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An attempt to explain the mechanism of the synthesis of α-tocopherol in the seedlings of *Pisum sativum* L.

Próba wyjaśnienia mechanizmu syntezy α-tokoferolu w kiełkach Pisum sativum L.

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INTRODUCTION

Green (1958) and Baszyński (1959) put forward the suggestion that α-tocopherol may arise from mono- and dimethyltocols in the process of their methylation.

The universality of the processes of the biological methylation is known both in the animal and in the plant kingdoms. The problem has been, to a great extent, explored by Du Vigneaud and associates who since 1939, working chiefly with animal material, have examined the process of the biosynthesis of the methyl groups, their transfer, degradation and removal from the metabolism.

Work on biological methylation in plants was also undertaken at this time, although the genesis of the methyl compounds, especially in fungi, had been known since the last hundred years or so (Schröter 1955).

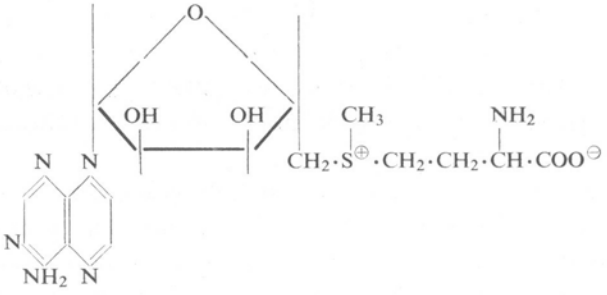
Research on the reactions of methylation in plants was started by Barrenscheen and Pany (1941). These authors stated that in etiolated seedlings of wheat, the enzymatic synthesis of creatine from guanidineacetic acid occurs without any particular donor of the methyl groups.

The genesis of the methyl groups is closely connected with the metabolism of compounds with an ability to split off fragments of C_1 units. The splitting-off of such active groups of C_1 occurs in the oxidation process in the formation of compounds of the formaldehyde type or formic acid. Active groups of C_1 ("formate") may arise from α-glicyne, glioxalic acid, β-serine, methanol, acetone, and certain atoms of the purine system or rings of imidazole histidine (Mothes 1959).

The synthesis of the methyl groups from "formate" require reduction, for which the action of folic acid and its derivates is essential.

From the later work of Du Vigneaud (c.f. Schröter 1958) it appears that methyl groups can be transferred from a donor to an acceptor without oxidation and reduction, and directly, in an unchanged form by the transmethylation reaction. This reaction is not dependent on folic acid but requires special donors of the methyl groups with "onium" structure, and of an unknown transmethylase system.

In many experiments the most active methylating agent in plants appears to be methionine. This compound, does not directly give up $—CH_3$ to the acceptor, but has first to be activated. This activation takes place with the action of ATP catalyzed by the methionine-activating enzyme (MAE) (Cantoni and Durrell 1957). This reaction leads to the formation of the "onium" compound S-adenosyl-methionine (AMe) (Baddiley and Jamieson 1954):



Only then "active methionine" gives up a methyl group transferred by its own methylase to the acceptor.

The methyl groups in plants are transferred above all to sulphur and nitrogen atoms, and also, which has not yet been observed in animals, to oxygen atoms (ricinine, protopine, lignin) (c.f. Schröter 1955).

Among the numerous acceptors of methyl groups mono- and dimethyltocols have not yet been counted, although Schwarz (c. f. Beckmann 1955) supposes that vitamin E may occur in the transmethylation reaction.

This possibility was not taken into consideration probably for two reasons:

(1) the unknown change in the tocopherol composition during the vegetative growth of the plant, which suggests the change of one of the tocopherols into another in the process of methylation or demethylation,

(2) the acceptance of $—CH_3$ by the carbon atom of the acceptor has been known only recently and so far in very few cases.

In 1954 in researches on *Bacillus subtilis* Rege and Sreenivasan showed, that the —CH_3 group, derived from glycine, serine, threonine or sarcosine, is substituted in C_5 thymine.

Since Alexander and associates (1958a, 1958b) observed the union of a methyl group to C_{24} in ergosterol of yeast, they assumed that this group derived from methionine is in the course of an unknown process of transmethylation, directly transferred from sulphur to carbon.

Bray and Shemin (c. f. Pawełkiewicz 1959) in their studies on the derivation of additional methyl groups in the corrin ring system of cyanocobalamin, observed the methylation of the carbon atom. They showed that the donor of these groups is methionine. From the experiments of these authors carried out on an *Actinomycetes* culture, it can be seen that the donor was not betaine or choline.

Bukin and Proniakowa (1959) state that the biosynthesis of vitamin B_{12} in *Propionibacterium shermani* is strictly connected with the processes of the methylation of carbon of the corrin ring system under the influence of methionine.

