from 25-HCC-26,27-3H according to the method of FRASER and KODICEK (9). Kidney was homogenized with 4 volumes of 0.32 M sucrose in 0.05 M Tris-HCl buffer (pH 7.4) (protein about 22 mg/ml). The incubation mixture contained 2 ml of kidney homogenate, 1.3 x 10$^{-10}$ moles (1.56 x 10$^{-7}$ Ci) of 25-HCC-26,27-3H, 8.4 µmoles of MgCl$_2$, 45 µmoles of L-malate, 90 µmoles of nicotinamide, 1.8 µmoles of NADP, 18 µmoles of G-6-P, and 2 I.U. of G-6-PDH (total volume: 4.5 ml). Incubation was carried out at 37°C for 60 min under oxygen. Two incubation media were combined and extracted by 4 volumes of chloroform: methanol (1:1, v/v). The residue was reextracted with 2 volumes of chloroform. Separation and purification of 1,25-DHCC were carried out by a Sephadex LH–20 column (2.4 x 34 cm) using the method of HOLICK and DELUCA (23). The column was eluted successively with chloroform: n-hexane (65: 35, v/v), and effluents of 5 ml were collected (flow rates 0.7 ml/min). Fifty µl was removed from each fraction to analyze radioactivity. Tubes containing 1,25-DHCC were collected, evaporated and then redissolved in small volume of methanol, which was rechromatographed in a Sephadex LH–20 column (1.1 x 40 cm) equilibrated with the same solvent. Each 2 ml were collected. Tubes containing the 1,25-DHCC were pooled and evaporated. The 1,25-DHCC obtained was further purified by thin layer chromatography using silica gel G developed with ethylacetate (solvent front 17 cm) (24).

Pronase was purchased from Calbiochem Co., U.S.A., and deoxyribonuclease (DNase) and ribonuclease (RNase) from Worthington Biochemical Co., U.S.A. Bovine serum albumin was obtained from Nutritional Biochemical Co. and cholesterol, 17-β-estradiol and testosterone from Teikoku Zoki Co. Vitamin D$_3$ was kindly supplied from Dr. Katsui, Eizai Co., after testing its biological activity.
RESULTS

1. Distribution of labeled vitamin D₃ and its metabolites in the intestinal mucosa after intracardial administration of vitamin D₃-U³H to the vitamin D deficient rat

Vitamin D deficient rats given 100 I. U. of vitamin D₃-U³H intracardially were killed by decapitation at 4, 8 and 16 hr after injection.

The radioactivity recovered in the intestinal mucosa was 5.8% and 3.5% at 8 and 16 hr after the injection, respectively. Distribution of these radioactivities in the subcellular fraction of the rat intestinal mucosa was 44% in the cytoplasmic fraction and 41% in the nuclear fraction 4 hr after the injection (Table 1). It was 24% in the cytoplasmic fraction and 52% in the nuclear fraction 16 hr after the injection. Radioactivity detected in the cytoplasmic fraction at the primary stage decreased later in contrast to that in the nuclear fraction. Radioactivity in the mitochondrial or microsomal fraction was about 10% which remained in the nearly same percentage at 4, 8 and 16 hr after the administration.

Table 1. Incorporation of radioactivity into subcellular fractions of rat intestinal mucosa after an intracardial dose of vitamin D₃-U³H.

Rats were fed a vitamin D deficient diet for 4 weeks prior to an injection of 100 I. U. of vitamin D₃-U³H (S.A. 0.05 μCi/100 I. U.) in 0.2 ml of propylene glycol. The rats were decapitated at 4, 8 or 16 hr after the injection, and their intestinal mucosa was removed. Fractionation and extraction procedures are described in the text. Results are expressed as radioactivity recovered in each subcellular fraction and as percentage of the radioactivity in the homogenate. The values are averages of three experiments at 4 and 8 hr and those of four experiments at 16 hr.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Recovered activity (dpm)</th>
<th>% of total recovered activity</th>
<th>Recovered activity (dpm)</th>
<th>% of total recovered activity</th>
<th>Recovered activity (dpm)</th>
<th>% of total recovered activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>5,830</td>
<td>100</td>
<td>6,380</td>
<td>100</td>
<td>3,850</td>
<td>100</td>
</tr>
<tr>
<td>Crude nuclei</td>
<td>2,390</td>
<td>41</td>
<td>3,130</td>
<td>49</td>
<td>2,000</td>
<td>52</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>410</td>
<td>7</td>
<td>700</td>
<td>11</td>
<td>420</td>
<td>11</td>
</tr>
<tr>
<td>Microsomes</td>
<td>470</td>
<td>8</td>
<td>770</td>
<td>12</td>
<td>510</td>
<td>13</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>2,560</td>
<td>44</td>
<td>1,780</td>
<td>28</td>
<td>920</td>
<td>24</td>
</tr>
</tbody>
</table>

