PRODUCTS OF PHOTOTRANSFORMATION OF PROVITAMINS D_4 OBTAINED FROM A MUTANT Saccharomyces cerevisiae YEAST. I. IRRADIATION IN ETHANOL

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The composition of the photolytic mixture formed on the irradiation of provitamin D_4 has been studied. Together with the main reaction products — vitamin D_4 and provitamin D_4 — a number of by-products were formed the structures of which have been established from their spectral characteristics.

Vitamins of the group D, which regulate the phosphorus and calcium metabolism in animal tissues [1, 2], play an important role in maintaining the homeostasis of mammalian and avian organisms. A deficiency of them leads to the appearance of rickets, osteoporosis, and a number of other diseases and makes it necessary to create diets enriched with the vitamins of this group. At the present time, the most readily available is vitamin D_2, as a source of which the yeast Saccharomyces cerevisiae is used. Other vitamins of the D group are products of chemical synthesis.

We have previously reported the production of mutant strains of the yeast Sacc. cerevisiae with a modified biosynthesis of sterols and an investigation of the composition of their sterol fractions with the aid of chromato-mass spectrometry [3, 4]. Some of these strains selectively accumulate cholesta-5,7,22-trien-3β-ol, cholesta-5,7,22,24-tetraen-3β-ol, and ergosta-5,7-dien-3β-ol, which are structural analogs of provitamins D_3 and D_4 [3]. The latter, as has been established, is promising as an antirachitic additive to the diet of agricultural animals and poultry [5]. The photochemical transformation of provitamins D into the vitamins that takes place under the action of UV light is characterized by complexity and is accompanied by the formation of a number of by-products [6]. Even slight preirradiation leads to the appearance of a multicomponent photolytic mixture containing toxic compounds. The main role in this process is played by the nature of the solvent, which may take part in some photoreactions and initiate the occurrence of others. In the present paper we report an investigation of the photolysis of provitamin D_4 in ethanol, since this is used most frequently as the reaction medium for the production of pharmaceutical antirachitic preparations.

The initial compound — provitamin D_4 — was isolated from the biomass of a mutant yeast by the procedure described previously [4] and was dissolved in ethanol. Irradiation was carried out in a quartz vessel by a high-pressure mercury lamp with periodic monitoring of the course of the phototransformations with the aid of UV spectrophotometry and gas-liquid chromatography (GLC).

The reaction was stopped in the late stages of conversion, when the most substantial formation of subsidiary photoderivatives takes place. The reaction products were fractionated with the aid of thin-layer chromatography (TLC). Each of the fractions obtained was separated further by high-performance liquid chromatography (HPLC) and the fractions corresponding to the peaks on the performance of HPLC were collected preparatively. The purity of the fractions was checked with the aid of GLC. The compounds isolated were identified mass-spectrometrically in the light of the results of UV spectrophotometry and relative retention times in GLC and HPLC. The results obtained are given in Table 1. The numbering of the compounds (Fig. 1) corresponds to that of the HPLC fractions.

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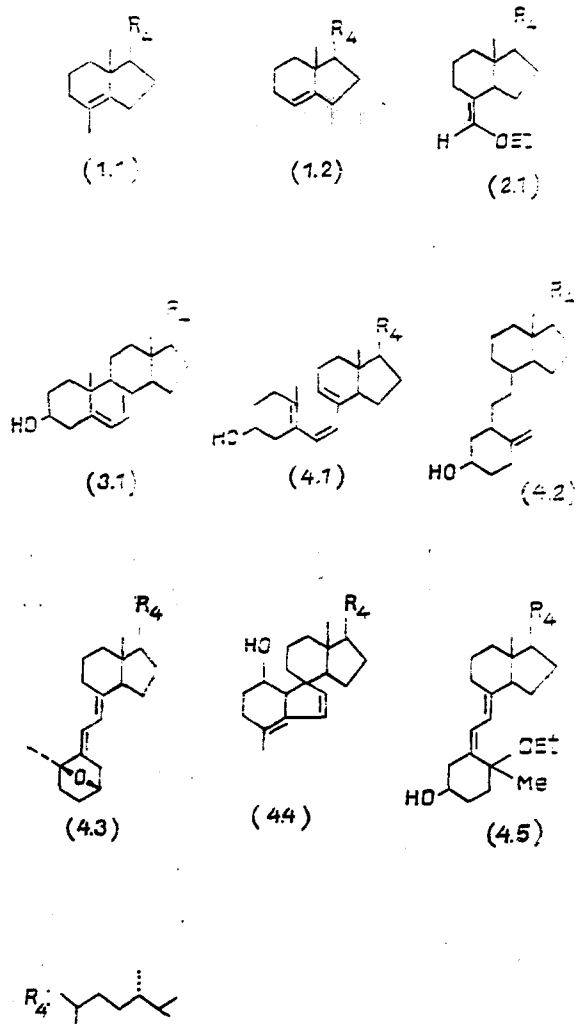


Fig. 1. Products of the photolysis of provitamin D_4 in ethanol.

