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Zużytkowanie racemicznego kwasu jabłkowego przez niektóre gatunki Aspergillus i Penicillium. — The utilization of racemic malic acid by Aspergillus and Penicillium species.

Mémoire

de M. F. GÓRSKI

présenté le 7 Février 1949, par M. W. Szafer m. t.

I

Our preceding paper (1947a) dealt with the utilization of optical isomers of dl-tartaric acid by 21 species of moulds. The present paper summarizes the results of our researches on the utilization of racemic (dl) malic acid by the same species. The references in literature on this subject are very scanty. According to MacKenzie and Harden (1903) a nutrient solution containing dl-malic acid becomes laevorotatory when *Asp. griseus* is cultivated on it. In one of our papers (1937b) we pointed out that *Asp. fumigatus* is able to absorb both the optical forms of malic acid, but since the (—)-acid is more rapidly used than the (+)-acid, a transitory dextrorotation appears in the nutrient solution.

There are however two difficulties which hinder the study of the metabolism of dl-malic acid, namely the very feeble rotatory power of this acid ($[\alpha]_D$ = about $\pm$ 2·5) and the lack of reliable chemical methods for its estimation.

1. The first difficulty is easily overcome by the method of polarimetric titrations described in detail in two of our preceding papers (1937a, 1947a). In this method 1 ml of a concentrated (15%) solution of ammonium molybdate is first added to a sample (10 ml) containing a mixture of the l-and d-form, with for instance an excess of the dextrorotatory isomer. The complex compound

thus formed displays a high rotatory power ($[\alpha]_D = \pm 850$, Patterson and Buchanan, 1932). Then a standard solution (m/10) of laevorotatory malic acid or of a l-malate is run in from a burette in an amount just sufficient to reduce to zero the optical activity of the sample. The exaltation of the rotatory power due to the presence of the molybdate is especially high in acid solutions, and for this reason the sample, if alkaline, must be acidified with acetic acid. The polarimetric readings are made in a tube fitted at both ends with lateral tubes permitting rapid filling and emptying. (Pellets tube). By this method the disturbing influences of several secondary factors (acidity, concentrations of the acid and of the molybdate, presence of alien ions etc) on the magnitude of the rotation are eliminated. When using a tube 50 cm in length it was possible to detect the presence of 0·2 mg of active malic acid in 15 ml.

2. The chemical methods used for the estimation of malic acid are either complicated and liable to cause losses or more simple but inaccurate. Thus, for instance, it is impossible to extract the acid with ether because its solubility in water is much greater than in ether. It is also impossible to precipitate the acid completely as a calcium salt. In our researches in which the concentration of the acid did not exceed 50 mg per 10 ml a formation of calcium malate could not be induced even after 20 or 30 ml of alcohol were added.

As all attempts to isolate the malic acid from the nutrient solution were unsuccessful. it was necessary to try an estimation of this acid by direct titration of the nutrient solution with sodium hydroxide (m/10). However, the nutrient solution contains two interfering substances, namely carbon dioxyde present chiefly as a carbonate and ammonia. By adding 1—5 ml of n/10 sulphuric acid to the solution, the carbon dioxyde is expelled from it. The amount which is added must be sufficient to make the sample acid, and therefere 2 or 3 drops of a convenient indicator (phenolphtalein or bromcresolblue) are added with the acid. The sample is gently boiled for a few minutes and then cooled. The expelled carbon dioxyde is replaced in the carbonates by sulphuric acid. The ammonia is eliminated by adding to the sample 0·5 ml of a neutral concentrated (40%) formaldehyde solution. The ammonia is converted into hexamethylenetetramine and the

equivalent quantity of malic acid is liberated (Ronchèse 1907). The sample is now ready for the titration with n/10 NaOH and phenolphtalein. Owing to the presence of formaldehyde and of phosphates in the solution the color change of the indicator is not very sharp. The quantity of malic acid left in the nutrient solution by the mould is calculated from the following data: a) from the amount of sodium contained in the nutrient solution, b) from the amount of n/10 H_2SO_4 added, c) from the amount of n/10 NaOH used for the neutralization. When this quantity and the excess of optically active acid are known, the rates of utilization of the laevo-(%l), of the dextro-acid (%d) and of the sum of both isomeric forms, %s = (%l + %d)/2 are readily calculated.