The radioactivity in the lipid fraction of the intestinal mucosa 16 hr after the administration of vitamin D₃-U³H was also chromatographed by a Sephadex LH-20 column (2.4×34 cm) (Table 2). The percent distribution of the radioactivity in the intestinal mucosa was 58% as 1,25-DHCC, 19% as 25-HCC and 20% as vitamin D₃, respectively. These results show that both 25-HCC, which was metabolized in the liver, and 1,25-DHCC, which was further metabolized in the kidney, were transported in the intestinal mucosa when vitamin D₃ was administered intracardially.
Table 2. Distribution of labeled vitamin D₃ and its metabolites in an intestinal mucosa 16 hr after an intracardial dose of vitamin D₃-U-³H to vitamin D deficient.

Vitamin D deficient rats were injected with 100 I.U. of vitamin D₃-U-³H (0.05 µCi/100 I.U.) intracardially 16 hr prior to killing. The extraction of total lipid from intestinal mucosa homogenate and the column chromatography procedure for the separation of vitamin D₃ and its metabolites in the lipid extract are described in the text. Lipid extracts from two rats were combined and applied to a Sephadex LH-20 column. All data are expressed as percentages of total radioactivity eluted from the column. Each value represents the average of three determinations.

<table>
<thead>
<tr>
<th></th>
<th>D₃ ester</th>
<th>D₃</th>
<th>25-HCC</th>
<th>1,25-DHCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of radioactivity recovered</td>
<td>3.3</td>
<td>17.4</td>
<td>16.8</td>
<td>52.7</td>
</tr>
<tr>
<td>dpm of radioactivity recovered</td>
<td>270</td>
<td>1,430</td>
<td>1,380</td>
<td>4,320</td>
</tr>
</tbody>
</table>

Total radioactivity recovered 8,200 dpm.

2. Subcellular distribution of radioactivity in the intestinal mucosa incubated with labeled vitamin D₃ or its metabolites in vitro

After the incubation of intestinal mucosa with labeled vitamin D₃, 25-HCC or 1,25-DHCC, the subcellular distribution of radioactivity was analyzed. During the 60 min of incubation, increases of the incorporation of each substrate into the cytoplasmic fraction were not observed. Incorporation ratio of 1,25-DHCC, 25-HCC and vitamin D₃ into the cytoplasmic fraction was about 8:6:1 (Table 3).

However, relatively large quantities of 1,25-DHCC were incorporated into the nuclear fraction and this incorporation increased as the incubation period increased. It was $5.91 \times 10^{-13}$ moles per mg of DNA at 2 min incubation. The increased rate of 1,25-DHCC incorporation into the nuclear fraction was about 1.8 and 3.5 times at 5 and 60 min of the incubation, respectively. The incorporation of 25-HCC into the nuclear fraction also showed an increase during the incubation, but its incorporation was about half the level of that of 1,25-DHCC. Only a little of vitamin D₃ was incorporated and its level was less than 15% of that of 1,25-DHCC. Both 1,25-DHCC and 25-HCC were incorporated into the nuclei of intestinal mucosa.

The translocation of radioactive vitamin D₃ and its metabolites into the nuclei of rat intestinal mucosa was further investigated. The intestinal mucosa was preincubated with labeled vitamin D₃ or its metabolites at 2°C for 10 min. This mucosa was washed, transferred to the fresh medium free from labeled substrates and incubated further at 37°C. Incorporation of 1,25-DHCC, 25-HCC and vitamin D₃ into the cytoplasmic fraction during preincubation was about 7:5:1 (Table 4). The incorporation of 1,25-DHCC into the cytoplasmic fraction rapidly declined up to 5 min and kept gradually decreasing until 60 min. The incorporation of 25-HCC decreased slowly up to 60 min. In the nuclear fraction, the incorporation of 1,25-DHCC and 25-HCC gradually increased, and concentrations of 1,25-DHCC and 25-HCC were $10.48 \times 10^{-13}$ and $6.43 \times 10^{-13}$ moles/mg.
Table 3. Incorporation of 1,25-DHCC, 25-HCC and vitamin D₃ into subcellular fractions of rat intestinal mucosa in vitro.