It may be that the transfer of —CH_3 to the carbon of the acceptor is not an isolated or an exceptional phenomenon. Because of this the author sets out in this paper to examine in vivo the possibility of the methylation of γ-tocopherol to α-tocopherol using methionine as donor of the methyl groups. If plant tissues control an enzymatic system capable of the synthesis of AMe and a system of transmethylases, one ought to allow the increase in α-tocopherol under the influence of this donor.

MATERIAL AND METHODS

In search for experimental material attention was drawn to the pea (*Pisum sativum* L.) According to Green (1958) peas contain α- and γ-tocopherols as well as insignificant traces of δ-tocopherol. This last disappears after the first day of germination and does not reappear till the beginning of the seed maturation. This is a very favourable circumstance since γ-tocopherol as a dimethyltocol requires the addition of only one —CH_3 group for the formation of α-tocopherol. However in the case where δ-tocopherol occurs (mono-methyltocol) one must look out for three products of substitution, which would confuse the interpretation of the results.

Before proceeding to the examination of the mechanism of the synthesis of α-tocopherol the fluctuation undergone by tocols in the germination process of the pea was determined and so also was their relation to the biosynthesis of carotenoids. After the determination of the changes in the composition of the tocopherols occurring in the germination process, research on the mechanism of the synthesis of α-tocopherol was carried

out on 2-day-old seedlings (2—3 cm long) since in this phase of growth they do not yet contain δ-tocopherol, or even traces of it, and are characterized still by a great quantity of tocopherol per unit of fresh matter.

All experiments were carried out in darkness (Baszyński 1959).

In these experiments peas (*Pisum sativum* L.) of the Victoria variety with a germinating percentage of 85 ± 4% were used. Seeds were supplied by the Agriculture Institute in Puławy.

Before germination the seeds were sterilized in a 0,1% of mercuric chloride for 30 minutes, and were than washed in distilled water.

Germination took place in a thermostat at a temperature of 28 ± 3°C on lignin moistened with distilled water.

For experiments on the number of changes of particular tocopherols in 7 days'growth, the seeds were kept on paraffinated gauze in Knop's five-times-dissolved nutrient solution. Material for analysis was collected after 1, 2, 3, 4, 5 and 7 days.

Methylation was carried out in 2-day-old cut seedlings in vivo by the infiltration method. Each time, 20 g of seedlings were taken for one estimation. Infiltration was carried out in a vacuum dessicator in beakers containing 100 ml of buffer solution with corresponding substrate. Air was removed from the dessicator by means of a vacuum pump, pressure was diminished to 40 mm Hg. After 30 minutes the tap was turned off, the pump was disconnected and pressure was restored in 2 minutes. After a quick and thorough rinsing with water the material was placed in Petrie dishes and put in a draught at a temperature of 30°C to drive off excessive water and to return the weight of the seedlings to the original state. Then they were put in dessicators (with open tube) and incubated in a thermostat at a temperature of 28 ± 3°C. Relative humidity in the dessicator was 85—90%.

The seedlings were incubated for 2, 10, and 20 hours. After the determination of the optimal time of incubation (10 hours) this time was adapted in all succeeding experiments.

As donor of the methyl groups, D,L-methionine (The British Drug Houses Ltd.) was always used, in the presence of ATP and magnesium ions. In the determination of the optimal concentration of methionine, it was used in concentrations of 0,02; 0,1 and 0,25 M. In all remaining experiments methionine was used in the concentration 0,1 M.

Adenosinetriphosphoric acid (The British Drug Houses Ltd.) was at first used in concentrations of $1 \cdot 10^{-3}$, $2 \cdot 10^{-3}$, and $5 \cdot 10^{-3}$ M, and later $1 \cdot 10^{-3}$ M.

$MgCl_2$ A. R. (FOCH Gliwice) was used in the concentration 0,02 M.

All experiments were carried out in a phosphate buffer at 0,02 M,

at pH 6,6. In the determination of the optimal concentration of hydrogen ions, phosphate buffer at 5,6 and 7,8 was used in addition.

All experiments were compared with a control (buffer, not containing substrates).

The biosynthesis of α-tocopherol was measured by the increase of this compound per unit of dry matter depending on time of incubation, the pH of the medium and the concentrations of methionine and ATP.

Tocopherols were estimated by Green's method, accepted by the British Analytical Methods Committee (1959).

The shoots were finely chopped in the Multi-Mixer homogenizer and prepared for chromatography according to the methods used in a previous paper by the author (1959). The unsaponifiable fraction was dissolved in benzene and chromatographed on a column filled with florisil activated according to the method of Glavinde, Kjelhede and Prange (c. f. Brown 1952). After paper chromatography the extinction of the eluate was measured on a Colleman photocolorimeter by the 8—209 filter (525 mμ). The identification of the tocopherol was made on the basis of the R_f and checking maximum absorption after eluating on filter paper in a spectrophotometer SF 4.

Carotene and xanthophyll were estimated by Worker's method (1957).

The results shown in the tables are arithmetical means of five repetitions.