The above method is obviously based on two assumptions: (1) that no organic acids are produced at the expense of malic acid, (2) that no optically active substances (other than malic acid) are formed during the growth of the mycelium. Though a direct proof of these assumptions is very difficult, yet the remarks below show that with a high degree of probability they may be considered as true. First it can be noticed that the amount of malic acid available from one culture is small, namely 268 mg (2 milliequivalents). As no other carbon source is available for the growth of the mycelium and for respiration, it is obvious that the amount of secondary acids formed at the expense of malic acid (if formed at all) must be very small too.

It is also probable, that owing to the simple structure of malic acid, the products of its desintegration cannot have a complicated structure, but are presumably simple compounds e. g. tartaric, malonic, oxalic, fumaric etc. acids. But as the addition of a calcium salt formed no precipitate, it was concluded that these acids are not formed in appreciable amounts during the growth of the fungus. Two exceptions however must be mentioned: a white precipitate of calcium oxalate was observed in the nutrient solutions on which *Asp. niger* and *Asp. Schiemanni* grew.

In our researches on the utilization of dl-tartaric acid, we were able to show that, with but two exceptions, no organic acids were formed at the expense of the racemic acid. For the estimation of this acid we could use two methods: first the method of Ronchèse mentioned above, and second the method

based on the precipitation of tartaric acid as acid potassium salt. The second method has the great advantage of permitting the extraction of only the tartaric acids from the nutrient solution. A comparison of the results obtained with both methods has shown that the observed differences are due to the presence of carbon dioxyde released during the process of respiration and converted with ammonia to carbonates. A great diminution of these differences was also observed if the sample destined for titration with NaOH and formaldehyde was previously heated, shaken or kept in vacuum. The differences disappeared almost completely if the carbon dioxyde was expelled by boiling with sulphuric acid.

In the course of the same researches we could show that no optically active substances are produced by the growing aspergilli. Owing to the possibility of isolating the tartaric acids from the nutrient solution it was also possible to compare the values of the rotatory power of the solution with the rotatory power of the pure acid isolated from it. In each case, within the limits of experimental errors, the results obtained were the same, and thus it can be concluded that no optically active compounds are secreted into the nutrient solution by the fungus.

From these observations and from the similarity of structure of malic and tartaric acid we have drawn the conclusion that also in the case of malic acid neither organic acids nor optically active compounds are produced by the growing mould. Thus the use of the above-described chemical and optical methods for the estimation of malic acid is justified and both may be expected to give reliable results.

The experimental conditions of the present researches were the same as in our investigation on the utilization of dl-tartaric acid. The culture flasks (50 ml) were kept in darkness at the temperature of $+30^{\circ}$ C. They were sterilized during two hours and inoculated the following day with a small quantity of spores. At given moments the flasks were taken for analysis. The nutrient solution separated from the mycelium on a Buchner filter was run directly into gauge flasks of 30 ml. The mycelium was washed with a few ml of water and the gauge flasks were filled up to the mark. Two samples, 10 ml each, were taken, the first for the chemical estimation, the second for the polarimetric rea-

dings. The composition of the nutrient solution is given in Table I. For reasons explained below the acid was neutralized in 55 or 45 percent by sodium hydroxide. Thus the nutrient solution contained approximatively acid dl-sodium malate. Also for other reasons the use of an acid salt instead of the free acid secures some advantages: (1) the growth of many species of *Aspergillus* is inhibited by the high acidity of solutions containing the free acid (pH = 2·7), (2) by the use of an acid salt data comparable with the results obtained in our investigations on the dl-tartaric acid are provided.