The intestinal mucosa of a vitamin D deficient rat was incubated with $8.5 \times 10^{-9}$ M of 1,25-DHCC-26,27-3H, 25-HCC-26,27-3H or vitamin D₃-1,2-3H at 37°C in a Krebs-Ringer-Bicarbonate buffer (pH 7.4) containing 10 μg/ml glucose for indicated periods. After washing the mucosa 3 times with the Krebs-Ringer-Bicarbonate buffer, the mucosa was subjected to subcellular fractionation and lipid extraction as described in the text. Each value represents the average of four determinations.

<table>
<thead>
<tr>
<th>Incubation times (minutes)</th>
<th>Cytoplasm $\times 10^{-16}$ moles/mg protein</th>
<th>Nuclei $\times 10^{-13}$ moles/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1,25-DHCC</td>
<td>25-HCC</td>
</tr>
<tr>
<td>2</td>
<td>1.75 ± 0.13</td>
<td>1.35 ± 0.14</td>
</tr>
<tr>
<td>5</td>
<td>1.91 ± 0.16</td>
<td>1.34 ± 0.10</td>
</tr>
<tr>
<td>15</td>
<td>1.87 ± 0.10</td>
<td>1.36 ± 0.064</td>
</tr>
<tr>
<td>60</td>
<td>1.71 ± 0.12</td>
<td>1.52 ± 0.081</td>
</tr>
</tbody>
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Table 4. Transport of 1,25-DHCC, 25-HCC and vitamin D₃ from cytoplasm into nuclei in rat intestinal mucosa in vitro.

The intestinal mucosa of a vitamin D deficient rat was reincubated with $8.3 \times 10^{-8}$ M of 1,25-DHCC-26,27-3H, 25-HCC-26,27-3H or vitamin D₃-1,2-3H for 10 min at 2°C in a Krebs-Ringer-Bicarbonate buffer (pH 7.4) containing 10 μg/ml glucose. After washing, the preincubated mucosa was transferred into a fresh medium without vitamin D₃ or its metabolites and incubated at 37°C for various times as indicated. Subcellular fractionation of nuclei and cytoplasm was carried out as described in the text. The values and their standard errors were obtained from four determinations.

<table>
<thead>
<tr>
<th>Incubation times (minutes)</th>
<th>Cytoplasm $\times 10^{-18}$ moles/mg protein</th>
<th>Nuclei $\times 10^{-13}$ moles/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1,25-DHCC</td>
<td>25-HCC</td>
</tr>
<tr>
<td>0</td>
<td>1.82 ± 0.11</td>
<td>1.21 ± 0.045</td>
</tr>
<tr>
<td>5</td>
<td>0.54 ± 0.047</td>
<td>1.09 ± 0.062</td>
</tr>
<tr>
<td>15</td>
<td>0.31 ± 0.024</td>
<td>0.94 ± 0.078</td>
</tr>
<tr>
<td>60</td>
<td>0.29 ± 0.031</td>
<td>0.87 ± 0.061</td>
</tr>
</tbody>
</table>
DNA over a period of 60 min incubation, respectively. However, little incorporation of vitamin D₃ into the nuclear fraction was observed, and the specific activity scarcely changed during the incubation.

From these results, it is considered that 1,25-DHCC and 25-HCC were first incorporated into the cytosol and then migrated to the nuclei.

3. Incorporation of vitamin D₃ metabolites from cytosol into nuclei in cell free system of rat mucosa

1,25-DHCC and 25-HCC were incubated with the cytoplasmic fraction in order to learn the role of cytosol in the process of translocation of vitamin D₃ metabolites into the nuclei. Incorporation of 1,25-DHCC and 25-HCC into the nuclei increased during incubation at 37°C (Fig. 1). 1,25-DHCC was also