The substances included in TLC fractions 1 and 2 were present in the photolytic mixture in very small amounts and consisted of low-boiling compounds with small molecular masses formed as the degradation of the main reaction products. Fraction 1 was separated with the aid of HPLC into two components. The compound constituting fraction (1.1) was identified as 2,4-dimethyl-7-R_4-bicyclo[4.3.0]non-1-ene. The m/z value of the molecular ion, M^+, was 276, corresponding to the cleavage of the molecule of previtamin D_4 [compound (4.1)] at the 6–7 bond. The mass spectrum contained a characteristic fragmentary ion with m/z 261, the appearance of which was connected with the elimination of a methyl group from the C_2 or the C_4 position, and two fragments with m/z 149 and 147 due to the splitting out of the side chain of the molecule – $[M - R]^+$ and $[M - R - 2H]^+$. This type of fragmentation occurs in sterols with Δ^7 double bonds [7]. The spectrum also contained an ion with m/z 109 which, apparently, appeared as a consequence of the allyl cleavage of C_4–C_7 and C_3–C_9, this apparently being analogous to the cleavage of ring D of steroids with Δ^7 double bond. It is obvious that the cyclopentane ring of the compound under investigation does not contain a double bond in the C_3–C_9 position, otherwise such decomposition would be blocked. Particular interest is presented by the question of the direction of the double bond at C_2. Its direction to atom 1 or atom 3 of the cyclohexane ring or to the carbon atom of the methyl group in position 2 is possible. The C_2–C_3 position is the most likely, as was confirmed by the high level of elimination of the methyl group due to allyl activation of the bond. Certain difficulties are also introduced by the known fact of the migration of a double bond on electron impact, which is the main problem in the identification of the positions of these bonds in steroid molecules [8].

The compound constituting fraction (1.2) was determined as 4-methyl-7-R_4-bicyclo[4.3.0] non-1-ene, similar in its chromatographic characteristics to (1.1). Its mass-spectrophotometric dissociation included the cleavage of the cyclopentane ring at the C_4–C_7 and C_3–C_9

TABLE 1. Products of the Photolysis of Provitamin D_4 in Ethanol

TLC		HPLC		GLC	UV spectrum, λ_{max}, nm	Amount**, %	Main ions in the mass spectrum, m/z (rel. int.)
fraction	$R_f \times 100$	fraction	$R_t \times 100$*	$R_t \times 100$*			
1	32	1.1	68	25	—	1.25	M^+ 276(65), 261(35), 147 (28), 109 (18), 43 (100)
		1.2	71	38	—	2.18	M^+ 262 (80), 247 (5), 135 (18), 133 (6), 95 (48), 43 (100)
2	37	2.1	99	69	—	2,61	M^+ 320 (18), 305 (19), 275 (65), 149 (42), 147 (18), 109 (25), 43 (100)
3	77	3.1	100	100	271,5; 282,0; 293,5	19,56	M^+ 398 (100), 383 (18), 380 (56), 365 (95), 339 (20), 271 (31), 253 (25)
4	80	4.1	113	112,5; 125	256	21,45	M^+ 398 (56), 383 (10), 380 (12), 365 (22), 271 (42), 253 (28), 176 (58), 158 (46), 136 (20), 118 (8), 109 (100)
		4.2	116	112,5; 125	265	18,34	M^+ 398 (65), 383 (11), 365 (25), 271 (39), 253 (30), 176 (15), 158 (24), 136 (68), 118 (100)
		4.3	117	112,5; 125	243,0; 252,0; 262,0	11,24	M^+ 398 (56), 383 (15), 353 (28), 271 (35), 247 (41), 176 (10), 136 (42), 133 (100), 118 (52)
		4.4	125	106	253	10,51	M^+ 398 (25), 380 (85), 378 (100), 365 (25), 271 (32), 253 (29), 176 (60), 136 (8), 118 (3)
		4.5	128	131	243,0; 250,5; 259,0	12,86	M^+ 444 (3), 442 (3.5), 429 (28), 398 (24), 396 (9), 378 (18), 365 (18, 355 (30), 339 (5), 271 (21), 253 (16), 176 (4), 158 (3), 149 (65), 136 (65), 118 (100)

*Relative to provitamin D_2.
**According to the GLC results.

bonds with the formation of a fragmentary ion having m/z 95, and practically all its fragmentation pathways were similar to those of compound (1.1).

