SYNTHESIS AND BIOLOGICAL ACTIVITY OF VITAMIN D_3 ACETATE

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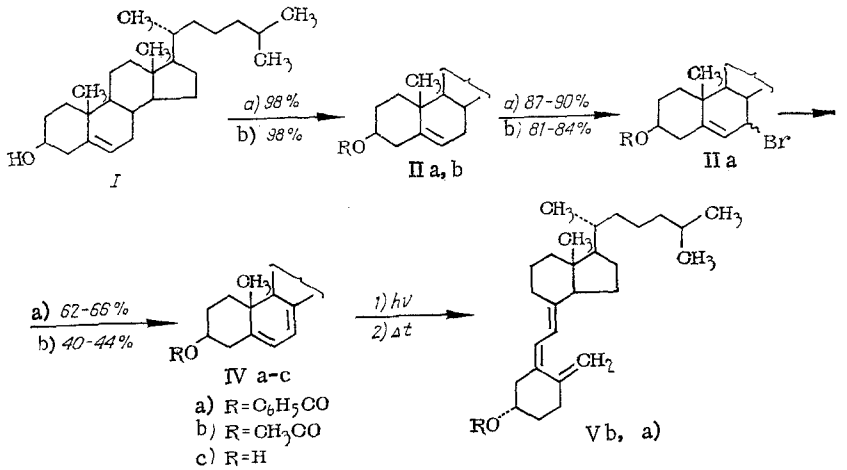
It has now been established that most of the vitamin D ethers and esters are devoid of biological activity. Only those that are readily hydrolyzed in the organism are active. The latter include the acetate, ethyl carbonate, phosphate, butyrate, sulfate, and palmitate. Biologically active esters of vitamins D_2 and D_3 are attracting the attention of many researchers by the fact that their chemical stability is far higher than that of nonsterified vitamins, and they may represent stable preparations of D vitamins for the needs of agriculture and possibly medicine as well [1-3].

The most accessible of the biologically active esters of vitamin D is the acetate. It is known that the biological activity of vitamin D_2 acetate in rats is equal to [4] or somewhat lower than (60-80%) [5] the activity of vitamin D_2. In an investigation of the toxic effect of vitamin D_2 and D_3 acetates, it was established [6] that the degree of calcification of the kidneys of heart in rats after the use of the acetate is lower than in rats that received vitamins D_2 and D_3.

To determine the advantages of the use of vitamin D_3 esters, we synthesized vitamin D_3 acetate (Vb) and studied the stability and biological activity of this ester in birds.

Vitamin D_3 acetate was produced by acetylation of crystalline vitamin D_3 (Vc) with acetic anhydride, and also by photochemical isomerization of the acetate of its provitamin — 7-dehydrocholesterol (IVb). The synthesis of IVb was performed analogously to the generally used method of producing 7-dehydrocholesterol (IVc). At the present time IVc is produced on the basis of cholesterol (I), which is benzoylated; cholesterol benzoate (IIa) is subjected to allyl bromination, the benzoate of the 7-bromo derivative (IIIa) obtained is dehydrobrominated, and the ester of 7-dehydrocholesterol (IVa) is saponified [7].



Acetylation of I with acetic anhydride, like the benzoylation, proceeds with a 98% yield. In the bromination of cholesterol acetate (IIb), the yield of the 7-bromo derivative (IIIb) is somewhat lower (81-84%) than that of IIIa (87-90%). The dehydrobromination of IIIb by sodium bicarbonate in the presence of catalytic amounts of α-picoline in boiling xylene [8, 9], leads to the production of IVb with a yield of 40-44% (according to the data of chromatospectrophotometric analysis [10]). The yield of IVa under these conditions is no less than 62%. The benzoate of IVa is precipitated by acetone from the reaction solution almost

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quantitatively (96%), while IVb, on account of its high solubility, is no more than 50% precipitated. Attempts to select conditions of more complete isolation of IVb from the reaction mixture were unsuccessful. The removal of xylene from the reaction mixture (which is rather hard to do under industrial conditions) and the precipitation of IVb from the residue with acetone leads to the production of a low-quality (less than 60%) ester of the provitamin. When it is recrystallized to improve the quality, substantial losses of the product are observed. Thus, the total yield of IVb is 22-26% of the initial I, while the yield of IVc, including the step of saponification of IVa, is 38%.

Photochemical isomerization of esters of provitamin D, leading to the production of esters of D vitamins, was carried out back in 1927 [4, 11]. It was established in this case that the photoconversion of esters proceeds analogously to the provitamins, but after saponification of the irradiated mixture, D vitamins are formed. In recent years several patents on methods of production of D vitamins by irradiation of esters of the provitamins (acetate, benzoate, propionate, valerate), followed by saponification of the reaction mixture, have been published. However, the conditions for the photoconversion of esters selected by the authors of the patents provided for a low (30-40%) yield of D vitamins [12-14].