Preliminary observations have shown that in all cases both the optical forms are utilized by the cultivated species, but with different rapidities. At the beginning of the growth the rotatory power of the solution is nil, then it increases, attains its maximum, begins to decrease and finally disappears completely if the fungus is allowed to grow long enough. This transitory appearance of the rotatory power proves that the given species displays a certain preference for one of the two optical isomers. This preference or selective power, as it is better to call it, is more or

TABLE I

The composition of the nutrient solution, pH = 3·3.

dl-malic acid	268 mg	$KH_2PO_4 + K_2HPO_4$	5 mg
sodium	40 or 51 „	$MgSO_4 7{\cdot}H_2O$	2·5 „
ammonia	10 „	water	25 ml

traces of Cd, Hg, Zn, Mn, Fe, Ba, Cu, Li, Bo, Mo. [1])

less pronounced according to the species under examination and the moment of analysis. Comparable results are obtained only with cultures which have reached the same stage in development i. e. which have used the same amount of the nutrient substratum. The experiments were therefore conducted in such a way that it was possible to obtain the rates of utilization of the laevo ($^0/_0$l) and of the dextro ($^0/_0$d) isomer corresponding to the moment when half of the substratum ($^0/_0$s = 50) was used. These rates calculated from the experimental data by means of interpolation are named the reduced rates of utilization. From the definition of $^0/_0 s = (^0/_0 l + {}^0/_0 d)/2$ results the following relation for the reduced

[1]) After Nielsen and Harteliu's, 1935.

rates: $^0/_0 l_{red} + {}^0/_0 d_{red} = 100$. The difference $^0/_0 l_r - {}^0/_0 d_r$ or the ratio $^0/_0 l_r : {}^0/_0 d_r$ forms a very convenient measure of the selective power.

The choice of 50 for the standard value of $^0/_0 s$ is only partly arbitrary. It was said above that the rotatory power of the nutrient solution (or the equivalent difference $^0/_0 l - {}^0/_0 d$) is nil at the beginning ($^0/_0 s = o$) and at the end ($^0/_0 s = 100$) of the growth. It is therefore probable that the difference $^0/_0 l - {}^0/_0 d$ will attain its maximum value for $^0/_0 s$ equal or close to 50. (Górski 1947b). But as there are some difficulties in stopping the growth of a culture just at the moment when the $^0/_0 s$ is 50, the following device was used to overcome them. The malic acid is neutralized to 55% with NaOH and an indicator (bromcresol-blue) the color change of which takes place at pH = 7 is added to the nutrient solution. During growth the mycelium, gradually uses the acid radical, while the base remains in the solution and neutralizes the remaining acid salt. When 45% of the acid has disappeared, the remaining acid salt is converted into a neutral salt, which is shown by the colour change of the indicator. At this moment the flask is taken for analysis and it is found that the value of $^0/_0 s$ approximates 50 by loss. If the analysis is made one or two days later, or if the nutrient solution is neutralized to 45% the corresponding value of $^0/_0 s$ exceeds 50%. From the data obtained of $^0/_0 s$, $^0/_0 l$ and $^0/_0 d$ the reduced rates of utilization are calculated by means of known methods of interpolation (for an example see Górski, 1947b).

