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SYNTHESIS AND BIOLOGICAL ACTIVITY OF 1α-HYDROXYVITAMIN D_3 1-BUTYRATE

N. A. Bogoslovskii, V. K. Bauman, M. Yu. Valinietse, D. A. Babarykin, and T. A. Kisel'nikova

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Vitamin D esters have aroused definite interest among researchers. Vitamins D_2 and D_3 are found in animal tissues [7] in the form of fatty acid esters, significant amounts of which are contained in fish liver [10]. The advantage of this form of ester is that it is marked by a greater stability compared with the free vitamin [17] and probably supplies a reserve source in the organism. A series of investigations has, however, established that esterification of D vitamins leads to a decrease in their biological activity. Thus, the antirachitic activity of the acetate, palmitate, and butyrate of ergocalciferol for growing rats corresponds to 59, 61, and 72% of the activity of ergocalciferol [5]. Similar results were obtained for rats, chickens, and Japanese quail in studies of the activity of cholecalciferol and its various fatty acid esters [14]. One of the main reasons for the decrease of biological activity of the vitamin after its esterification may be its weakened capability for absorption in the small intestine of animals [6]. This conclusion is supported by data [8] according to which vitamin D_3 palmitate or the free vitamin, when introduced parenterally, shows equal activity. There is little published data regarding the esters of vitamin D metabolites and their analogs.

There are indications that the active hormonal form of vitamin D_3 – 1α,25-dihydroxyvitamin D_3, as well as its analogs 1α-hydroxyvitamin D_3 and 1α,24,25-trihydroxyvitamin D_3 – are converted into esters in the organism [12, 15]. The structure of these esters, however, is not clear, and their biological activity has not been investigated.

To address the question of whether blockage of the 1α-hydroxy group by a radical of fatty acid preserves the biological activity of the secosteroid, we synthesized 1α-hydroxyvitamin D_3 1-butyrate (I) and studied its biological activity.

In studying the effect of this ester on chickens, we compared it with the effect of 1α-hydroxyvitamin D_3 (1α-OH-D_3), taking into consideration changes in the process of ab-

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sorption. Thus, in our experiments we used two methods — oral and intramuscular — of administering the preparation to the organism, bypassing the intestines.

EXPERIMENTAL CHEMICAL

1α-Hydroxyvitamin D_3 1-Butyrate. This was obtained of 1α-hydroxyvitamin D_3 1,3-dibutyrate. We dissolved 150 mg of 1α-hydroxyvitamin D_3 (mp 136-138°C) in 1 ml of pyridine and added 0.2 ml of butyric anhydride. After 24 h, 20 ml of water was added and 20 ml of ethyl ether was used for extraction. The ether layer was washed out with 10% H_2SO_4 (2 × 20 ml) and 1% KOH (2 × 20 ml). To the ether solution was added 7.5 ml 0.1 N KOH in abs. alcohol. The solutions were maintained for 2 h at 20°C and for another 18 h at −12°C. The alkali was then washed out with water, and the solution was concentrated to 5 ml and applied to a column containing 15 g of silica-gel (Silicagel L 40/100, Chemapol, Czechoslovakia). A 1:3 mixture of ethyl ether—hexane was used to elute 30 mg of 1α-hydroxyvitamin D_3 3-butyrate, λ_{max} 265 nm; a 2:3 mixture of ethyl ether—hexane was used to elute 31 mg of 1α-hydroxyvitamin D_3 1-butyrate, oil, UV spectrum, λ_{max}, nm (log ε).(C_2H_5OH): 244 (4.164), 268 (4.153). PMR spectrum ($CDCl_3$), δ, ppm: 0.53 (3H, s, 18-H), 0.88 (6H, I 6 Hz, 26, 27-H); 4.23 (1H, m, Iβ-H); 5.12, 5.42 (2H, AB, q, I 1.5 Hz, 19-H); 5.60 (1H, m, 3α-H); 6.04, 6.49 (2H, AB, q, 6-H, 7-H, I 11.4 Hz).

EXPERIMENTAL BIOLOGICAL

The biological activity of I and of 1α-OH-D_3 was studied in white leghorn roosters. The chickens were raised in cages without access to ultraviolet light on a basic feed deprived of vitamin D. After three weeks, pronounced signs of rickets were noted: decrease in rate of weight gain; hypocalcemia; low mineralization of bone tissue, etc. At this time the chickens were divided into five experimental groups, with 12 chickens in each group. The chickens in the first group continued to receive the basic ration. They suffered from rickets and served as a negative control group. The chickens in the second and third groups, in addition to the basic ration, received preparation I, 0.65 nmole per head, orally or intramuscularly, in 0.1 ml of propylene glycol. The chickens in the fourth and fifth groups received 0.65 nmole of 1α-OH-D_3 orally or intramuscularly. The concentrated alcohol solutions of preparations I and 1α-OH-D_3 were synthesized at the Scientific-Production Union "Vitaminy" and diluted in corresponding volumes of propylene glycol.