In all statistical counts, Student "t" method was applied; only in tables 4 and 5 the influence of the concentration of the substrate (methionine and ATP) was determined by Fisher's method.

In the tables the value of the confident interval was calculated with a 5% standart error only in cases where there was a real influence of this factor ($t^0 > t_{0,05}$; $F^0 > F_{0,05}$).

RESULTS

1. Content of tocopherols in the germinating pea seed and quantitative changes in their composition

In etiolated shoots of the pea, tocopherols occur in significant quantities. The total content of tocopherols calculated on the basis of dry matter falls in the period of growth. After seven days it is 40,3% of what it was on the first day of germination. The absolute content of tocopherols (in 10 seedlings) at first rises insignificantly and from the third day of germination a fall in tocopherol content can be observed until the end of the seven-day experiments.

Table 1

Content of tocopherols and carotenoids in seedlings of *Pisum sativum* L. growing in darkness

Day of growth	Dry matter %	Tocopherols												Carotenoids in μg					
		per g of fresh weight							per g of dry weight	in 10 seedlings				per g of fresh weight		per g of dry weight		in 10 seedlings	
		α		γ		δ		total	total	α	γ	δ	total	carotene	xanthophyll	carotene	xanthophyll	carotene	xanthophyll
		μg	%	μg	%	μg	%	μg	μg	μg									
1	30,4	6.6 ±0.10	12.1	45.5 ±0.45	83.1	2.6 ±0.10	4.8	54.7	179.9	0.5	3.4	0.2	4.1	—	—	—	—	—	—
2	19.9	10.4 ±0.97	32.4	21.2 ±2.14	67.6	traces		31.6	158.9	1.5	3.2	—	4.3	traces					
3	17.2	11.2 ±0.40	42.3	15.0 ±0.23	57.7	—	—	26.0	151.2	1.7	2.3	—	4.0	traces					
4	14.7	12.0 ±0.70	58.2	8.6 ±0.77	41.8	—	—	20.6	140.2	2.2	1.6	—	3.8	0.1 ±0.02	0.4 ±0.08	0.7	2.7	0.03	0.13
5	10.6	8.8 ±0.67	65.7	4.6 ±0.36	34.3	—	—	13.4	126.8	2.2	1.2	—	3.4	0.2 ±0.01	0.4 ±0.07	1.9	3.8	0.5	1.0
7	6.3	3.7 ±0.55	80.4	0.9 ±0.17	19.6	—	—	4.6	72.4	2.3	0.6	—	2.9	0.5 ±0,05	0.6 ±0.12	6.3	9.5	1.5	2.3

Etiolated pea seedlings contain three tocols (α, γ, δ). δ-Tocopherol forms the smallest percentage of the total tocopherols (4,8%); on the second day only slight traces can be observed. With further growth δ-tocopherol disappears completely. Changes in tocopherol content involve diminution of the amount of γ-tocopherol (from 83,1% on the first day of germination to 19,6% after seven days) and augmentation of the amount of α-tocopherol (to 80,4% on the last day of the experiments) (see table 1).

At first carotenoids do not appear at all. Only traces of these compounds can be observed on the second and third days of the experiments. The first measurable quantity of carotenoids was observed on the fourth day of germination. The biosynthesis of carotenoids occurs more and more intensively as the seedlings develops. During the whole period of the research there is more xanthophyll that carotene. The biosynthesis of carotene occurs more quickly than that of xanthophyll: this can be seen in the change of the relation of xanthophyll to carotene reaching 4,0 on the fourth day of the experiments and 1,5 after seven days.

2. Dependence of the synthesis of α-tocopherol on the incubation-time of the seedlings

Since there were no data on the speed of the synthesis of α-tocopherol in pea-shoots, an analysis was carried out 2, 10, and 20 hours after the time of infiltration (table 2). A suitable time had to be chosen in which a fall in the total of tocopherols would not occur, and the increase of α-tocopherol was as big as possible. Infiltration by buffer alone had no real influence on lowering the total of tocopherols after 2 hours, and only to a very slight degree after 10 hours. However, after 20 hours the decrease in total of tocopherol is very significant, reaching 1/3 of what it was in the beginning.

Infiltration with methionine (+ATP and Mg^{++}) also did not affect the total of tocopherols in the first two periods of incubation, and the decrease after 20 hours is also very significant.

The content of α-tocopherol after infiltration of the seedlings with buffer (without methionine) scarcely changes after 2 hours; 10 hours of incubation causes a rise in the amount of α-tocopherol, while 20 hours'incubation caused a big fall in the amount.

Methionine in comparison with the sample before infiltration in the two first cases raised the quantity of α-tocopherol in proportion to the time of incubation. After 20 hours'incubation methionine causes a decrease of this compounds. In comparison with the control methionine gives higher results in all cases.

