

## Water-soluble Derivatives of Vitamin D<sub>2</sub>

### I. Preparation and Properties of Vitamin D<sub>2</sub>-phosphoric Acid Ester

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1. An improved method for the phosphorylation of vitamin D<sub>2</sub> with phosphorus oxychloride in ether and pyridine has been described. Two reaction products, compounds I and II, have been isolated and studied.

2. Compound I, which was the main product, had no antirachitic effect *in vivo*. The infrared spectrum of this substance indicated that the vitamin had been modified during phosphorylation.

3. In *in vivo* studies, compound II showed antirachitic effects comparable to those of unphosphorylated vitamin D<sub>2</sub>. When compound II was administered to rachitic rats, the time interval between dosing and the appearance of a demonstrable effect on calcium absorption and skeletal deposition was as long as the interval for the unphosphorylated vitamin. Hydrolysis to phosphoric acid and vitamin D<sub>2</sub> may occur prior to the exertion of antirachitic activity of compound II *in vivo*.

The insolubility of vitamin D in water prohibits the use of this vitamin in aqueous solutions for the study of its action *in vitro*. To overcome this difficulty, Zetterström<sup>1,2</sup> prepared the water-soluble phosphoric acid ester of calciferol (vitamin D<sub>2</sub>), using the same method as Karrer and Bussman<sup>3</sup> had earlier used for the preparation of the phosphoric acid ester of tocopherol (vitamin E). Phosphorylated vitamin D<sub>2</sub> is of interest for both *in vitro* and *in vivo* studies of the action of vitamin D.

When we attempted to synthesize phosphorylated vitamin D<sub>2</sub> with this method we were not successful. We have therefore modified the method in order to increase its reproducibility, its yield and the purity of the phosphorylated compound. The product obtained was found to be heterogeneous. Two components have been separated and the chemical and biological properties of these studied separately.

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## MATERIALS AND METHODS

*Vitamin D<sub>2</sub>* (calciferol), crystalline, was obtained from Fluka AG (Buchs, Switzerland). It was stored in the dark at  $-20^{\circ}\text{C}$  in nitrogen-filled, sealed glass ampoules. Before use the purity of the vitamin was checked chromatographically (see below).

*Organic solvents* were dried and re-distilled before use.

*Radioactive substances* were obtained from the Radiochemical Centre (Amersham, England).  $^{45}\text{CaCl}_2$  had a specific activity of 5–10 mC per gram Ca, and  $^{32}\text{POCl}_3$  of about 65 mC per gram P.

*Preparation of phosphorylated vitamin D<sub>2</sub>*. 500 mg (1.3 mmoles) of crystalline vitamin D<sub>2</sub> were dissolved in 50 ml of ethyl ether in a glass-stoppered flask, which was wrapped with aluminium foil (to exclude light) and chilled with crushed ice. 6 ml (75 mmoles) of pyridine were added, and the contents of the flask well mixed. Then 2.3 ml (26 mmoles) of phosphorus oxychloride ( $\text{POCl}_3$ ) were added slowly with careful mixing. The flask, still in the ice-bath, was then shaken automatically during the reaction, the course of which was followed by thin layer chromatography of 5  $\mu\text{l}$  aliquots of the reaction mixture, using system A (see below). After 2 h the reaction was practically complete. The reaction mixture was then transferred to a red glass separatory funnel, and the flask was washed out with 50 ml each of water, ethanol, and heptane, which were then added to the separatory funnel. The water had to be added slowly, to avoid too vigorous reaction.

After separation of the phases, the upper phase was washed with two consecutive 25 ml portions of pre-equilibrated acid lower phase (the lower phase from a mixture of equal amounts of ethyl ether, ethanol, heptane and water, acidified to pH 3 with 1 N HCl). The lower phase was in the corresponding way shaken with two 25 ml portions of pre-equilibrated acid upper phase. During these separations the pH was checked with indicator paper and adjusted to 3 with 1 N HCl if necessary. All of the lower phases were discarded. The upper phases (which now contained both the phosphorylated and the remaining unphosphorylated vitamin) were combined, dried with  $\text{Na}_2\text{SO}_4$  and concentrated at room temperature and reduced pressure to a small volume.