The length of the experiment was 10 days. At the end of this period, all the chickens were weighed and separated into groups of six for further study. After they were killed, their parathyroid glands were removed and weighed. The relative mass of these glands was calculated for 100 g of living chicken mass. We determined the level of calcium in the blood serum on an atomic-absorption spectrophotometer. Inorganic phosphorus (P_i) and the activity of alkaline phosphatase, used as substrate for the enzyme sodium β-glycerophosphate, were measured [16]. To determine the mineralization of the bone tissue, the tibia was incinerated in a muffle furnace at 600°C after it was dried and the fat removed. The amount of vitamin D dependent on the calcium-bound protein (CaBP) was measured in the mucous membrane of the duodenum by radial immunodiffusion in agar [2]. CaBP was calculated for 1 mg of dissolved protein, which was determined by the biuret reaction [9].

In another experiment with rachitic chickens 21-30 days old, we studied the dynamics of CaBP in the mucous membrane of the duodenum after a one-time intramuscular injection of 3.25 nmole of I or 1α-OH-D_3 in 0.1 ml of propylene glycol. The chickens were killed one, three, six, and nine days after the preparation was injected. Six chickens were used in each variant of the experiment. CaBP was determined by the above-mentioned method.

RESULTS AND DISCUSSION

The structure of the 1α-hydroxyvitamin D_3 1-butyrate synthesized was confirmed by NMR spectrum. The structure of the synthesized esters — 1α-hydroxyvitamin D_3 1-butyrate and 3-butyrate — was further confirmed by their chromatographic mobility, a mobility determined by their absorption energy in silicagel. In the presence of a polar eluent, the adsorption energy A of molecules with similar polar groups is approximately inversely proportional to the projection area made on the surface of the adsorbent by the hydrophobic part of the molecule. (For simplicity, it can be assumed that in an adsorbed molecule the hydroxyl group in ring A is positioned equatorially, and that the direction of the bonds C—O is perpendicular to the surface of the adsorbent [4]). The value of A is significantly greater for 1α-hydroxyvitamin D_3 3-butyrate than for 1α-hydroxyvitamin D_3 1-butyrate, in accordance with the greater chromatographic mobility of 1α-hydroxyvitamin D_3 3-butyrate.

TABLE 1. Biological Effects of 1α-OH-D_3 1-Butyrate and 1α-OH-D_3 on Rachitic Chickens

Group No.	Preparation	Method of administration	Weight gain, g	Relative mass of parathyroid gland, mg/100 g mass	Ash content of tibia, %	Blood serum			CaBP, μg/1 mg protein
						Ca, mg/100 ml	P_i, mg/100 ml	alkaline phosphatase activity, unit	
1st	Control		37,4±3,2	4,61±1,12	25,43±0,69	7,92±0,22	4,01±0,19	30,76±1,13	0
2nd	1α-OH-D_3 1-butyrate	Oral	41,3±2,8	2,24±0,51	30,01±1,22	10,45±0,40	4,60±0,10	22,73±0,44	30,89±4,79
3rd	1α-OH-D_3 1-butyrate	Intramuscular	71,2±5,7	1,10±0,26	35,65±0,82	12,03±0,16	4,85±0,16	16,00±1,05	61,98±8,15
4th	1α-OH-D_3	Oral	57,2±4,3	1,89±0,29	33,62±0,53	12,41±0,17	4,60±0,10	20,15±0,58	52,76±6,47
5th	1α-OH-D_3	Intramuscular	66,0±4,5	1,51±0,25	34,97±1,1	12,43±0,20	4,97±0,18	18,71±0,87	60,16±5,49

Notes. 1) The unit of activity of alkaline phosphatase expresses the quantity of P_i (mg) eliminated from sodium β-glycerophosphate calculated on 100 ml of blood serum incubated for 30 min at 37°C. 2) Preparations administered to each chicken orally or intramuscularly, 0.65 nmole 24 h, over a period of 10 days.

In contrast to the vitamin D_3 esters in the hydroxyl group in position C-3, the UV spectrum of 1α-hydroxyvitamin D_3 1-butyrate has two peaks at 244 and 268 nm [13].

When both steroids were intramuscularly injected into the chickens of the third and fifth groups, they showed high antirachitic activity. This is demonstrated by the results of our study of the biological activity of I and 1α-OH-D_3. These results are shown in Table 1. Within 10 days, complete disappearance of signs of rickets was observed in the injected chickens, as compared with the control group of chickens (first group). The rate of weight gain increased by a factor of 1.8-1.9, the calcium level became normal, the activity of the alkaline phosphatase in the blood serum sharply decreased, and the relative mass of the thyroid gland decreased significantly (by a factor of 3-4). The data for CaBP Vitamin D obtained from our study of the mucous membrane of the duodenum confirm the high activity of the preparations and fulfillment of the necessary requirements for vitamin D_3 in chickens [3].