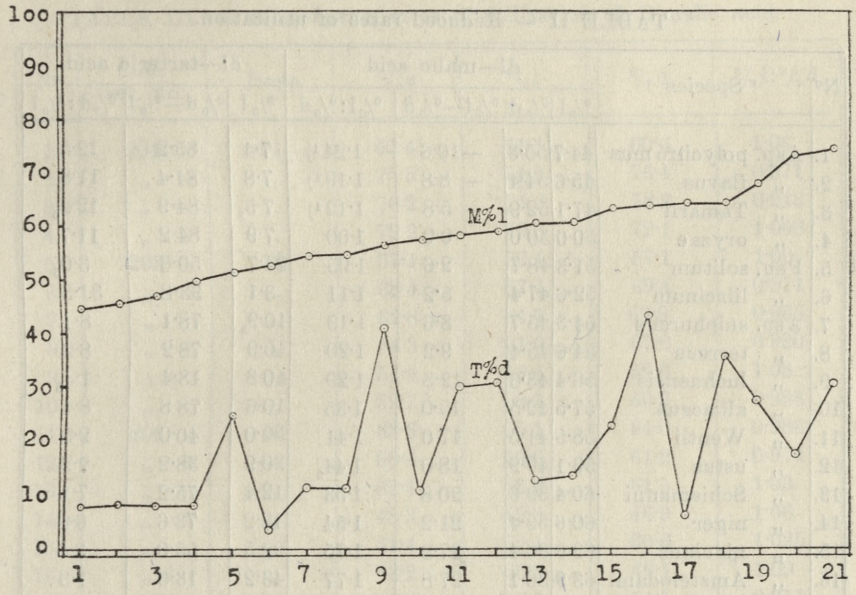
II

The results of our investigations are summarized in Table II. Columns 3 and 4 refer to the reduced rates of utilization; columns 5 and 6 indicate the differences $^0/_0 l_r - {}^0/_0 d_r$ and the ratios $^0/_0 l_r : {}^0/_0 d_r$. The species are arranged according to the increasing values of $^0/_0 l$. As we intend to compare the results obtained with the results recorded previously with dl-tartaric acid (Górski 1947a) we have added in columns 7, 8 and 9 the reduced rates, the differences $^0/_0 d - {}^0/_0 l$ and the ratios $^0/_0 d_r : {}^0/_0 l_r$ referring to the dl-tartrate. The data of columns 3 and 7 are graphically presented in graph 1. Owing to the relation $^0/_0 l_r + {}^0/_0 d_r = 100$, the line

referring to $^0/_0 l_r$ represents also $^0/_0 d_r$, provided that the scale of ordinates is inverted.

The data of the table may be interpreted as follows:

1. In all cases both the optical isomers are readily attacked by the species used in our researches, which means that their selective power towards malic acid is slight. For 18 out of 21



Graph 1. — Reducuced rates of utilization (ordinates) of l-malic acid (M%) and d-tartaric acid (T%d) for different species of moulds. The differences 100 — %l and 100-%d of the ordinates represent the reduced rates of utilization of d-malic acid and l-tartaric acid.

1 — Asp. polychr.	8 — Asp. terreus	15 — Asp. nidulans
2 — „ flavus	9 — „ luchuens.	16 — „ Amstelod.
3 — „ Tamarii	10 — „ alliac.	17 — „ fumigat.
4 — „ oryzae	11 — „ Wentii	18 — „ gracilis
5 — Pen. solitum	12 — „ ustus	19 — „ versic.
6 — „ lilacin.	13 — „ Schieman.	20 — „ Sydowi
7 — Asp. sulphur.	14 — „ niger	21 — Pen. meleagr.

species the ratio $^0/_0 l : ^0/_0 d$ lies between 1 and 2 and does not exceed 3 for the remaining three aspergilli. In this respect the above results differ notably from the results recorded with the dl-tartaric acid. In the case of this acid some aspergilli as for

inst. *Asp. luchuensis* or *Amstelodami* are able to attack both the isomeric forms ($^0/_0$l = 40, $^0/_0$d = 60), while others, for inst. *Asp. fumigatus* or *terreus* are almost unable to use the l-acid ($^0/_0$l < 10). The ratio $^0/_0$l:$^0/_0$d for the majority of the cultivated species is 3—10. The mean ratio is 6·17, which if compared with the mean ratio (1·74) for the malate, corresponds to a fairly high value.

TABLE II — Reduced rates of utilization.