The product was dissolved in 50 ml of upper phase, transferred to a separatory funnel, 50 ml of pre-equilibrated neutral lower phase (ethyl ether, ethanol, heptane and water, but *no* HCl) was added. 1 N NaOH was added drop-wise until the pH was about 9, as measured with indicator paper. The upper phase was shaken with two consecutive 25 ml portions of pre-equilibrated alkaline lower phase (ethyl ether, ethanol, heptane and water, adjusted to pH 9 with 1 N NaOH). The lower phase was in the corresponding way shaken with two 25 ml portions of pre-equilibrated alkaline upper phase. The pH was checked, and if necessary adjusted to 9 with 1 N NaOH. The upper phases were discarded.

The alkaline lower phases (containing the sodium salt of phosphorylated vitamin) were combined, acidified with 1 N HCl and again extracted with 40 ml of pre-equilibrated acid upper phase. From this upper phase the phosphorylated vitamin was then again extracted with 20 ml of pre-equilibrated alkaline lower phase, using 1 N NaOH to adjust the pH to about 9.

The solution was concentrated under reduced pressure and then lyophilized. The amorphous powder obtained was stored in a nitrogen atmosphere over  $\text{P}_2\text{O}_5$  in darkness at  $-20^{\circ}\text{C}$ .

In one experiment  $^{32}\text{POCl}_3$  was used in order to obtain  $^{32}\text{P}$ -labeled vitamin D<sub>2</sub>-phosphoric acid ester.

*Analytical thin-layer chromatography*. For the analysis of unphosphorylated and phosphorylated vitamin D<sub>2</sub>, thin layer plates with a size of  $6.5 \times 6.5$  cm were used. They were covered with a layer of adsorbent 0.25 mm thick. Two different systems were used, which henceforth will be named system A and system B.

- 1) *System A*. *Adsorbent*: 20 g Kieselgel G (from Stahl, Merck AG., Darmstadt, Germany) and 38 ml water. Activated at  $120^{\circ}\text{C}$  for 30 min.  
*Solvent*: Petroleum ether: ethyl ether: acetic acid, 69:30:1 (v/v).
- 2) *System B*. *Absorbent*: 10 gram hydroxylapatite, prepared as described by Anacker and Stoy,<sup>4</sup> 0.8 g of gypsum (calcium sulphate dihydrate, heated at  $150^{\circ}\text{C}$  over night) and 40 ml of water. Activated at  $120^{\circ}\text{C}$  for 60 min.  
*Solvent*: Isoamyl acetate: propionic acid: butanol: water, 40:30:20:10 (v/v). (Hofmann, personal communication).

For the detection of the spots one of the following reagents was used:

1) *Phosphomolybdic acid reagent*. 10 g phosphomolybdic acid dissolved without heating in 100 ml of absolute ethanol and filtered. After spraying, the plate was heated at 100°C for 5–10 min. Green spots appeared on a yellow background.

2) *Sulphuric acid–nitric acid reagent*. Concentrated sulphuric acid, concentrated nitric acid and water were mixed in the proportions 40:30:30 (v/v). After spraying, the plate was heated with a flame. Brown spots appeared on a beige background.

*Preparative thin-layer chromatography*. For the preparative separation of the two different compounds formed on phosphorylation of the vitamin, chromatography with system B was performed on 4.0 × 20 cm glass plates, covered with a 0.75 mm thick layer of adsorbent. 3–4 mg of the crude phosphorylated vitamin D<sub>2</sub> were applied to each plate. After development of 8 plates simultaneously, every second plate was sprayed with the phosphomolybdate reagent. On the other plates the adsorbent was removed in the regions corresponding to the two phosphorylated compounds, dissolved in 25 ml 2 N HCl and the compounds extracted with 20 ml of pre-equilibrated acid upper phase. The upper phase was extracted with 6 consecutive 20 ml portions of pre-equilibrated acid lower phase. The lower phases were discarded. Then 10 ml of pre-equilibrated alkaline lower phase were added, and 1 N NaOH to give a pH of about 9. The upper phase was extracted with a further 10 ml portion of pre-equilibrated alkaline lower phase, and then discarded. The two lower phases were concentrated under reduced pressure and lyophilized.