Tabie 2

Effect of incubation time on the synthesis of α-tocopherol

	Before infiltration	After infiltration					
		2 hours		10 hours		20 hours	
		C	S	C	S	C	S
Total of tocopherols in μg/1 g of dry matter	144.0	141.8	142.5	140.2	139.2	51.5	61.6
Increase after incubation		—2.2	—1.5	—3.8	—4.8	—92.5	—82.4
confident interval		—	—	3.83	4.62	12.88	9.78
Increase under the effect of methionine for the given time of incubation		0.7		—1.0		10.1	
confident interval		—		—		—	
α-tocopherol in μg/1 g of dry matter	55.8	55.3	59.3	61.2	70.4	31.1	41.2
Increase after incubation		—0.5	3.5	5.4	14.6	—24.7	—14.6
confident interval		—	—	2.91	4.58	8.20	7.40
Increase under the effect of methionine for the given time of incubation		4.0		9.2		10.1	
confident interval		—		2.29		9.52	
% of α-tocopherol	38.7	39.0	41.5	43.6	50.6	60.5	66.8
Increase after incubation		0.3	2.8	4.9	11.8	21.74	28.04
confident interval		—	1.9	1.4	2.9	2.8	3.3
Increase under the effect of methionine for the given time of incubation		2.66		6.92		6.30	
confident interval		2.42		2.04		1.40	

In each sample infiltrated 20 g of etiolated pea seedlings in 100 ml 0,02 M of phosphate buffer: *C* — control; *S* — sample containing 0,1 M D,L-methionine, $1 \cdot 10^{-3}$ M ATP, 0,02 M $MgCl_2$ in solution. Time of incubation respectively 2, 10, 20 hours.

The percentage content of α-tocopherol increases in all three combinations. The most marked influence of methionine on the synthesis of α-tocopherol was achieved 10 hours after the time of infiltration. Essential differences also occur after 2 and 20 hours. Although the greatest percentage increase of α-tocopherol occurred after 20 hours incubation, this experimental variant was not applied in further experiments because of the considerable decrease in the total of tocopherols.

3. Influence of the pH on the synthesis of α-tocopherol

In order to follow the synthesis of α-tocopherol in relation to the pH of the medium and to determine the optimum pH for the synthesis, phosphate buffers of pH 5,6; 6,6 and 7,8 were used for infiltration. Having regard to the significant buffering properties of plant cells, in the choice of suitable concentration of hydrogen ions, the author attempt to apply a relatively wide scale of pH. The results of this series of experiments can be seen in table 3.

The total amounts of tocopherols in infiltrated pea seedlings after 10 hours of incubation undergoes a slight decrease for all tested pH values. This applies as much to the samples infiltrated with buffer alone as to those with methionine.

The content of α-tocopherol increases after infiltration for all pH values used, both in samples without methionine and in those with methionine. There are statistically important differences in the content of α-tocopherol for pH 5,6 and 6,6, while for pH 7,8 there are no essential differences.

The increases of α-tocopherol after infiltration without methionine reach, correspondingly 4,3 μg, 6,4 μg, and 1,5 μg, with the increasing pH values. In comparison with the state before infiltration this is 8,3%, 12,4%, and 2,8%.

After infiltration with methionine these increases are markedly higher and reach 6,7 μg, 17,5 μg, 1,9 μg correspondingly, which is 13,8%, 33,8%, and 3,7%.

These figures shown that the greatest increase in α-tocopherol is achieved at pH 6,6, when the sample infiltrated by methionine gives an increase 2,7 times greater than the control (without methionine).

The percentage content of α-tocopherol in relation to the total of tocopherols is also highest at pH 6,6.

4. Effect of the methionine concentration on the synthesis of α-tocopherol

As can be seen from table 4, the content of the total of tocopherols in 1 g of dry matter in seedlings infiltrated by various concentrations of

Table 3

Effect of pH on the synthesis of α-tocopherol

	Before infiltration	After infiltration and incubation					
		pH					
		5,6		6.6		7.8	
		Concentration of methionine in M					
		0	0.1	0	0.1	0	0.1
Total of tocopherols in μg/1 g of dry matter	144.1	142.1	141.0	141.4	140.3	140.9	140.0
Increase after infiltration and incubation		—2.0	—3.0	—2.7	—3.8	—3.2	—4.1
confident interval		—	2.18	1.69	—	—	3.90
Increase under the effect of methionine at a given pH		—1.0		—1.1		—0.9	
α-tocopherol in μg/1 g of dry matter	51.8	56.1	58.5	58.2	69.3	53.3	53.7
Increase after infiltration and incubation		4.3	6.7	6.4	17.5	1.5	1.9
confident interval		2.64	4.08	4.34	4.71		
Increase under the effect of methionine at a given pH		2.4		11.1		0.4	
confident interval		—		3.00		—	
% of α-tocopherol	36.0	39.6	41.5	41.2	49.5	37.8	38.4
Increase after infiltration and incubation		3.6	5.5	5.2	13.5	1.8	2.4
confident interxal		1.7	2.7	3.1	4.0		
Increase under the effect of methionine at a given pH		1.9		8.3		0.6	
confident interval				3.00			

In each sample infiltrated 20 g etiolated pea seedlings in 100 ml 0,02 M of phosphate buffer at pH respectively 5,6, 6,6, 7,8. Samples with D, L-methionine (0,1 M) contain $1 \cdot 10^{-3}$ M ATP and 0,02 M $MgCl_2$. Time of incubation 10 hours

methionine differs slightly from the amounts of these compounds before infiltration. These differences are chance differences except for the concentration 0,02 M of methionine.