N°	Species	dl—malic acid				dl—tartaric acid		
		$^0/_0$l	$^0/_0$d	$^0/_0$l—$^0/_0$d	$^0/_0$l:$^0/_0$d	$^0/_0$l	$^0/_0$d—$^0/_0$l	$^0/_0$d:$^0/_0$l
1.	Asp. polychromus	44·7	55·3	—10·6	1·24[1])	7·4	85·2	12·5
2.	„ flavus	45·6	54·4	— 8·8	1·19[1])	7·8	84·4	11·8
3.	„ Tamarii	47·1	52·9	— 5·8	1·12[1])	7·5$_5$	84·9	12·2$_5$
4.	„ oryzae	50·0	50·0	0·0	1·00	7·9	84·2	11·7
5.	Pen. solitum	51·3	48·7	2·6	1·05	24·7	50·6	3·0$_5$
6.	„ lilacinum	52·6	47·4	5·2	1·11	3·1	93·8	31·3
7.	Asp. sulphureus	54·3	45·7	8·6	1·19	10·9$_5$	78·1	8·1
8.	„ terreus	54·6	45·4	9·2	1·20	10·9	78·2	8·2
9.	„ luchuensis	56·4	43·6	12·8	1·29	40·8	18·4	1·4$_5$
10.	„ alliaceus	57·5	42·5	15·0	1·35	10·6	78·8	8·4
11.	„ Wentii	58·5	41·5	17·0	1·41	30·0	40·0	2·3
12.	„ ustus	59·1	40·9	18·0	1·44$_5$	30·9	38·2	2·2
13.	„ Schiemanni	60·4	39·6	20·8	1·53	12·4	75·2	7·1
14.	„ niger	60·6	39·4	21·2	1·54	13·2	73·6	6·6
15.	„ nidulans	63·6	36·4	27·2	1·75	22·5	55·0	3·4
16.	„ Amstelodami	63·9	36·1	27·8	1·77	43·2	13·6	1·3
17.	„ fumigatus	64·3	35·7	28·6	1·80	5·9	88·2	16·0
18.	„ gracilis	64·5	35·5	29·0	1·82	35·8	28·4	1·8
19.	„ versicolor	68·1	31·9	36·2	2·13	27·5	45·0	2·6
20.	„ Sydovi	73·3	26·7	46·6	2·75	17·2	65·6	4·8
21.	Pen. meleagrinum	73·7	26·3	47·4	2·80	30·5	39 0	2·3

[1]) Ratio $^0/_0$d:$^0/_0$l. The corresponding values of the ratio $^0/_0$l:$^0/_0$d are 0·808, 0·838, 0·890.

Particularly from columns 5 it is evident that none of the species used is suitable for the isolation of pure dextrorotatory malic acid, while laevorotatory tartaric acid is easily prepared by cultivating *Asp. fumigatus* or *Pen. lilacinum* on the racemic compound.

2. Another difference between the metabolism of the two acids is demonstrated by the fact that with the dl-tartrate the $^0/_0$d was always higher than the $^0/_0$l, all the species showing a more or less pronounced preference for the d-acid. In the case

of malic acid however 17 species display a preference for the laevo isomer, 3 for the dextro one and one species absorbs both the optical forms at the same rate. Yet it is interesting to notice that even in the last case each particular culture shows a very slight preference, either for the left or for the right isomer (table III). The reason of this is as yet unknown. However it

TABLE III — *Asp. oryzae*, rates of utilization of dl-malic acid.

N°	Cult. ser. no	Days	%s	%l	%d	%l:%d
1.	204	21	62·4	64·3	60·6	1·06
2.	„	„	75·3	74·2	76·4	0·971
3.	„	„	76·2	74·2	78·2	0·948
4.	„	„	72 2	72·3	72·1	1·003
5.	205	12	57·1	57·1	57·1	1·00
6.	„	„	68·4	67·4	69·4	0·971
7.	„	„	62·8	61·7	63·9	0·966
8.	„	„	64·3	61·6	67·0	0·920
9.	„	„	54·6	56·5	52·6	1·08
10.	„	„	59·7	58·7	60·6	0·968
11.	206	10	63·6	65·1	64·0	0·986
12.	„	„	60·4	59·6	61·2	0·974
13.	„	„	62·4	63·2	61·5	1·03
14.	„	„	48·3	49·8	46·9	1·06
15.	„	„	61·4	62·1	60·6	1·025
16.	„	„	48·2	50·3	46·1	1·09
17.	207	11	49·8	48·5	51·1	0·951
18.	„	„	41·4	40·6	42·2	0·964
19.	„	„	42·5	41·8	43·1	0·972
20.	„	„	38·6	39·7	37·6	1·055
21.	„	„	40·8	40·8	40·8	1·00

seems that neither the age of a culture nor the corresponding set have an influence on the value of the ratio %l:%d. The data contained in the table seem to indicate that the appearing of positive and negative preferences for the laevo isomer is a matter of pure chance. Indeed in 11 cases the ratio %l:%d is greater than unity, in 8 cases less than unity and in 2 cases %l = d%. The mean of the ratios is 0·9997 or unity. A distribution made at random would not be different.