*Measurement of the concentration of phosphorylated and unphosphorylated vitamin D<sub>2</sub>*: Vitamin D<sub>2</sub> has an absorption peak at 265 m $\mu$ . In the extraction procedures described above, and in preparing solutions of the phosphorylated vitamin for other experiments, the concentration was calculated from the optical density of the solution at this wavelength. The phosphorylated vitamin was assumed to have the same molar extinction coefficient as the unphosphorylated vitamin.

*Elementary analysis*: Assays of carbon and hydrogen were performed at the Chemical Department, University of Lund. Assay of phosphorus was performed with the method of King.<sup>5</sup>

*Absorption curves*. (1) *In the ultraviolet region*: Vitamin D<sub>2</sub> or the compounds isolated on phosphorylation were dissolved in 50 % ethanol in 1 cm light pathway quartz cuvettes, and the absorption curves obtained with an automatically recording Beckman DK-2 spectrophotometer. (2) *In the infrared region*: Recording of the absorption spectra in the infrared region was performed at the Department of Chemistry, University of Lund, using tablets prepared with potassium bromide. Dr. R. Larsson gave us kind help with the evaluation of the curves.

*Breeding of rachitic rats for biological tests*. Albino rats of the Sprague-Dawley strain were used. Pregnant female rats were fed Bill's diet<sup>6</sup> for one week before the birth of the litter and then during the nursing period.

*Bill's diet*

Ground yellow corn (with shells)	57
Whole milk powder	25
Linseed meal	12
Crude casein	3.7
Alfalfa leaf meal	1.5
Iodized table salt	0.4
Calcium carbonate	0.4
	<hr/> 100.0

The rats were weaned at 28–31 days after birth and placed in stainless steel metabolic cages in a room where all daylight was excluded. A maximum of seven weanlings were placed in each cage. The weanlings were fed the rachitogenic diet D 40 of Brunius, as quoted by Hjertquist.<sup>7</sup>

*Brunius' rachitogenic diet D 40*

Ground yellow corn (with shells)	86
Egg albumin	10
Calcium carbonate	3
Sodium chloride	1
	<hr/>
	100.0

Both the mothers and the weanlings were fed their diet *ad libitum*, and they had free access to distilled water. The weanlings were marked individually and weighed weekly to make certain that an adequate rate of growth occurred. The depletion period on diet D 40 lasted 21–23 days. The initial weight of the weanlings was 44–62 g (mean 52.9), their final weight 55–108 g (mean 74.9). Rats which weighed less than 40 g at the end of the nursing period, or did not increase in weight during the subsequent depletion period, were discarded.

At the end of the depletion period the rats had swollen joints, and roentgenography (kindly performed by Dr. O. Norman, Roentgenological Department, University of Lund) revealed the classical picture of rickets. We also found a positive line test with the method described in the U.S. Pharmacopeia (Ed. XIV). The rats were immediately used for the biological tests of antirachitic activity.