UV irradiation of IVb was performed by the method that we developed, using erythema lamps with É-2 or É-3 luminophore (emission maximum in the region of 302-312 nm) in a continuous action apparatus [7]. Under these conditions the degree of conversion of the provitamin can be increased to 90-95%, in contrast to the generally accepted 40-50%. Just as in the case of IVc, the basic properties of photolysis were the acetate of the provitamin (yield 75-80% of the converted IVb) and lumisterol acetate (10-15%). After thermal isomerization of the acetate of the provitamin to Vb, the yield of the latter was no less than 70%, i.e., was higher than the yield of Vc (about 60%). This is due to the fact that in photochemical isomerization of IVb, there is practically no oxidation of the provitamin and its photoderivatives (oxidation products are absent on the chromatogram), while in thermal isomerization of the acetate of the provitamin, the equilibrium is more shifted in the direction of Vb than in the case of the nonesterified provitamin.

The acetate of Vb, isolated from the mixture of products of photochemical and thermal isomerization after chromatography on aluminum oxide (II degree of activity) in the system benzene—petroleum ether (60:40), represents a colorless resin. The same product was obtained in the acetylation of crystalline Vc with acetic anhydride in pyridine. We were unable to obtain Vb in crystalline form. The resinous product has $[\alpha]_D^{22}$ +38.4° (acetone); UV spectrum (ethanol): γ_{max} 265 nm, ε 18,100; IR spectrum (carbon tetrachloride): 2960, 1745, 1650, 1475, 1445, 1380, 1245, 1035 cm^{-1}.

Samples of Vb were placed for storage at 5°C. The product was stored in a flask with a ground stopper in the presence of air. Each six months the specific rotation and molar extinction coefficient were determined, along with the chromatographic purity of Vb. Over a period of 2.5 years of storage, no change in the constants was observed, while on a Silufol UV-253 plate in chromatography in benzene, after development with concentrated sulfuric acid, only the spot of Vb (R_f 0.55) and traces of the acetate of the provitamin (R_f 0.62) are present.

The antirachitic activity of Vb was studied in comparison with crystalline Vc in experiments on chicks. Two series of investigations were conducted. In experimental series I, one-day-old chicks were divided into three groups (10 in each) by the method of analogs. The chicks of the first group received only the basic diet (BD); the chicks of the second group received 10 IU Vc against a background of the BD; those of the third group received 10 IU Vb. In the experimental series II, the chicks received the BD from the first days of their life; they were divided into three groups at the age of two weeks. The chicks of the first group continued to receive the BD, those of the second 10 IU Vc, those of the third 50 IU Vb.

The preparations were administered internally, daily: in series I for 30 days, in II for 10 days. At the age of one month, the chicks were weighed, sacrificed, and biochemical investigations conducted. The criteria of activity were the content of calcium, inorganic phosphorus, and the alkaline phosphatase activity in the blood serum [15-17], the ash content of the tibia [8], and the content of calcium-binding protein in the duodenal mucosa [19].

From an analysis of the data obtained it is evident (Table 1) that Vb has a definite influence on the mineral metabolism in the organism, whereas its growth-stimulating effect is very weakly manifested. Considering the special role of the skeleton as a reserve of

TABLE 1. Antirachitic Activity of Vb (M ± m)

Index	Experimental series I			Experimental series II		
	Group of animals					
	1	2	3	1	2	3
Increase in live weight in 10 days, g	37,6±1,4	57,2±3,8	41,1±4,3	8,2±3,4	43,8±6,7	26,8±7,4
Ash content of tibia, %	42,8±0,3	52,9±0,5	51,9±0,6	24,7±0,6	36,2±0,6	31,0±0,7
Calcium concent in blood serum, g/liter	0,095± ±0,004	0,123± ±0,003	0,124± ±0,004	0,083± ±0,007	0,102± ±0,002	0,095± ±0,005
Phosphorus content in blood serum, g/liter	0,048± ±0,002	0,063± ±0,003	0,060± ±0,001	0,051± ±0,015	0,082± ±0,005	0,071± ±0,003
Alkaline phosphatase activity of blood serum, units	50,2±1,7	29,3±1,0	26,6±0,4	29,8±1,6	18,5±1,6	22,9±0,9
Calcium-binding protein in duodenal mucosa, μg per mg protein	—	—	—	0	86,7±9,3	36,1±3,4