3. The most striking difference recorded between the results obtained with dl-malic acid and dl-tartaric acid is of a stereo-

chemical nature. The (+)-malic acid and the (+)-tartaric acid appear to have the same spatial configuration (Freudenberg-Brauns & Siegel 1922, Kortüm 1932, Lowry 1935). If in the (+)-tartaric acid (see p. 12) a hydroxyl group is replaced by an atom of hydrogen (+)-malic acid is obtained. Basing the reasoning on the above and on the facts recorded for the dl-tartrate it could be expected that the utilization of the d-malic acid should be higher than that of the laevo isomer.

4. When comparing the rates of utilization of malic and tartaric acids there arises the question concerning the correlation between the corresponding selective powers shown by the different species. All the correlation data are recorded in the table IV.

TABLE IV

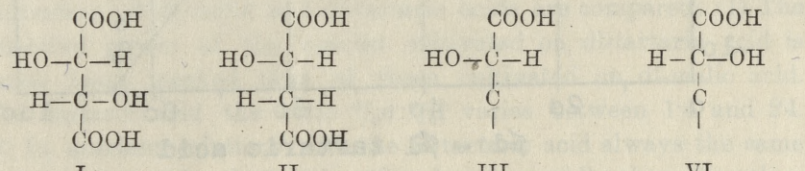
Data for the correlation between the selective powers referring to the dl-malic and dl-tartaric acids.

acid	mean diff. %l—%d or %d—%l	σ	equations of regression
dl-malic	16·8	± 16·0	$y = -0{\cdot}303x + 35{\cdot}35$
dl-tartaric	61·4	± 20·5	$x = -0{\cdot}497y + 69{\cdot}68$
	correlation coefficient	$r = -0{\cdot}388 \pm 0{\cdot}186$	

In the corresponding graph 2 are plotted %d—%l for the dl-tartrate against %l—%d for the dl-malate for each species. The absolute value of the correlation coefficient is small (0·388) but perhaps greater than could be expected at a first glance at the graph 2. A still more interesting feature is its negative sign: this means that in most cases the species which show a marked selective power when cultivated on dl-tartaric acid lose this property when grown on dl-malic acid. In the graph the highest values of %l—%d for the malate correspond to the middle values of %d—%l for the dl-tartrate.

5. Thus we see that when estimating the utilization of the two acids by the aspergilli, in spite of the similarity of structure, only disparities are recorded, We must therefore look for the reasons of these disparities in the slight structural differences of the two acids. The molecular structure of malic acid differs from that of tartaric acid in three respects: (1) In malic acid a hydroxyl group is replaced by an atom of hydrogen (see formulas I and II). (2) An asymmetric carbon atom is lost by this substi-

tution. (3) The degree of symmetry of malic acid is less than that of tartaric acid. Obviously the greatest importance must be attributed to the second point, for it provides some explanation for the disparities in question. We must however make some supplementary assumptions: (1) the enzymes attack the most oxidized group of the molecule i. e. the carboxylic group COOH, (2) the probability of collision with the enzymic system is the same for both the COOH groups, and (3) the configuration corresponding to formula III is readily assimilated by the given species of mould, while the configuration represented by formula IV is completely unattackable. Now considering a certain number of racemic malic acid molecules it is obvious that the l-molecules (configuration III) are rapidly utilized by the mould. The d-molecules disappear also but more slowly, from the nutrient solution. Indeed, if the COOH group linked with