The concentration of the methionine plays a part in the synthesis of α-tocopherol and has a real effect. The biggest increase of the compound

Table 4

Effect of methionine on the synthesis of α-tocopherol

	Before infiltration	After infiltration and incubation				
		concentration of methionine in M				Effect of dosages of methionine
		0	0.02	0.10	0.25	Confident interval
Total of tocopherols in μg/1 g of dry matter	149.5	147.4	143.5	146.7	142.7	3.32
Increase after infiltration and incubation		—2.1	—6.0	—2.8	—6.8	
confident interval		—	5.63	—	—	
α-tocopherol in μg/1 g of dry matter	52.2	61.1	61.8	69.6	63.1	1.51
Increase after infiltration and incubation		8.9	9.6	17.4	10.9	
confident interval		2.47	1.77	1.70	2.88	
% of α-tocopherol	34.9	41.6	43.2	47.5	44.3	1.40
Increase after infiltration and incubation		6.7	8.3	12.6	9.4	
confident interval		2.19	2.31	2.10	2.07	

Infiltrated 20 g etiolated pea seedlings in 100 ml 0.02 M phosphate buffer containing D, L-methionine (0; 0,02 0,1; 0,25 M) + $1 \cdot 10^{-3}$ M ATP + 0,02 M $MgCl_2$. Time of incubation 10 hours.

was observed at 0,1 M of samples infiltrated without the addition of methionine increased itself nearly twice over, forming 33,3% in relation to the quantity of α-tocopherol before infiltration.

Taking the percentage content of α-tocopherol into account against the background of the total of tocopherols, it can be shown that the increase in the synthesis of α-tocopherol is related to concentrations of methionine in the order

$$0 < 0{,}02 < 0{,}25 < 0{,}1 \text{ M}.$$

5. Effect of ATP on the synthesis of α-tocopherol

Treating the synthesis of α-tocopherol as a result of the methylation of γ-tocopherol, a check had to be made to find whether ATP, which is

Table 5

Effect of ATP on the synthesis of α-tocopherol

	Before infiltration	After infiltration and incubation					
		control	0,1 M Methionine				
			concentration ATP in M				Efect of APT dosage
			0	$1 \cdot 10^{-3}$	$2 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	confident interval
Total of tocopherols in μg/1g of dry matter	145.0	144.9	142.7	141.5	139.9	139.9	—
Increase after infiltration and incubation		—0.1	—2.3	—3.5	—5.1	—5.1	
confident interval		—	—	—	—	—	
Increase under the effect of methionine with various dosages of ATP			—2.2	—3.4	—5.1	—5.0	
confident interval			—	—	3.7	3.6	
α—tocopherol in μg/1g of dry matter	53.5	61.1	64.7	72.0	68.6	65.9	1.78
Increase after infiltration and incubation		7.6	11.2	18.5	15.1	12.4	
confident interval		2.66	3.67	4.70	5.43	5.41	
Increase under the effect of methionine with various dosages of ATP			3.6	10.9	7.5	4.8	
confident interval			2.46	3.87	4.60	4.25	
% of α-tocopherol	36.9	42.2	45.4	51.0	49.1	47.2	1.90
Increase after infiltration and incubation		5.3	8.5	14.0	12.2	10.3	
confident interval		2.7	3.0	4.8	5.1	5.1	
Increase under the effect of methionine with various dosages of ATP			3.2	8.8	6.9	5.0	
confident interval			2.0	3.4	3.7	3.1	

Infiltrated 20 g of etiolated pea seedling in 100 ml 0.02 M phosphate buffer. In the case of using 0.1 M D, L-methionine ATP was used in the concentrations 0; $1 \cdot 10^{-3}$; $2 \cdot 10^{-3}$; $5 \cdot 10^{-3}$ M. Time of incubation 10 hours.

the methionine-activating agent, has an effect on this process. The results of this series of experiments are to be seen in table 5.

From them it appears that the infiltration lowers the total of tocopherols in all combinations. Comparing the addition of ATP and methionine with the control, a reduction in the total of tocopherols is observed. In two cases out of three, where the ATP concentration reaches $2 \cdot 10^{-3}$ M and $5 \cdot 10^{-3}$ M a marked decrease in tocopherols is noticed.

As regards the samples before infiltration the differences in the total of tocopherols could arise accidentally.