TABLE 2. Influence of Vb, c on the Productive and Reproductive Quality of Chickens (M ± m)

Index	Conditions of experiment		
	control (BD)	BD + 0.025 g Vc per ton of combined feed	BD + 0.025 g Vb per ton of combined feed
Conservation of flock, %	86,6	88,8	93,3
Rate of egg laying, %	51,7±4,4	67,1±4,3	68,1±4,3
Average weight of one egg, g	56,4±1,0	58,1±1,0	57,3±0,8
Live weight of layer at end of experiment, kg	1,67±0,03	1,77±0,04	1,82±0,04
Fertilizability of eggs, %	79,2±9,1	94,8±1,5	97,0±0,7
Hatchability of fertilized eggs, %	72,5±7,7	91,0±1,0	91,4±1,13

mineral salts in birds [20], we tested Vb on laying hens, since during the formation of the egg shell, a substantial amount of calcium passes directly from their bone tissue. The experiment was conducted by the method of groups (50 in a group) on young laying hens, equalized in live weight, age, and productivity. The time of the experiment was 10 months. Additions of Vb, c were introduced into the combined feed by a stepwise method: once every three days a triple daily dose, calculated on the basis of 0.025 g per ton of combined feed. The following indices were considered during the experiment: 1) conservation of the flock; 2) live weight of hens; 3) average weight of eggs; 4) reproductive qualities of hens (fertilizability and hatchability of eggs).

As can be seen from Table 2, Vb had the same influence on the rate of egg laying, weight of eggs, and their fertilizability as Vc, while the conservation of the flock and live weight of the laying hens were higher when Vb was given. No noteworthy changes occurred in the biochemical indices of the blood when Vb was fed to the hens. Our investigations show that Vb can be used in the feed of laying hens to intensify egg laying and preserve the flock.

EXPERIMENTAL

Acetylation of I. A 100 g portion of I in 400 ml of piperidine was added; 100 ml of acetic anhydride was added to the solution obtained in small portions during cooling, and the mixture was left for 12-14 h at room temperature. The end of the acetylation reaction was determined by thin-layer chromatography according to the absence of the spot of I (R_f 0.1) on the Silufol UV-253 plate and the presence only of the spot of IIb (R_f 0.5) (benzene, development with concentrated sulfuric acid). After the end of the reaction, 750 ml of water was added to the mixture, and it was mixed well and filtered. The precipitate IIb was washed on the filter with water, with a 2% solution of hydrochloric acid, again with water to a neutral pH (according to an indicator), and with chilled acetone. Yield 109 g (98.5%), mp 115-116°C, $[\alpha]_D^{20}$ −47.4° (chloroform). According to the literature data, mp 116°C, $[\alpha]_D^{20}$ -47° (chloroform) [21].

Bromination of IIb According to the Method of [8]. To a boiling solution of 10g IIb in 55 ml carbon tetrachloride (temperature of oil bath 105°C), a suspension of 3.7 g of pulverized 1,3-dibromo-5,5-dimethylhydantoin and 60 mg azoisobutyrodinitrile in 10 ml carbon

tetrachloride were added. The reaction proceeds vigorously and ends in 2-3 min. The mixture was cooled, filtered, and the amount of IIIb in solution determined by an argentometric method [22]. Yield of IIIb 81-84%.

Dehydrobromination of IIIb According to the Method of [8]. A mixture of 40 ml of o-xylene, 10 g pulverized sodium bicarbonate, and 0.2 ml α-picoline was heated on a bath to 120°C. At a temperature of the mixture 100-110°C, a solution of IIIb in carbon tetrachloride was poured in and the bath temperature raised to 165°C. After 30 min the reaction ended; the salt precipitate was filtered off and washed with hot o-xylene. We obtained 18-20 ml of a xylene solution containing a mixture of products of the dehydrobromination reaction. The amount of IVb was determined by a chromatospectrophotometric method [10]. The yield of IVb was 40-44%. The xylene solution was evaporated to dryness under vacuum, 15 ml of acetone was added, and it was left for 12 h at -15°C. We obtained 4.5 g of a product containing 57% IVb. Yield of IVb (calculated on the basis of IIb) 26%.

A 4.5 g portion of the product was dissolved in 7 ml of acetone with heating and left at 0°C for 12 h. We obtained 3.2 g of a product containing 68% IVb. After several crystallizations from acetone, we obtained IVb with mp 126°C, $[\alpha]_D^{20}$ -90.5° (benzene); UV spectrum: λ_{max} 272, 282, 293 nm, ε 11,090, 11,800:6700. According to the literature data, mp 130-130.5°C, $[\alpha]_D^{21.5}$ -87 ± 2° (benzene) [23].