According to HPLC and GLC (Table 1), TLC fraction 2 contained the single compound (2.1), which was characterized as 2-ethoxymethylene-4-methyl-7-R_4-bicyclo[4.3.0]nonane. It consisted formally of the product of addition of a molecule of the solvent (ethanol) to the C_2 position of compound (1.1), although it is not excluded that (2.1) was formed through the cleavage of the dienic system of the ethoxy derivative of vitamin D_4. Compound (2.1) was relatively unstable to electron impact: The intensity of the M^+ peak was 18% of the density of the maximum peak of the spectrum. The primary processes in the breakdown of the molecular ion were the loss of methyl and hydroxyethyl groups, and the resulting fragmentary ion $[M - C_2H_6O]^+$ dissociated in a similar manner to compound (1.1).

The study of fractions 1 and 2 permitted the conclusion that in the course of irradiation the reaction products underwent degradation mainly through the cleavage of the C_6–C_7 bond of the secosteroid linkage. The mechanism of this process is similar to the breakdown of olefins in the gas phase [9].

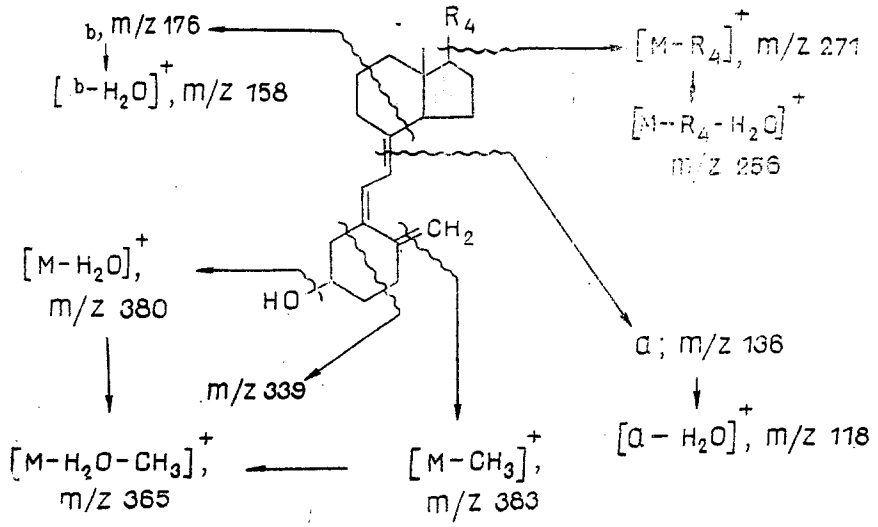


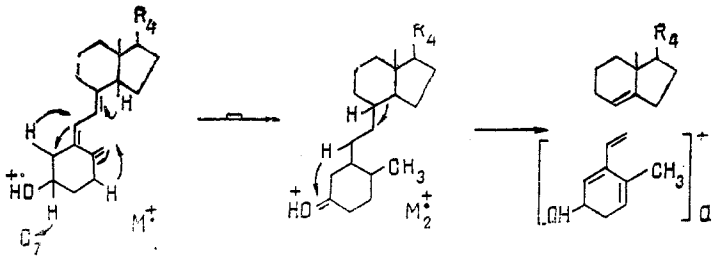
Fig. 2. Scheme of the fragmentation of vitamin D_4 under the action of electron impact.

According to HPLC and GLC results, TLC fraction 3 contained a single compound the chromatographic parameters of which coincided with those of the initial ergosta-5,7-dien-3β-ol (provitamin D_4). The results of UV spectrophotometry and mass spectrometry corresponded completely with those known for this compound [4]. Thus, this fraction was formed by provitamin D_4 that had not undergone conversion.