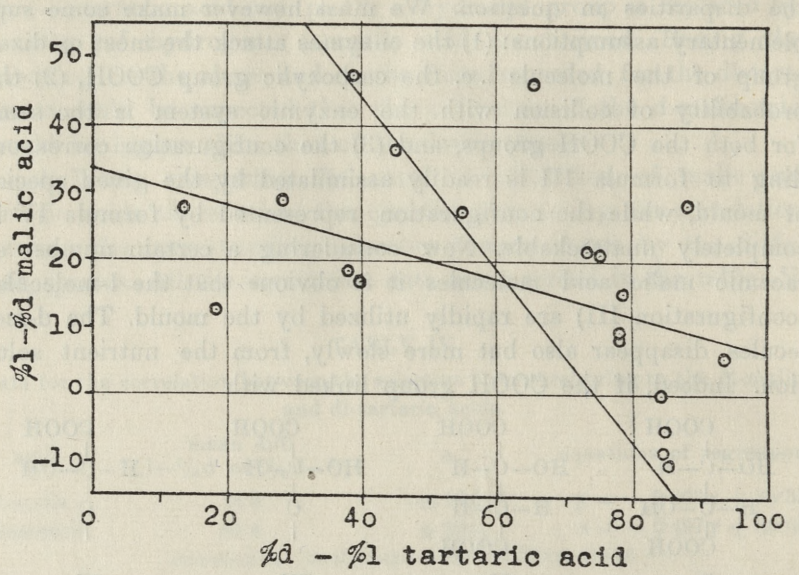


I l-tartaric acid — II l-malic acid — III laevo- — VI dextro-

the asymmetric carbon atom is involved in a collision with the enzyme the molecule will remain unbroken. On the contrary the molecule will desintegrate if the COOH group removed from the asymmetric carbon atom meets the enzyme. In this case no spatial configuration, due to the presence of an asymmetric centre, can protect this part of the molecule from decomposition. Yet sooner or later every d-molecule must collide in this way and in consequence the dextro acid will disappear slowly but completely. But before this happens the solution will show temporarily a dextro-rotation.

The above explanation cannot be applied to dl-tartaric in which both the carboxylic groups are linked to asymmetric atoms. We may therefore suppose that this acid is than assimilated in a different way by the moulds from the malic acid. This supposition is corroborated by the facts stated above in sections 3 and 4. The view that dl-malic and dl-tartaric acids are attacked in different ways is strongly supported by the lack of close and posi-

tive correlation between their selective powers. Still, more convincing perhaps is the preference which the majority of the species displays for the dextro tartrate and its stereochemical



Graph 2. — Correlation between the selective powers referring to malic and tartaric acids. The selective powers are measured by the differences $\%l - \%d$ (malic acid, ordinates) and $\%d - \%l$ (tartaric acid, abscissae). $r = -0{\cdot}388$.

opposite the laevo malate. But it is better to postpone a more detailed discussion of these remarkable facts until new experimental evidence is at our disposal.

Summary

1. 18 *Aspergillus* and 3 *Penicillium* species were cultivated on racemic malic acid (1% solution) in order to investigate their ability to utilize the laevo and dextro optical isomers.

2. The culture flasks were kept in darkness at the temperature of $+30^{\circ}$ C. The non-utilized acid was estimated by titrating the nutrient solution with natrium hydroxide in the presence of formaldehyde. The polarimetric estimations were made by the method of polarimetric titrations worked out by the author.

3. The results are expressed in reduced rates of utilization,

which means that the rates of utilization of the left ($^0/_0$l) and and right ($^0/_0$d) acids are estimated at the moment when half of the sum of the two optical isomers has been utilized by the moulds. The reduced rates of utilization satisfy the equation $^0/_0 l_{red} + {}^0/_0 d_{red} = 100$. The ability shown by a species to utilize one of the two optical isomers to a greater extent than the other is called the selective power. The difference $^0/_0$l — $^0/_0$d or the ratio $^0/_0$l:$^0/_0$d are measures of the selective power.

4. The selective power of the cultivated species for the isomers of dl-malic acid is slight, both the isomeric forms being easily attacked. The values of the ratio $^0/_0$l:$^0/_0$d varies from 0·86 to 3·07; 17 species used the laevo acid more rapidly than the dextro, 3 species the dextro more easily than the laevo acid and one assimilated both the optical isomers at the same rate.