A comparative analysis of various indicators in chickens receiving preparations I and 1α-OH-D_3 demonstrate the absence of real differences in the antirichitic actions of the steroids studied. It should be noted, however, that there is a definite tendency toward a stronger effect for I (third group) than for 1α-OH-D_3 (fifth group) with respect to increase in chicken weight, decrease in alkaline phosphatase activity, and mass of the parathyroid gland.

Particularly important is the fact that the antirachitic effects of the preparations change significantly depending on the method used in administering them to the chickens. Thus, when I was intramuscularly injected (third group), signs of rickets in the chickens completely disappeared and the concentration of CaBP indicated that vitamin D requirements were completely satisfied. When the preparation was orally administered (second group), the concentration of CaBP was twice as low, a fact which accords with the lower rate of weight gain and the higher activity of alkaline phosphatase in the blood serum of chickens in the second group. After intramuscular injection of I, significant changes occurred in the parathyroid gland. Its relative mass decreased fourfold, while after oral administration of I, it decreased twofold. Depending on the method of its administration, differences in the activity of 1α-OH-D_3 are less pronounced. If that activity is determined according to the amount of CaBP present, it is only 15% less after oral administration than after intramuscular injection.

As we have shown [1], the activity of vitamin D in the preparations is most accurately measured by the concentration of CaBP in the mucous membrane of the duodenum 24-72 h after a one-time administration of these preparations to rachitic chickens in which CaBP has not been synthesized. This test is based on a direct correlation between the dose of vitamin D and the concentration of CaBP [2]. In these studies (see Fig. 1), we determined the following: 72 h after one-time intramuscular injections of 3.25 nmole of vitamin D_3, of I, and of 1α-OH-D_3, the amounts of CaBP synthesized in the mucous membrane of the duodenum were, respectively, 65, 81, and 83 μg/1 mg protein. These concentrations, expressed on a standard curve in vitamin D_3 units of activity, would comprise 50, 60, and 62 IU. Thus, the biological activity of steroid I in the chickens is 1.25 times higher than the activity of vitamin D_3, but it is almost equal to the activity of 1α-OH-D_3.

Furthermore, certain features in the dynamics of the activity of the two preparations, dependent on time elapsed after their one-time administration, can be seen in Fig. 1. Thus, the concentration of CaBP is higher after 24 h in chickens receiving 1α-OH-D_3 ($P < 0.01$). In the succeeding 48 h, the concentration of CaBP increases in chickens receiving 1α-OH-D_3 as it does for chickens receiving I, and both groups of chickens reach approximately the same maximum. On the sixth and ninth days, the concentration of CaBP decreases, but in chickens receiving I, this decrease occurs more slowly. Nine days after the preparations were administered, CaBP comprises approximately 18 μg/mg protein in chickens receiving I, while only a trace of CaBP is found in chickens receiving 1α-OH-D_3 (about 4 μg/mg).

Thus, our data demonstrate that the blocking of the 1α-hydroxy group in 1α-OH-D_3 with butyric acid does not prevent high biological secosteroid activity. Evidently, what first occurs in the organism is hydrolysis of the ester group and the formation of free 1α-OH-D_3 which is then quickly hydroxylated in the liver, forming the active metabolite 1α,25-dihydroxyvitamin D_3. This can be explained by the lower activity of I compared with that of 1α-OH-D_3 in the first 24 h after administration, which reflects a certain lag in the formation of 1α-hydroxyvitamin D_3 from the ester forms of I. However, the activity of both forms of 1α-OH-D_3, equal and high after 72 h, points to the fact that the rate of their hydrolysis is sufficiently high to ensure both the required quantity of active metabolite and its physiological effect in the intestinal epithelium by CaBP synthesis.

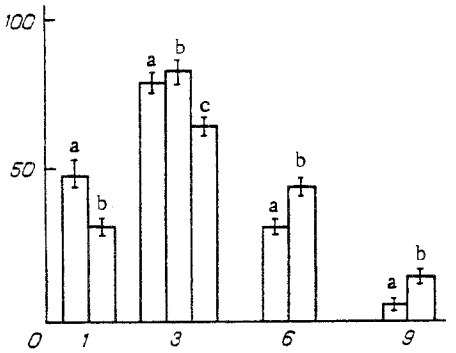

Fig. 1. Concentration of CaBP in mucous membrane of duodenum at different intervals after one-time intramuscular injection of rachitic chickens with 3.23 nmole 1α-hydroxyvitamin D_3 (a), 1α-hydroxyvitamin D_3 1-butyrate (b), and vitamin D_3 (c). Abscissa: days after administration of vitamin; ordinate: concentration of CaBP μg/mg protein.