The main components of the mixture analyzed proved to be the substances forming TLC fraction 4. When it was separated (HPLC), five individual substances were isolated. On analysis by the GLC method, each of fractions (4.1) and (4.2) issued identically on the chromatogram in the form of two peaks (Table 1) with a constant ratio of 2:1. A comparison of the retention times of fractions (4.1) and (4.2) with standards permitted the assumption that the first of them contained previtamin D_4 and the second vitamin D_4. The observation of double peaks with retention times identical for the first and second cases is a consequence of the well-known fact of the thermal cyclization both of previtamins and of vitamins D in the injector of the chromatograph, leading to the formation of the corresponding pyro and isopyro components [10]. The ratio of their concentration remains constant over a wide temperature interval, and therefore in GLC a vitamin and a previtamin D always issue in the form of double peaks in a constant proportion.

The UV spectra of compound (4.1) recorded a single absorption band with λ_{max} 256 nm, which is characteristic for previtamins D [11]. The m/z value of the molecular ion, 398, coincided with that of provitamin D_4 which showed that (4.1) belonged to the group of photoisomers which includes, in addition to previtamin and vitamin D_4, a broad group of so-called toxisterols and a number of other photoderivatives. In an analysis of the mass spectrum it was established that the main dissociation pathway of the molecular ion was the cleavage of ring C at the C_8–C_{14} and C_{11}–C_{12} bonds with the formation of the so-called ion **b** with m/z 176 (Fig. 2) and its subsequent degradation, accompanied by the appearance of the intense peak of the $[b - H_2O]^+$ peak with m/z 158. Fragmentation of this type predominates for endocyclic trienes and, in particular, for previtamin D_4, which is due to the high stability of the $\Delta^{5(10),6,8}$-system of double bonds [12]. All that has been said above permitted the identification of (4.1) as 9,10-secoergosta-5(10),cis-6,8-trien-3β-ol (previtamin D_4).

The UV spectrum of fraction (4.2) consisted of a single absorption band (λ_{max} 265 nm) which is typical for the absorption of the triene system of vitamins D [11]. The mass spectrum of this fraction was likewise characteristic of this group of compounds (see Table 1). It is known that in isomeric trienes competition takes place between two pathways of fragmentation under the action of electron impact: the cleavage of the C_7–C_8 bond to form ion **a** with m/z 136 and with the opening of ring C to form ion **b** (see Fig. 2) [12]. The first pathway predominates in secosteroids having double bonds exocyclic in relation to the cyclohexane ring, which include vitamin D_4, while the second, as already mentioned, takes place in endocyclic trienes. The cleavage of the C_7–C_8 bond precedes the migration of the double bond under the action of electron impact. The following scheme is suggested for this process:



Then the degradation of ion a takes place with the formation of the $[a - H_2O]^+$ ion with m/z 118, which gives the basic peak of the spectrum, this obviously being due to its aromaticity [12].

A general scheme of the fragmentation of vitamin D_4 is given in Fig. 2. In comparison with the initial provitamin D_4 (3.1) a decrease in the intensity of the molecular ion is observed because of the low stability of (4.1) under the action of electron impact. The fall in the size of the $[M - H_2O]^+$ peak for vitamins is connected with competition on the part of the reaction $M^+ \rightarrow a$. It is also interesting to note the low intensity of the $[M - H_2O - CH_3]^+$ peak, which is connected both with the predominance of fragmentation of types a and also with the considerable consumption of energy in the elimination of the methyl group in position 19. A comparison of the mass spectrum of compound (4.2) with those known for vitamins D_2 and D_3 permitted the conclusion that all the differences observed in fragmentation were connected with the structure of the side chain. For example, the intensities of the peaks of ions with m/z 271 and 253 corresponding to the elimination of the side chain were lower for vitamin D_4 than for vitamin D_2, which is due to the presence of a Δ^{22}-bond in the side chain of the latter in the β-position to the point of cleavage.

The substances in addition to the previtamin (4.1) and the vitamin (4.2) composing the TLC fraction consisted of structural isomers of vitamin D_4 having the trivial name of "toxisterols." Fraction (4.3) consisted of toxisterol D (3,10-epoxy-9,10-secoergosta-5,7-diene). The nature of the UV spectrum of this compound (see Table 1) indicated the presence of a dihydrotachysterol chromophore structure of the molecule [13]. During GLC we observed peaks with retention times coinciding with those of vitamin D_4; their molecular weights are coincided. However, the nature of the fragmentation had a number of peculiarities. Thus, the mass spectrum did not contain the peaks of dehydrated ions, which is a well-known distinguishing feature of dihydrotachysterol, an isomer of vitamin D, and products related to it [14]. The presence in the spectrum of the peak of a fragmentary ion with m/z 247, which is a characteristic feature of the breakdown of toxisterols D, was also observed. The mechanism of its formation is not clear.