*Biological test of antirachitic activity.* The rats were lightly anesthetized with ethyl ether and fed an appropriate amount of a solution of the substance to be tested in a volume of 0.5 ml by a stomach tube. Both vitamin D<sub>2</sub> and the phosphorylated vitamin were given in 50 % ethanol. The control group was fed the same volume of the pure solvent. After the required time interval, during which the rats were fed diet D 40, they were again anesthetized and fed 2–5  $\mu$ C of <sup>45</sup>Ca, together with 20 mg of calcium lactate as a carrier, all dissolved in 1.0 ml of 0.01 N HCl. 18 h after the administration of the <sup>45</sup>Ca the rats were sacrificed. The upper ends of both tibiae and fibulae were removed, cleaned and ashed overnight in an electric furnace at 800°C in porcelain crucibles. The ash was dissolved in 5.0 ml of nitric acid, *d* 1.10, and the radioactivity of the solution measured. The radioactivity was also measured in a diluted aliquot of the solution fed, and the amount of <sup>45</sup>Ca found in the upper ends of the tibiae and fibulae was calculated as percentage of the dose fed. This gives an estimate of the amount of calcium deposited in the skeleton, which is strongly influenced by vitamin D.<sup>8,9</sup>

*Measurement of <sup>45</sup>Ca.* The radioactivity of the nitric acid solution of the bone ash was measured with an apparatus similar to that used by Carlsson.<sup>8</sup> The Geiger-Müller tube was equipped with an anticoincidence circuit to reduce the background counts, which in our experiments were 12–13 cpm. With a dilution series of the radioactive calcium we found the cpm measured to be proportional to the amount of isotope present up to over 5500 cpm. This figure was never exceeded in our samples. The presence of non-radioactive calcium or dissolved bone ash did not influence the counts given by the radioactive calcium in these control experiments.

*Dephosphorylation of the phosphorylated vitamin by a homogenate of small-intestinal mucosa.* A normal rat, weighing 200 g, was killed with a blow on the head. The small intestine was removed, rinsed with about 10 ml of saline and cut open. The mucosa was scraped off with a glass slide and homogenized in an Ultra-Turrax homogenizer with 10 ml of 0.05 M sodium phosphate buffer pH 6.3. During the homogenization the tube was chilled with crushed ice. The homogenate was diluted with buffer to a total of 20 ml.

A 10 ml portion of the homogenate was mixed with 0.2 ml of a solution of <sup>32</sup>P-labelled isolated compound I in 0.05 M sodium phosphate buffer pH 6.3. This solution contained 130  $\mu$ g of compound I labelled with <sup>32</sup>P to give a radioactivity of 4 800 000 cpm with the equipment used.

Another 10 ml portion of the mucosal homogenate was mixed with 0.2 ml of a solution of <sup>32</sup>P-labelled isolated compound II in the same buffer. This solution contained 181  $\mu$ g of compound II and 5 850 000 cpm of <sup>32</sup>P.

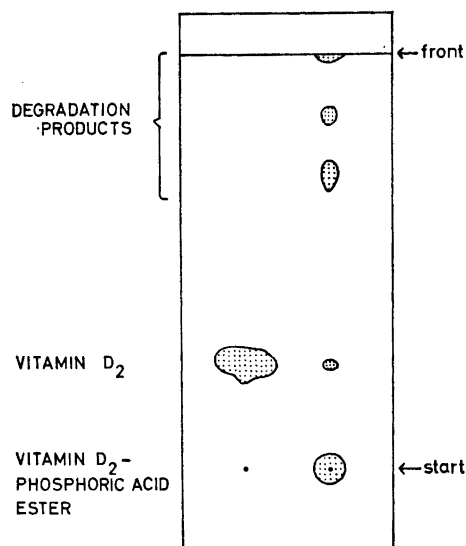
The two samples were incubated at 37°C and at intervals 2.0 ml aliquots were withdrawn and mixed with 2.0 ml each of ethanol, heptane, and ethyl ether. 1 N HCl was added drop-wise to give a pH of about 3 as measured with an indicator paper. After

shaking and separation into two phases, 2.0 ml of the upper phase was transferred to a counting vessel, the solvent was evaporated, the residue dissolved in 10 ml of dioxane with scintillator substance added, and the radioactivity measured in a Packard liquid scintillation counter. Inorganic phosphate formed on hydrolysis of the vitamin ester is extracted into the lower phase with this method.