![Fig. 1. Incorporation of 1,25-DHCC and 25-HCC from cytoplasm into nuclei with cell free system of rat intestinal mucosa. The purified nuclei were suspended in 9 volumes of 0.25 M sucrose in Tris-HCl buffer (pH 7.4). One ml of the nuclear suspension was incubated with 2×10⁻¹² moles of 1,25-DHCC-26,27-³H or 25-HCC-26,27-³H in the presence of a cytoplasmic fraction at 37°C or 2°C. After incubation, the mixture was immediately chilled and centrifuged for 20 min at 2,000×g at 2°C. The resulting pellet was washed with 2 ml of 0.25 M sucrose-TKM and extracted with 4 volumes of chloroform: methanol (1:1).]
incorporated to a greater extent and more rapidly than that of 25-HCC, as observed in the experiment using the intestinal mucosa. The amount of 1,25-DHCC incorporated into the nuclei was $8.0 \times 10^{-12}$ moles and that of 25-HCC was $4.5 \times 10^{-13}$ moles during 30 min of incubation. However, the incorporation of 1,25-DHCC and 25-HCC into the nuclei was not increased when the cytoplasmic fraction was substituted by 0.25 M sucrose or bovine serum albumin solution in which the protein concentration was equal to the cytoplasmic fraction (Fig. 2). The incorporation of 1,25-DHCC and 25-HCC into the nuclei was also not observed during the incubation at 2°C.

From these results, it was confirmed that the cytoplasmic fraction should be necessary for the translocation of vitamin D₃ metabolites into the nuclei in the rat intestinal mucosa.

4. Isolation of binding proteins for 1,25-DHCC and 25-HCC in cytoplasmic fraction of the rat intestinal mucosa

The cytoplasmic factor which induces migration of vitamin D₃ metabolites into the nuclei in intestinal mucosa was identified by means of sucrose density gradient ultracentrifugation. The cytoplasmic fraction was incubated with radioactive vitamin D₃ metabolites for 20 min at 2°C. After the incubation, the cytoplasmic fraction was layered in a tube which had a gradient concentration of sucrose ranging from 5 to 20%. The tubes were centrifuged at 216,000×g for 12 hr at 2°C. The peak of radioactivities corresponding to 1,25-DHCC was found at the 8th fraction from the top of the tube (Fig. 3A). When 25-HCC was
Fig. 3. Identification of cytoplasmic binding factors for 1,25-DHCC and 25-HCC by sucrose density gradient ultracentrifugation. 0.2 ml of cytoplasmic fraction containing about 5.5 mg/ml protein were reacted with $3.1 \times 10^{-12}$ moles of 1,25-DHCC-26,27-3H or 25-HCC-26,27-3H for 20 min at 2°C. The reaction mixture was layered on a 5-20% sucrose gradient and then centrifuged at 216,000×g at 2°C for 12 hr. The centrifuged tube was punctured at the bottom, and 12 drops each were collected in a scintillation vial. Bovine serum albumin was used as a standard.

incubated with the cytoplasmic fraction, a radioactive peak was found at the 11th fraction from the top of tube (Fig. 3B). When labeled vitamin D₃ was incubated with cytoplasmic fraction, a radioactive peak was not observed (Fig. 3C). These results suggest that both 1,25-DHCC and 25-HCC have their own specific binding factors in the cytoplasmic fraction of rat intestinal mucosa.

The cytoplasmic binding factors of 1,25-DHCC and 25-HCC were also analyzed by Sephadex G-200 column chromatography (1.4×55 cm). Radioactivity of 1,25-DHCC was found at the fraction where the $V_e/V_o$ ratio was 2.4. There are two other radioactive peaks at void volume and at the fraction of $V_e/V_o$ ratio 1.7 (Fig. 4A). These small radioactive peaks increased when a large amount of 1,25-DHCC was applied to the column. The last radioactive peak was confirmed to be a free form of 1,25-DHCC, since radioactive 1,25-DHCC was eluted at the
Fig. 4. Elution patterns of 1,25-DHCC and 25-HCC binding factor of rat intestinal cytoplasmic fraction in a Sephadex G-200 column. Two ml of rat intestinal cytoplasmic fraction (about 5.5 mg/ml protein) were incubated with $3.1 \times 10^{-11}$ moles of 1,25-DHCC-26,27-3H or 25-HCC-26,27-3H at 2°C for 20 min. After incubation, each reaction mixture was applied to a Sephadex G-200 column (1.4×55 cm) equilibrated with 0.01 M Tris-HCl (pH 7.4). Elution was carried out with the same buffer, and each 3 ml of effluent were collected. 0.2 ml of each fraction were examined for protein and 0.5 ml for radioactivity. ○--○ absorbency at 750 mμ per 0.2 ml of effluents, ●●● radioactivity (cpm) per 0.5 ml of effluents.