Another compound belonging to the class of toxisterols, 4,8-cyclo-9,10-secoergosta-5(10),6-dien-3β-ol (toxisterol A), made up fraction (4.4). Its retention time in GLC was close to that of provitamin D_4 and differed considerably from those of previtamin and vitamin D_4. Its UV spectrum contained no band characteristic for a $\Delta^{5,7}$-dienic system of a sterol and had a considerable absorption in the 250-255 nm region which is typical for the heteroannular dienic grouping —CH=CH—C=CMe [13]. The main fragmentary ions in the mass spectrum of compound (4.4) had mass numbers coinciding with the "provitamin" fragments but differing in intensity. The following features were noted for the fragmentation of compound (4.4): 1) The molecular ion has a relatively low intensity, which is connected with the absence of a conjugated $\Delta^{5,7}$-dienic system from the structure; and 2) the peak of the fragmentary ion $[M - CH_3]^+$ has a low intensity, which is connected with the unsuitability of the vinyl elimination of the methyl group in the C_{10} position and shows the saturated nature of rings C and D of the molecule, since the presence of multiple bonds facilitate the splitting out of a methyl group in the C_{13} position [8]. The highly intense peaks of fragmentary ions with m/z 380 and 378 are connected with the dehydration of the molecular ion and with its simultaneous dehydration and dehydrogenation, respectively. In this process, the aromatization of ring A takes place, and the structure arising acquires stability to electron impact. The same factors explain the presence in the spectrum of the peak of the $[M - CH_3 - H_2O]^+$ ion, which, in spite of the unsuitability of the elimination of a methyl group, is extremely intense because of its stability. In the breakdown of compound (4.4), the dissociation $M^+ \rightarrow b$ predominates, this being characteristic for the fragmentation of the heteroannular dienic system present in the molecule [12].

Fraction (4.5) contained 10-ethoxy-9,10-secoergosta-5,7-dien-3β-ol (toxisterol F) which is the product of the photoaddition of ethanol to vitamin D_4. Its UV spectrum coincided

with that known for toxisterols formed in the photolysis of provitamins D_2 and D_3. The m/z value of the molecular ion corresponded to the hypothesis of the addition of an ethoxy group, and the low intensity of the peak of this ion indicated the instability of substance (4.5) under electron impact. The initial breakdown of the molecule takes place through the elimination of the methyl and ethoxy substituents and water from ring A, and the peaks of the fragmentary ions $[M - CH_3]^+$, $[M - H_2O]^+$, $[M - C_2H_6O]^+$ and $[M - C_2H_6O - H_2O]^+$ appear in the spectrum. The formation of the latter, as in the case of vitamin D, can take place both on the successive and on the simultaneous elimination of water and ethanol. After the elimination of the ethoxy group from compound (4.5), a fragmentary ion is formed that repeats the structure of vitamin D_4 and the dissociation of which, in the main, takes place similarly to that of the latter.

The biological properties of the compounds accompanying the formation of the vitamins of the D group remain little studied. However, the available information [13] indicates that their presence lowers the value of antirachitic drugs.

EXPERIMENTAL

As the producing agent of provitamin D_4 we used a prototrophic strain of the yeast Saccharomyces cerevisiae 78-PT21 [15]. Cultivation was carried out under the conditions described previously [3, 4]. The sterol fraction was isolated by the method of Breivik and Owades in Woods' modification [16].

The provitamin D_4 was irradiated in a quartz test tube in an atmosphere of argon with its continuous bubbling through the solution at room temperature. Ethanol was used as the solvent, the concentration of the solution being 2.5 mg/ml. The source of radiation was a RL-250 high-pressure mercury lamp with a linear emission spectrum [17]. The lamp was set up at a distance of 10 mm from the irradiated surface. The course of the reaction was monitored with the aid of GLC and by UV spectrophotometry.

TLC was performed at Silufol plates impregnated with $AgNO_3$ in the solvent system chloroform—acetone (95:5 by volume). UV spectra were taken on a Specord M 40 spectrophotometer in ethanol.

GLC was conducted on a Tsvet 100 chromatograph in a 0.2 × 500 cm column with 3% of OV-17 on Chromaton N Super at a temperature of 280°C. The carrier gas was helium at a rate of flow of 35 ml/min. Flame-ionization detector.