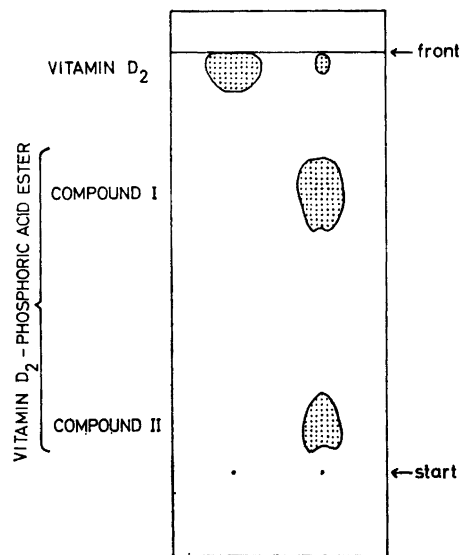
## RESULTS

*Phosphorylation of vitamin D<sub>2</sub>.* The course of the phosphorylation reaction was followed on thinlayer chromatograms, using system A. In this system the phosphorylated reaction products did not move from the starting line (Fig. 1). After 2 h only a faint spot of unchanged vitamin remained. During the course of the reaction some spots appeared which moved somewhat more rapidly than vitamin D<sub>2</sub>. These spots probably contain degradation products of the vitamin. These as well as the remainder of unphosphorylated vitamin were removed during the subsequent extraction procedure.

When the free acid of phosphorylated vitamin D<sub>2</sub> was isolated (from an acid upper phase) a slightly yellow oil was obtained. Usually it was isolated as the sodium salt (from an alkaline lower phase) which on lyophilization



*Fig. 1.* Thin-layer chromatography, system A, of vitamin D<sub>2</sub> (left) and the crude product of the phosphorylation of vitamin D<sub>2</sub> (right). The remainder of unphosphorylated vitamin and the faster-moving components, which probably are degradation products, in the crude phosphorylated preparation are removed during the subsequent extraction procedure.



*Fig. 2.* Thin-layer chromatography, system B, of vitamin D<sub>2</sub> (left) and the crude product of the phosphorylation of vitamin D<sub>2</sub> (right). Compare Fig. 1.

gave a white amorphous powder. When the powder was stored at  $-20^{\circ}\text{C}$ , protected from light, in a desiccator over  $\text{P}_2\text{O}_5$  and in a nitrogen atmosphere, it was stable for months. If these precautions were not taken, it rapidly turned brown, and at the same time degradation products were formed appearing as a series of spots which moved ahead of vitamin  $\text{D}_2$  in the thin-layer chromatography system A. No spot with the mobility of the unphosphorylated vitamin was seen either in the freshly prepared phosphorylated vitamin or in the deteriorated preparations. The sensitivity of the thin-layer chromatography was sufficient to exclude the presence of more than 1 % of unphosphorylated vitamin  $\text{D}_2$ .

From 500 mg of vitamin  $\text{D}_2$  420 mg of the sodium salt of the phosphorylated vitamin was obtained, representing a yield of 77 %. (Found: C 61.0; H 9.11; P 5.18. Calc. for  $\text{C}_{28}\text{H}_{43}\text{O} \cdot \text{PO}(\text{ONa})_2$ : C 64.7; H 8.65; P 5.96.) The optical density at  $265 \mu$  was 86 % of the value expected assuming the same molar extinction coefficient for the phosphorylated as for the unphosphorylated vitamin.

*Fractionation of the phosphorylated vitamin.* When the phosphorylated product of vitamin  $\text{D}_2$  was chromatographed on thin layer plates using system B, two distinct spots appeared, with,  $R_F$  values 0.7 and 0.1, respectively (Fig. 2). The faster one of these two spots will henceforth be named compound I, and the slower one compound II.

Small amounts of compound I and compound II were isolated on thin layer plates as described in the method section. On re-chromatography each of the isolated compounds formed a single spot with the expected  $R_F$  value. In spite of the repeated extractions with acid lower phase the preparations isolated from the thin layer plates contained large amounts (80–90 %) of inorganic salts. The recovery of compound I was 50–60 % and of compound II 20–30 %, of the crude phosphorylated vitamin  $\text{D}_2$  applied to the thin layer plate.