The content of α-tocopherol increases in all samples in comparison with the amount before infiltration, and the results are statistically significant; the differences between the α-tocopherol content in samples infiltrated with various concentrations of ATP and methionine, and the control (infiltration with buffer alone), are also borne out by statistics. The most intensive synthesis of α-tocopherol is indicated at $1 \cdot 10^{-3}$ M.

This also refers to the percentage increase in α-tocopherol content. The results mentioned indicate that ATP is an important factor in this synthesis and has essential effect.

DISCUSSION

The synthesis of tocopherols in the pea during its period of germination was examined fragmentarily (Zacharowa 1954, Green 1958). The experiments carried out by me on the quality and quantity of tocopherols in the first days of germination at daily intervals have not been described in literature.

During the peas'growth the synthesis of the tocols increases for the first three days and then goes in decreasing as far as the absolute amount of these compounds is concerned. Zacharowa, however, has reported an increase in tocopherol content in the germinating pea throughout the whole 20-days-period of her experiments. The method used by Zacharowa to estimate tocopherols did not permit the separation of non-tocopherol reducing substances, of which there are a relatively considerable number in the pea. Thus we do not know if this increase refers to tocopherols or also to these reducing substances.

Chattophadyay and Banerjee (1952) observed increase in the synthesis of tocopherols in leguminous and cereal plants during germination. The same objections as were made in Zacharowa's case may be applied to the work of these authors too, because of the method used.

The results here obtained show a greater correspondence with Green's work (1958). This author also observed a tendency to decrease

in the total of tocopherols, after the initial increase. A comparison of results leads, however to certain difficulties because of the different conditions of germination, such as temperature, humidity, and (connected with it) the different rate of growth of plants.

In Green's experiments, the increase of total tocopherol content was visible still on the fourth day of germination, while in mine a decrease began on the third day. It should be mentioned that 3-days-old seedlings in my experiments were about 3 cm long, while in Green's this phase of growth was reached on the 8th day, and in this time the decrease in tocopherol content is also marked in the experiments of that author.

My researches confirm Green's results as to the occurrence of three tocols in the pea (α, γ, δ). δ-Tocopherol was found in traces by him in the whole 8-days-period of observation. It was not possible to discover it after the second day of germination in my experiments, but it should be remembered that the analysed plant material had reached a later stage of development than Green's seedlings at his time.

During the time of the experiments synthesis of α-tocopherol and percentage increase in content were noted. After 7 days, α-tocopherol formed 80,1% of the tocols present. This value also departs from Green's, although in my experiments 7-days-old seedlings were 10—12 cm long and have had the first pair of leaves, while in Green's 8-days-old plants were scarcely 3 cm long.

In the field experiments carried out by Green such a percentage of α-tocopherol was reached after 21 days.

There is no possibility of comparing my results with Green's data on the basis of days of germination. The only course is to compare the phase of growth of the plant and correlate the results to this.

The synthesis of tocopherols, when the seedlings are germinating in darkness, diminishes. This may be connected, on one hand with the using-up of the phytol in the etiolated plant, which is vitaly necessary for the synthesis of tocopherol, and on the other with the increase in synthesis of carotenoids — the probably rivals of the tocopherols for the phytol. The second possibility seems more likely since the tocopherol total decreases systematically with the appearence of the carotenoids. Such an absolute decrease of tocopherols has not been observed in maize (Baszyński 1959).

It appears that α-tocopherol arises from γ-tocopherol. The decrease of γ-tocopherol brings an even more marked increase in α-tocopherol than in maize.

Thus etiolated pea seedlings are more suitable for research on the

biosynthesis of α-tocopherol as a process of the methylation of mono- and dimethyltocols, than maize.

Since in the first days of growth I did not observe essential differences in the total amount of tocopherols, whereas the percentage content of α-tocopherol markedly rose with the growth of the pea, the process of transmethylation most probably took place. When I began my experiments, this type of reaction based on the AMe synthesis was well known in vertebrates (Cantoni 1956), and had recently been noted in micro-organisms also (Mudd and Cantoni 1958, Schlenk, Dainko and Stanford 1959).

The optimum incubation time of infiltrated seedlings for the synthesis of α-tocopherol, as recorded by me, is 10 hours. This times agrees with the intensivity of the AMe synthesis in *Saccharomyces cerevisiae* and *Torulopsis utilis*. Schlenk and associates were able to show that the AMe synthesis reached its maximum after 10 hours, and holds this level for the 40-hour duration of the experiments. The fact that after 20 hours'incubation I noted a considerable fall in the tocopherol total is probably the results of difficulty in preserving the sterility of the seedlings and the decomposition process occurring in this connection.

The concentrations of hydrogen ions on the total tocopherol did not appear to be critical. The optimum synthesis of α-tocopherol was achieved at pH 6,6. It is very likely that this pH is the optimum, either:

(1) for the enzyme responsible for the AMe synthesis, or.

(2) for the transmethylase transferring $—CH_3$ from AMe to γ-tocopherol.