HPLC was carried out on a Du Pont chromatograph. Ultraviolet detector, λ_{max} 263 nm. Zorbax Sil 4.6 × 250 ml column with chloroform—acetone (200:2, by volume) as the mobile phase at a rate of passage of 1.25 ml/min. Mass spectra were obtained on a Varian MAT 311A instrument, samples being admitted to the ion source by direct introduction after separation with the aid of HPLC. The temperature of the sample evaporator was raised from 50 to 300°C at the rate of 1°C/min. The mass spectra were scanned every second. The ionizing voltage was 70 eV.

The results of the separation and analysis are given in Table 1.

SUMMARY

The composition of the photolytic mixture formed on the irradiation of provitamin D_4 has been studied. Together with the main reaction products — vitamin D_4 and previtamin D_4 — a number of by-products are formed the structures of which have been established from their spectral characteristics.

LITERATURE CITED

1. R. I. Yakhimovich, The Chemistry of the D Vitamins [in Russian], Kiev (1978).
2. H. F. De Luca and H. K. Schnoes, Annu. Rev. Biochem., 52, 411 (1983).
3. A. V. Andreev, A. V. Moseichuk, K. A. V'yunov, et al., Khim. Prir. Soedin., 841 (1985).
4. A. V. Andreev, A. V. Moseichuk, K. A. V'yunov, and N. P. Mikhailova, Khim. Prir. Soedin., 196 (1986).
5. N. P. Mikhailova, M. Yu. Valnietse, V. K. Bauman, and K. A. V'yunov, Prikl. Biokhim. Mikrobiol., 24, No. 1, 98 (1988).
6. M. F. Holick, J. A. MacLaughlin, and S. H. Doppelt, Science, 211, No. 4482, 590 (1981).
7. C. Djerassi, Pure Appl. Chem., 21, No. 2, 205 (1970).

8. C. Djerassi, Pure Appl. Chem., 50, No. 3, 171 (1978).
9. T. Kobayashi, S. Yoshimoto, and M. J. Yashimura, Nutr. Sci. Vitaminol., 23, No. 2, 281 (1977).
10. H. Ziffer, W. J. A. Van den Heuvel, E. O. A. Haahti, and E. C. Horning, J. Am. Chem. Soc., 82, No. 24, 6411 (1960).
11. H. F. De Luca and J. W. Blunt, Meth. Enzymol., 18, 709 (1971).
12. Z. V. I. Zaretski, Adv. Mass Spectrom., 8, 590 (1980).
13. A. G. M. Barrett, D. H. R. Barton, R. A. Russell, and D. A. Widdowson, J. Chem. Soc. Perkin Trans. I, No. 6, 631 (1977).
14. Z. V. I. Zaretskii, Recent Dev. Mass Spectrom. Biochem. Med. Environ. Res., 7, 227 (1980).
15. N. P. Mikhailova, Z. A. Zhakovskaya, A. V. Andreev, and K. A. V'yunov, Khim.-farm. Zh., No. 2, 1490 (1987).
16. R. A. Woods, J. Bacteriol., 108, No. 1, 69 (1971).
17. Handbook on Photochemistry [in Russian], Moscow (1983), p. 78.

PROTEIN KINASE FROM COTTON SEEDS

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A Ca^{2+}-dependent protein kinase C, active at pH 6.5-8.0 has been found in cotton seeds for the first time. The localization of the enzyme in the seeds has been established and some of its properties are described (stability in various media, capacity for performing the phosphorylation of various substrates, activation by calcium ions). Highly active preparations of cottonseed protein kinase C have been isolated by biospecific chromatography.

Phosphorylation-dephosphorylation reactions are the main link in the transformation of a hormonal signal into specific metabolic responses [1, 2].

The enzyme protein kinase C performs the phosphorylation of cell proteins, thus mediating the action of hormones on the effector systems of the cells. In animal tissues, this enzyme plays the main role as part of the transmembrane "signal systems" [3] and controls many cell functions [4-6].

In plant cells, as in animal cells, protein kinase C is a mediator and enhancer of the action of hormones [7-9]. In spite of the factual material that has accumulated indicating the importance of the phosphorylation of proteins for the control of metabolic processes in higher plants [10-12], investigations in this field are only in their initial stage.

We have established the presence in cotton seeds of an enzyme with protein kinase activity. The dependence of the enzymatic activity on the pH was investigated in the extract obtained after the centrifugation of a suspension of three-day cotton seedlings in a suitable buffer (1:5). The phosphorylation of casein took place with the maximum rate at pH 6.5-8.0. The enzymatic activities were concentrated almost completely in the soluble fraction.

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