Photochemical Isomerization of IVb. To obtain a 0.1% solution, 20 g of IVb was dissolved in 20 liters of ethanol with heating to 35-40°C. After complete dissolution of Vb, the solution was cooled to 18-20°C and purged for 30 min with an inert gas to remove atmospheric oxygen. Irradiation of the alcohol solution of IVb was performed in a continuous action apparatus, consisting of 20 erythema lamps with luminophore possessing emission maximum at 302-305 nm. The irradiated solution was passed directly along surface of the erythema lamps, enclosed in a metallic casing, at a regulated rate (about 15-18 liters/h), ensuring 90-95% conversion of IVb. The solution obtained after irradiation was evaporated 50-fold (to 400 ml) in a vacuum evaporating apparatus at the boiling point of ethanol for 2.5 h for thermal isomerization of the acetate of the previtamin to Vb and concentration of the irradiated solution.

Quantitative determination of the products obtained was performed by a chromatospectrophotometric method by separation on Silufol UV-253 plates in a benzene—petroleum ether (60:40) mixture; the substances were eluted with ethanol, followed by spectrophotometric determination according to the value of the optical density at γ_{max}. The content of the basic components in the alcohol concentrate obtained is: Vb 68%, acetate of the provitamin 10%, lumisterol acetate 12%, unconverted IVb 8%.

Production of Vb. A. From an alcohol concentrate. A 100 ml portion of the alcohol concentrate was evaporated under vacuum to resins, and the residue obtained (5 mg) was chromatographed on 100 g of aluminum oxide, II degree of activity, in a benzene—petroleum ether (60:40) mixture. After removal of the acetate of the provitamin (0.46 g), fractions containing Vb were collected, the solvent evaporated, and a resinous residue of Vb (3.2 g) obtained, ε 16,500. Repeated chromatographic purification gave 2.9 g Vb, $[\alpha]_D^{22}$ +38.5° (acetone).

B. By acetylation of Vc. A 1 g portion of crystalline Vc (mp 84-85°C) was dissolved in 2 ml of pyridine, 2 ml of acetic anhydride was added, and the mixture left in darkness for 12 h at room temperature. We added 10-15 ml of water to the solution, extracted Vb with benzene, washed the extract with a 2% solution of hydrochloric acid and with water, and dried with sodium sulfate. After the solvent was distilled off, we obtained a resinous residue, which was purified by chromatography on a column with aluminum oxide. We obtained 0.73 g Vb in the form of a colorless resin, ε 18,100 $[\alpha]_D^{22}$ +38.4° (acetone).

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SYNTHESIS OF PROVITAMIN D_5 FROM PLANT RAW MATERIAL

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It has been shown previously [1] that on heating phytosterol with chloride salts of divalent metals, complex compounds of β-sitosterol are formed with the metal chlorides which then may be readily decomposed into the sterol and inorganic components. This affords a real basis for converting phytosterol obtained on treating wood [2, 3] or the resin of peat wax [4] into provitamin D_5 by the scheme: phytosterol→complex of β-sitosterol with metal chloride→β-sitosterol→β-sitosterol benzoate→7-bromo-β-sitost-5-en-3β-ol benzoate→β-sitosta-5,7-dien-3β-ol benzoate→β-sitosta-5,7-dien-3β-ol→provitamin D_5→vitamin D_5. The antirachitic activity of vitamin D_5 was studied previously in [5, 6]. In practice provitamin and vitamin D_5 may be obtained by this scheme from various samples of phytosterol with a β-sitosterol (I) content of 40% and more.

Data are given in the present work on methods of obtaining provitamin D_5 from phytosterol isolated from the byproducts of cellulose sulfate manufacture. Phytosterol from the Kekhrassk Paper Combine [3] was used in the work and had mp 80-125°C containing 65% (I) determined by the digitonin method [7].

The first and second stages of the synthesis, the preparation of a molecular complex of calcium chloride with (I) and decomposition of the molecular complex to sterol and inorganic portions and isolation of (I), were carried out under conditions close to those described previously [1]. With this aim phytosterol was mixed with an organic solvent with heating, the insoluble portion was filtered off, and calcium chloride, dissolved in 96% ethyl alcohol, was added to the solution heated to 40°C. After standing for a day the molecular complex of calcium chloride with (I) of composition $(C_{29}H_{50}O)_2 \cdot CaCl_2 \cdot 6H_2O$ was filtered off and boiled in alcohol. On cooling the alcohol solution (I) crystallized out. More pure (I) was obtained when decomposition of the addition product of (I) with calcium chloride was carried out in the presence of activated carbon.

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