The increased concentration of CaBP in the next six days in chickens that received I as against those that received 1α-OH-D_3 can be explained by the slower degradation of the ester form in the organism as against the free form. A comparison may be drawn with the increased biological activity in chickens receiving 24,25-difluoro-1,25-dihydroxyvitamin D_3, whose high chemical stability has been linked to that increase in activity [11].

These results of our studies show that I possesses two specific biological activities in comparison with 1α-OH-D_3: 1) high activity when intramuscularly administered and a sharp decrease in activity when orally administered; 2) prolonged action after one-time administration. Both characteristics of I are determined by its ester form. Its weak activity when orally administered is apparently due to its lower capacity for absorption in the intestines, a fact that has been similarly noted for the ester form of the vitamin [5, 6, 8, 14]. It has been shown that cholesterol esters cannot be absorbed without first being hydrolyzed by pancreatic juice enzymes, which are also necessary for hydrolyzing esters of vitamin D_3 [6]. Apparently, the rate of hydrolysis for esters of vitamin D and its analogs is insufficient for their rapid absorption. When I is intramuscularly administered, it is doubtful whether direct interaction between the ester and the specific receptor for 1α,25-dihydroxyvitamin D_3 takes place in the intestinal mucous membrane. But hydrolysis of the ester bond can take place in the liver. Molecules of 1α-OH-D_3 are slowly released, oxidized in position C-25, and converted into the active form of vitamin D_3.

Our data on the significant difference in the activity of preparation I underscore the fact that in determining the biological activity of preparations of vitamin D groups, it is important to take into consideration their ability to be absorbed in animal intestines. Despite the high antirachitic activity of preparation I when it is intramuscularly injected, it is its low biological activity when orally administered that severely limits its use in animal husbandry.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF CHLORO-SUBSTITUTED 1.5-DIKETONES AND THEIR CYCLIZATION PRODUCTS

V. G. Kharchenko, S. N. Chalaya, L. K. Kulikova, and O. V. Litvinov

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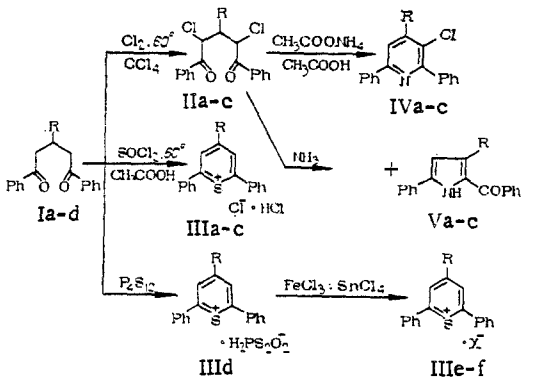
With the aim of preparing new biologically-active compounds, we synthesized chloro-substituted 1,5-diketones and their cyclization products, such as benzoylpyrroles, chloropyridines, and thiopyrilium salts, previously unknown, which serve as the bases for biologically-active preparations [2, 3].

The chlorination of the 1,5-diketones Ia-c was carried out with chlorine or thionyl chloride. The action of chlorine on 1,5-diketones leads to the electrophilic substitution of the hydrogen in the α-position to the carbonyl group to give the 2,4-dichlorosubstituted 1,5-diketones IIa-c [4]. Use of thionyl chloride instead of chlorine resulted in the cyclization of ketones Ia-c to give the corresponding thiopyrilium chlorides IIIa-c:



Ia—Va: R=H; Ib—Vb R=Me; Ic—Vc: R = Ph; Id, IIId-f: R = $C_6H_4NMe_2$-p.
IIIe: X = $FeCl_4$; IIIf: X=$SnCl_6$.

Structural features of these chloro-substituted 1,5-diketones IIa-c are shown specifically in their reactions with nucleophilic reagents [5]. Reaction of chlorodiketones IIa-c with ammonium acetate in acetic acid led to nucleophilic attack of the reagent on the carbonyl group and cyclization with formation of the 3-chloropyridines IVa-c. Reaction of IIa-c with ammonia also gave IVa-c as well as the 2-benzoylpyrroles Va-c produced as a result of nucleophilic attack of a chlorine atom and subsequent cyclization by reaction of the amino group with the carbonyl.

With the aim of introduction of dimethylamino groups, which strongly increase the biological activity of compounds, cyclization of diketone Id with phosphorus polysulfide resulted in the preparation of the thiopyrilium phosphate IIId. The thiopyrilium tetrachloroferrate (IIIe) and the hexachlorostannate (IIIf) were obtained by exchange reaction of salt IIId with ferric chloride and stannic chloride, respectively.

The structure of the compounds obtained was substantiated by data from elemental analysis and IR and NMR spectroscopy (Table 1).

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