Compound I and compound II had the same absorption spectrum in the ultraviolet region as vitamin  $\text{D}_2$ , with one peak at 210 and one at  $265 \mu$ .

In the infrared region vitamin  $\text{D}_2$  has a series of sharp absorption peaks at 800–1100  $\mu$ , smaller peaks at 1100–1650  $\mu$ , and three very large peaks at 2800–3400  $\mu$ . The absorption spectra of compound I and II were difficult to evaluate because of the strong interference from inorganic salts present. One clear difference could, however, be seen: a sharp absorption peak at 960  $\mu$ , which was present both in the unphosphorylated vitamin  $\text{D}_2$  and in compound II, was absent in compound I.

In the experiment in which  $^{32}\text{P}$ -labelled phosphorus oxychloride was used for the phosphorylation of vitamin  $\text{D}_2$ , the radioactivity of compound I and compound II was measured separately, after their isolation as described above. Compound I had an activity of 37 000 cpm/ $\mu\text{g}$  and compound II, 32 400 cpm/ $\mu\text{g}$  (the concentration of compound I and compound II was measured by the optical density at  $265 \mu$ ). The two compounds thus seem to have approximately the same ratio vitamin  $\text{D}_2$  to phosphorus.

*Antirachitic effect of compound I and compound II.* Table 1 shows the results of one experiment in which groups of rats were fed the isolated compound I and compound II. Each rat was fed 0.75  $\mu\text{g}$  of the compound to be

*Table 1.* Biological test for antirachitic effect of the isolated compound I and compound II. Each rat was fed 0.75  $\mu$ g of the substance to be tested and 72 h later 20 mg of calcium lactate with 3  $\mu$ C of <sup>45</sup>Ca. After a further 18 h the rats were sacrificed.

Weight of animal, g	<sup>45</sup> Ca uptaken in upper ends of tibiae and fibulae, (% of dose fed)	Mean value of % <sup>45</sup> Ca uptake
Control group		
81	0.59	0.80
74	0.50	
62	1.58	
76	0.77	
76	0.56	
Fed compound I		
91	0.64	0.66
87	0.72	
58	0.49	
84	0.95	
86	0.51	
Fed Compound II		
91	1.61	1.89
68	2.18	
85	1.87	
75	(Died during the anaesthesia)	

*Table 2.* Time-response curve for the effect of compound II on the skeletal deposition of <sup>45</sup>Ca. Rachitic rats were fed 0.75  $\mu$ g of compound II, or in one group the same amount of unphosphorylated vitamin D<sub>2</sub>. The control groups were fed only the vehicle. After varying time intervals they were fed 20 mg of calcium lactate with 3  $\mu$ C of <sup>45</sup>Ca, and after a further 18 h the animals were sacrificed.

No. of animals in each group	Time interval between administration of vitamin and <sup>45</sup> Ca, h	Mean value of <sup>45</sup> Ca uptake in upper ends of tibiae and fibulae (% of dose fed)
Control groups		
3	24	0.68
5	72	0.89
Fed compound II		
4	4	0.69
3	8	0.78
3	12	0.74
4	18	1.25
9	24	1.21
5	48	1.69
5	72	2.22
Fed Vitamin D <sub>2</sub>		
4	72	1.91

*Table 3.* The hydrolysis of compound I and compound II by a homogenate of rat small-intestinal mucosa.  $^{32}\text{P}$ -labelled compound I and compound II have been incubated with the mucosal homogenate at  $37^\circ\text{C}$  in 0.05 M sodium phosphate buffer pH 6.3, and the amount of unsplit vitamin phosphate remaining after different time intervals has been measured as the radioactivity which can be extracted with organic solvents from the acidified solution.