The biochemical nature of both cytoplasmic binding factors for 1,25-DHCC and 25-HCC was studied. The cytoplasmic fraction, which was treated with either pronase, DNase or RNase, was combined with 1,25-DHCC or 25-HCC, and then was analyzed to learn their binding activities by means of sucrose density gradient ultracentrifugation. The radioactivity bound to cytoplasmic binding factor for 1,25-DHCC decreased to 14% and the factor for 25-HCC also decreased to 17% after the pronase treatment. However, effects of DNase or RNase were
observed (Table 5). Both binding factors were composed only of protein without DNA and RNA. Subsequently, the cytoplasmic fraction heated at 50°C for 5 min decreased the binding activities of 1,25-DHCC and 25-HCC to about one-third of the control. These two binding factors were heat unstable.

Table 5. Effect of pronase, DNase, RNase and heat treatment on the binding of 1,25-DHCC and 25-HCC to rat intestinal cytoplasmic fraction.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1,25-DHCC</th>
<th>25-HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/binding fraction</td>
<td>cpm/binding fraction</td>
</tr>
<tr>
<td>Untreated</td>
<td>1,790</td>
<td>2,020</td>
</tr>
<tr>
<td>Pronase</td>
<td>250</td>
<td>340</td>
</tr>
<tr>
<td>DNase</td>
<td>1,810</td>
<td>1,900</td>
</tr>
<tr>
<td>RNase</td>
<td>1,770</td>
<td>1,980</td>
</tr>
<tr>
<td>Heat</td>
<td>540</td>
<td>520</td>
</tr>
</tbody>
</table>

From these results, it was demonstrated that binding proteins specific to 1,25-DHCC and 25-HCC from the cytosol into the nuclei.
Table 6. Effect of steroids on the binding of 1,25-DHCC or 25-HCC to cytoplasmic fraction of rat intestinal mucosa.

0.2 ml of rat intestinal cytoplasmic fraction containing 5.5 mg/ml protein was preincubated with $3.1 \times 10^{-12}$ moles of steroids for 20 min at 2°C and then incubated with $3.1 \times 10^{-12}$ moles of 1,25-DHCC-26,27-3H or 25-HCC-26,27-3H for 20 min at 2°C. The incubated mixture was layered on a 5–20% sucrose gradient and centrifuged as described in Fig. 3. Data are expressed as total radioactivity of 1,25-DHCC or 25-HCC binding fractions. Each value represents the average of three determinations.

<table>
<thead>
<tr>
<th>Steroid in incubated medium</th>
<th>1,25-DHCC</th>
<th>25-HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>1,770</td>
<td>1,870</td>
</tr>
<tr>
<td>Vitamin D$_3$</td>
<td>1,810</td>
<td>2,080</td>
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<td>Cholesterol</td>
<td>1,900</td>
<td>1,830</td>
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<tr>
<td>17-β-Estradiol</td>
<td>1,720</td>
<td>2,020</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1,840</td>
<td>1,960</td>
</tr>
</tbody>
</table>

DISCUSSION

It is now generally accepted that vitamin D$_3$ is converted to 25-HCC in the liver (5, 6) and, subsequently, to 1,25-DHCC in the kidney (7–10) before initiating intestinal calcium transport.

When a physiological dose (10 I.U.) of vitamin D$_3$ was administered to the chick, the radioactivity was concentrated in the crude nuclear fraction of the intestinal mucosa (21). When 100 I.U. of radioactive vitamin D$_3$ were administered to vitamin D deficient rats, 3.5% of the radioactivity was distributed in the intestinal mucosa and half of this amount located in the nuclear fraction 16 hr after the administration (Table 1). In this experiment, 60% of the radioactivity in the intestinal mucosa was obtained as 1,25-DHCC and 20% as 25-HCC (Table 2).