If such a hypothesis is accepted, the first case would not accord with the optimum indicated by Mudd and Cantoni for MAE from Fleischmann baker's yeast, which accepts a fairly wide range of pH values, around pH 7,6. The second alternative would be more likely, since Shapiro (1958) has shown that extracts obtained by the disintegration of cells of *Escherichia coli, Aerobacter aerogenes, Saccharomyces cerevisiae* and *Torulopsis utilis* with ultrasonic waves catalyzed the transfer of $—CH_3$ at pH 6,5—7,0.

The lack of essential changes in the α-tocopherol content at pH 7,8 is perhaps conditional on the increased activity of hydrolyzing enzymes of the pyrophosphate bounds of ATP (e.g. of nucleotide pyrophosphatase at optimum pH 6,5—8,5) occuring in plants (Lassota 1958). The deprived tissues would thus become a substrate for the AMe synthesis, and on the other hand the liberated pyrophosphate (PP) could act as inhibitor of MAE which was demonstrated in rat liver (Cantoni and Durell 1957) and later also in yeast (Mudd and Cantoni 1958).

We also known, from Mudd and Cantoni's work, that AMe is very unstable at pH around 8,0.

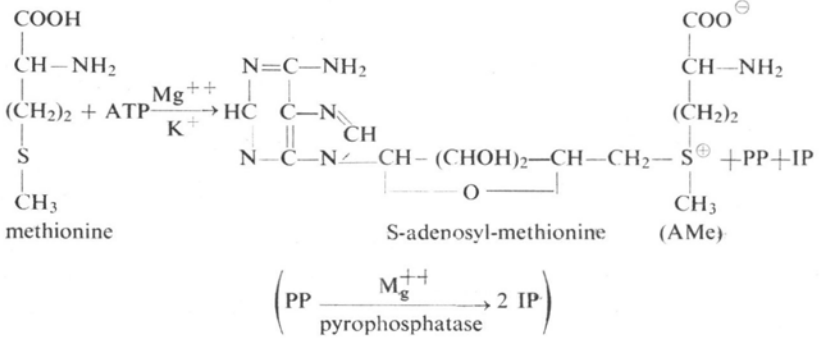
If the inhibiting action of PP also extends to MAE in plants, the somewhat adventageous effect of pH 5,6 on the synthesis of α-tocopherol could explain the often-noted decomposition of PP under the effect of plant tissues at this pH value (c. f. Lassota 1958).

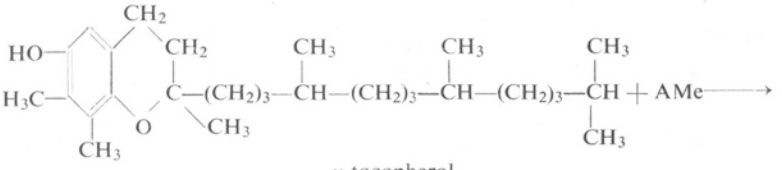
Pierpoint (1957a, 1957b) indicated such an action in extracts from pea seedlings at pH 5,0. The decomposition of PP, if it occurs in such conditions, would act against the inhibition of MAE and AMe would have better conditions for the synthesis.

The quicker synthesis of α-tocopherol in samples infiltrated with methionine and with methionine plus ATP is an argument in favour of the transmethylation hypothesis.

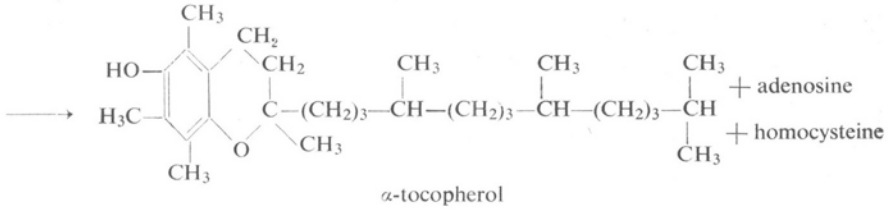
The concentrations of methionine used besides 0,02 M are relatively strong. It can be seen from my predecessors'work that only an inconsiderable part of the given methionine plays a part in the AMe synthesis. Schlenk and associates (1959), because of the ease with which *Saccharomyces* and *Torulopsis* synthesize AMe, obtained a yield of the order of 10—20%. According to these authors the presence of other amino acids has the effect of lowering this output. There is a relatively large number of free amino acids in pea seedlings and thus in this case the yield may be even more reduced (in Schlenk's case 30—60%). These authors, however did not observe the formation of S-adenosyl-methionine at any time; normally one stereoisomer formed, most frequently L. In my own experiments I used D,L-methionine and this fact was also not without effect on the yield of α-tocopherol.

The cooperation of ATP in reinforcing the synthesis of α-tocopherol is probably connected with the AMe synthesis. In the light of the results obtained, the following scheme of the methylation of γ-tocopherol might be accepted:





γ-tocopherol



α-tocopherol

This scheme also includes the action of AMe, the presence of which in the pea was not observed until now.