Time of incubation hours	Lipid-soluble $^{32}\text{P}$ , % of original Compound I	Compound II
0	100	100
0.5	95.5	96.2
1.5	81.6	80.4
2.5	75.2	—
24	51.9	43.5

tested (measured from the optical density of the solution at 265  $m\mu$ ), corresponding to 30 International Units of vitamin  $\text{D}_2$ , 72 h before the administration of the calcium. With the unphosphorylated vitamin this dose is sufficient to give a maximal effect. Compound II gave a response similar to that of the corresponding amount of unphosphorylated vitamin  $\text{D}_2$ , but compound I had no effect.

In the next experiment, the effect of varying the time interval between compound II dosing and  $^{45}\text{Ca}$  dosing on the  $^{45}\text{Ca}$  uptake was studied. It is known that after the administration of unphosphorylated vitamin  $\text{D}_2$  to rachitic rats, the ability to absorb calcium increases slowly and does not reach its maximum until 72 hours later.<sup>9</sup> Compound II exerts its effect equally slowly (Table 2). The values obtained agree closely with those obtained by Lindquist<sup>9</sup> for free vitamin  $\text{D}_2$ .

*Dephosphorylation of compound I and compound II in vitro:* When  $^{32}\text{P}$ -labelled compound I and compound II were incubated with a small-intestinal mucosal homogenate at pH 6.3, the results indicated hydrolysis of both substances at approximately the same rate (Table 3).

#### DISCUSSION

The method for the preparation of phosphorylated vitamin D described here differs in some details from that of Zetterström<sup>1</sup> both as to the conditions of the phosphorylation and the purification of the product. We carried out the reaction in ether-solution using less pyridine and more phosphorus oxychloride in relation to the vitamin. The purification of the product has been achieved by a solvent partition system instead of by crystallization. With these modifications the product formed could be separated from unchanged vitamin D easily and reproducibly. The product was found to be heterogeneous by thin-layer chromatography, consisting of two compounds designed I and II. These compounds have not previously been separated and characterized. Both of them contain phosphorus, as shown in the experiment in which  $^{32}\text{P}$ -labelled vitamin phosphate was prepared and by the elementary analysis, and both have the same ultraviolet absorption spectrum as vitamin



D<sub>2</sub>. The infrared absorption spectrum indicates a modified structure of the vitamin component of compound I (which is the one obtained in the largest amounts), but not of compound II. This also fits well with the finding that compound II, but not compound I, exerts vitamin D activity.

Phosphorylated vitamin D<sub>2</sub> is of interest not only as a water-soluble vitamin D derivative which can be studied in *in vitro*-systems, but also as a possible intermediary metabolite of the vitamin. Phosphorylated vitamin D<sub>2</sub> has been found to activate alkaline phosphatase *in vitro*,<sup>2</sup> although other authors have claimed that the effect is only the reversal of the inhibitory action of borate.<sup>10</sup> It has also been found to increase the oxygen consumption of palatal mucosa from rachitic rats *in vitro*.<sup>11-14</sup> The latent period between the administration of vitamin D<sub>2</sub> to rachitic rats and its effects on calcium absorption and deposition has never been explained. We had speculated that phosphorylated calciferol might be an active intermediary metabolite in the activation or transport of vitamin D, but the time-response relationships shown in Table 2 do not support this hypothesis. Since we also found that compound II could be dephosphorylated by a homogenate of intestinal mucosa, the possibility exists that the administered compound II is dephosphorylated before it exerts its antirachitic effect.

Compound I was dephosphorylated by the mucosal homogenate at the same rate as was compound II. The lack of antirachitic effect of compound I therefore gives further evidence that the vitamin in this ester has been modified and inactivated during the phosphorylation. Since about two-thirds of the phosphorylation product of vitamin D<sub>2</sub> was the inactive compound I, and since a large-scale separation of compound I and compound II from each other seems difficult to achieve, we have, in preliminary experiments, prepared the sulfate ester of the vitamin. This compound was also found to have antirachitic effect.

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