We have demonstrated that both 1,25-DHCC and 25-HCC were incorporated into the nuclei of the rat intestinal mucosa in vivo and in vitro. However, little vitamin D$_3$ was incorporated into the nuclei. These results indicate that the migration of 1,25-DHCC and 25-HCC into the nuclei requires the specific binding proteins in the cytoplasmic fraction. The molecular weights of these specific binding proteins for 1,25-DHCC and 25-HCC were approximately 82,000 and 106,000, as determined by the method of MARTIN and AMES (25) with bovine serum albumin (4.3 S, M. W. = 67,000) as a standard. These binding proteins were heat unstable and contained neither DNA nor RNA. In the previous papers (30, 31), we have shown, by Sephadex G–200 column chromatography, that the specific protein which binds to vitamin D$_3$ is detected in the cytoplasmic fraction of the rat intestinal mucosa. However, when the protein bound vitamin
D$_3$ was assessed by means of sucrose density gradient ultracentrifugation, the existence of vitamin D$_3$ binding protein was not observed. This discrepancy may result because several other proteins in the cytoplasmic fraction may aggregate during the Sephadex G–200 column chromatography, and will be bound to vitamin D$_3$.

The estrogen receptor in the uterine cytosol is well known as the specific protein participating in the transport of 17-β-estradiol from the cytosol into the nuclei (15, 16). The estrogen receptor dissociates into several subunits in 0.3–0.4 M KCl, and estrogen binds to one subunit. These subunits aggregate to make the native protein of 8–9 S when KCl is removed (32–35). When the cytoplasmic fraction of the intestinal mucosa is treated with 0.3 M KCl during both homogenization and Sephadex G–200 column chromatography, binding activity to vitamin D$_3$ is diminished regardless of the same binding activity of both 1,25-DHCC and 25-HCC. Further, the sterol carrier protein, which participates in the cholesterol biosynthesis in the liver, has been proposed to aggregate with large protein molecules through heat treatment (36). Estrogen receptor has been established to dissociate into several subunits, the 4 S protein of which binds to estrogen at 37°C (37). When the cytoplasmic fraction was treated at 50°C for 5 min, the molecular weight of individual binding proteins to 1,25-DHCC and 25-HCC remained constant, regardless of the remarkable decrease of binding capacity to them. However, no binding capacity to vitamin D$_3$ was observed under the same conditions. Therefore, the vitamin D$_3$ binding protein described in the previous papers (30, 31) would be the aggregating form of other cytoplasmic proteins.

Several authors have reported evidence that 1,25-DHCC should be considered one of the most active metabolites of vitamin D$_3$ and which functions more rapidly than 25-HCC or vitamin D$_3$ (38). It has been also demonstrated that polar metabolites of vitamin D$_3$ preferentially accumulate in the chromatin fraction and that the physiological function of the intestinal mucosa is initiated by 1,25-DHCC in the chromatin fraction (14). In our experiments, 25-HCC as well as 1,25-DHCC was also incorporated into the nuclei in vivo and in vitro. Furthermore, the specific binding protein of 25-HCC was observed in the cytoplasmic fraction of the rat intestinal mucosa, and its properties were different from that of 1,25-DHCC. These specific binding proteins obtained from the rat intestinal cytoplasmic fraction play a role in the transport of 1,25-DHCC and 25-HCC from the cytoplasmic fraction into the nuclei. These facts suggest that 1,25-DHCC and 25-HCC play a physiological role in the intestine.

TRUMMEL et al. have found the direct effect of 25-HCC, but not vitamin D$_3$, on calcium release from the bone without addition of 1,25-DHCC in vitro (39). MCNUTT and HAUSSLER have further observed that 25-HCC is significantly more effective than vitamin D$_3$ in the stimulation of body weight gain, and produce more weight gain than 1,25-DHCC when vitamin D$_3$ or its metabolites are
continuously given (40). On the other hand, CORRADINO has demonstrated that in the embryonic chick intestine, maintained in organ culture, 1,25-DHCC was by far the most potent inducer of the calcium binding protein, but vitamin D$_3$ itself is also a necessary inducer of calcium absorption (41). Hence, it would be possible that 25-HCC itself can also stimulate calcium transport in the intestine.

In our observations, 25-HCC was incorporated into the nuclei of the rat intestinal mucosa, and the specific binding protein which transports 25-HCC to the nuclei by a two-step mechanism was found in the cytoplasmic fraction. Therefore, it is suggested that not only 1,25-DHCC but also 25-HCC are responsible for the mechanism of intestinal transport of calcium. However, further information on the function and mechanism of 25-HCC in the intestine remains to be investigated.

The outline of this report was presented at the IV International Congress of Endocrinology on June 23, 1972 in correcting the presented abstract. During the preparation of this manuscript, papers on the same subject appeared by BRUMBAUGH and HAUSSLER (42) and TSAI and NORMAN (43).

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REFERENCES

BINDING PROTEINS FOR VITAMIN D₃ METABOLITES