However, M u d d (1960a, 1960b) has recently shown that extracts from barley and millet seedlings may contribute to the methylation of alkaloids; what is more, that in higher plants AMe is synthesized and fills the role of donor of the methyl groups. These two recent papers supply the basis for accepting the pathway indicated for the synthesis of α-tocopherol in the germinating pea. The results obtained are in agreement with the supposition, the final answer to how far my conclusions are correct can only be given by carrying out experiments using the labelled methionine-methyl C^{14}.

CONCLUSIONS

1. The occurrence of α, γ, and δ-tocopherols in the first days of germination of the pea (*Pisum sativum* L.) has been confirmed. δ-Tocopherol disappears when the seedlings has reached a length of about 3 cm.

2. An increase in the total of tocopherol in the first days of germination, and a decrease from the third day to the end of the 7-days experiments, were observed. This is conditional on the increase of intensivity in the synthesis of carotenoids.

3. An increase in α-tocopherol content with the growth of the plant and occurring with a simultaneous decrease in γ-tocopherol, was shown to exist.

4. Methionine and ATP reinforce the synthesis of α-tocopherol. The optimal concentration of methionine for this synthesis is 0,1 M and ATP is $1 \cdot 10^{-3}$ M.

5. It was determined that the optimum time of incubation for pea-seedlings infiltrated with methionine and ATP, in the synthesis of α-tocopherol, is 10 hours.

6. The most intensive synthesis of α-tocopherol under the influence of methionine was observed at pH 6,6.

7. A scheme of the synthesis of α-tocopherol in vivo in young pea-seedlings was formulated, leading to the methylation of γ-tocopherol. While not embarking on the problem of the synthesis of tocopherols in the whole life-cycle of plants, the author thinks that α-tocopherol arises principally in the transmethylation of non-α-tocopherols in the germination period of the pea.

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STRESZCZENIE

Green w badaniach nad składem i lokalizacją tokoferoli w okresie wegetacji roślin sugerował możliwość demetylacji α-tokoferolu jako etapu w syntezie pozostałych tokoli, a także brał pod uwagę proces odwrotny (1958).

Niezależnie od Greena w swojej poprzedniej pracy autor wysunął przypuszczenie, że α-tokoferol może powstawać z jedno- i dwumetylotokoli na drodze ich metylacji (1959).

W niniejszej pracy postanowiono przebadać możliwość metylacji γ-tokoferolu do α-tokoferolu z wykorzystaniem metioniny jako donatora grup metylowych.

Badania nad biosyntezą α-tokoferolu przeprowadzono na 2-dniowych odciętych kiełkach grochu odmiana Victoria in vivo metodą infiltracji. Biosyntezę α-tokoferolu mierzono przyrostem tego związku na jednostkę suchej masy w zależności od czasu inkubacji, pH środowiska, stężenia infiltrowanej metioniny i ATP.

Przed przystąpieniem do badań nad mechanizmem syntezy α-tokoferolu ustalono, jakim fluktuacjom ulegają tokole w procesie kiłkowania grochu i jaki jest ich stosunek do syntezy karotenoidów.

Tokoferole oznaczano według metody Greena zaakceptowanej przez Angielski Komitet Metod Analitycznych (1959).

Karotenoidy oznaczano metodą Workera (1957).

WNIOSKI

1. Potwierdzono występowanie w pierwszych dniach kiełkowania α, γ, δ-tokoferoli. δ-Tokoferol zanika po osiągnięciu przez kiełki około 3 cm długości.

2. Stwierdzono wzrost sumy tokoferoli przez pierwsze dni kiełkowania, a od 3 dnia jej spadek przez cały okres 7-dniowych doświadczeń. Uwarunkowany jest on prawdopodobnie wzrostem intensywności syntezy karotenoidów.

3. Wykazano wzrost zawartości α-tokoferolu z wiekiem rośliny zachodzący z równoczesnym spadkiem γ-tokoferolu.

4. Metionina i ATP wzmagają syntezę α-tokoferolu. Optymalne ze stosowanych stężeń metioniny dla tej syntezy wynosi 0,1 M, a ATP $1 \cdot 10^{-3}$ M.

5. Ustalono, że optimum czasu inkubacji infiltrowanych metioniną i ATP kiełków grochu przy syntezie α-tokoferolu wynosi 10 godzin.

6. Najintensywniejszą syntezę α-tokoferolu pod wpływem metioniny stwierdzono przy pH 6,6.

7. Zaproponowano schemat syntezy α-tokoferolu in vivo w młodych kiełkach grochu, sprowadzający się do metylacji γ-tokoferolu. Nie wchodząc bliżej w zagadnienie syntezy α-tokoferolu w pełnym cyklu życiowym roślin, wydaje się, że w okresie kiełkowania grochu α-tokoferol powstaje głównie na tej drodze.

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