5. Very striking differences are noticeable if the rates of utilization of dl-malic and dl-tartaric acids are compared: (1) The selective power of the species cultivated on dl-tartaric acid is much more marked than of those cultivated on dl-malic acid. For tartaric acid the ratio $^0/_0$d:$^0/_0$l varies between 1·4 and 24. (2) In nutrient solutions with the dl-tartaric acid always the same isomeric form (the dextro) is utilized more rapidly than the other. (3) Although the laevo malic acid and the laevo tartaric acid have the same spatial configuration the optical isomers more readily used by the majority of the cultivated species are the laevo malic acid and the dextro tartaric acid. (4) The correlation between the selective powers for the malic and tartaric acids is slight and negative, $r = -0{\cdot}388$. (5) The probable conclusion is that the malic and tartaric acids are attacked by the aspergilli in two different ways.

6. None of the species used in the researches is convenient for the biological preparation of pure dextrorotatory or pure laevorotatory malic acid.

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Naegeliella flagellifera Correns w Polsce. — Naegeliella flagellifera Correns in Poland.

Mémoire

de M^lle^ *J. SIEMIŃSKA*

présenté le 7 Mars 1919 par M^lle^ J. Wołoszyńska m. c.

In May 1943 I found some colonies of alga, brown in colour, in the ponds of the Fishery Experimental Station of the Jagiellonian University in Mydlniki near Kraków. The colonies were growing on slides placed in the ponds for the purpose of investigating epiphytic algae. The alga mentioned was found to belong to the genus *Naegeliella*. The first description of this genus was given by Correns (1892) who found it in Tübingen in Würtenberg. It was then found by Scherffel (1927) near Iglós (Hungary) and by Godward (1933) in Loughton (England). *Naegeliella* belongs to *Chrysophyceae*; Pascher (1925) placed it among *Chrysocapsales*, in the family *Naegeliellaceae*.

Naegeliella flagellifera was discovered by Correns epiphytic on the threads of *Cladophora* and it was further observed by Scherffel on *Vaucheria*; *Naegeliella britannica*, found by Godward, was growing in the same way on *Nitella*, *Oenanthe fluviatilis* and on immersed glass slides. The last species differs from the former by the presence of protoplasmic threads, enclosed in mucilage hairs. *Naegeliella natans* described by Scherffel is a planctonic form and differs in many respects from both the species mentioned. It is highly probable that this species belongs to the genus *Naegeliella*.

Naegeliella is a very rare alga; I was unable to find any further references in the literature respecting its occurrence. It has never been recorded in Poland.

The alga is found every year in the ponds of the Station, but in varying quantities. The largest quantities of this alga were found in the pond »Stare Koryto« in June 1943 and July 1947; the colonies covered over 40% of the surface of the immersed slides. Together with *Naegeliella* were found the following epiphytic algae: *Achnanthes minutissima*, *Coleochaete orbicularis*, *Stigeoclonium tenue*, *Chaetopeltis orbicularis*, *Oedogonium* sp. and others. In 1944 the first colonies of *Naegeliella* were found at the end of April and the last ones were found in small numbers even at the beginning of November. During the winter months not one colony was found. In the next year (1945) the alga was found both in ponds emptied for the winter and in those which were filled with water during the whole winter. Each of the ponds has its separate inflow of water from the river. This is why we may suppose that the alga is carried into the ponds with the water from the river, or that it develops from spores resistent to lack of water in the winter. No colonies of *Naegeliella* were found on the slides immersed in the river. It must be added that the current of the river is relatively quick. The alga was also found on the leaves of *Potamogeton* sp., which grew in the ponds, on *Chara foetida*, and on the surface of piles and all sorts of timber immersed in the pond water.

The colonies of *Naegeliella* develop from a single cell. The subsequent divisions of this cell give rise to a colony which is one, two and sometimes even three layers of cells thick. The base of the colony is rounded in shape, or irregular, if the alga is growing among other epiphytic organisms or crystals of calcium carbonate. The base of the colony reaches 100 μ in diameter.

The cells, when viewed from above, are elongated and ellipsoid shape; they are about 5—15 μ in length, 4—8 μ in width (at the time of cell-division even 14 μ in width), and 6—9 μ in height, on the average. Each cell has one V-shaped chromatophore, which is golden-brown in colour. The margin of the chromatophore is for the most part not quite smooth, but shows sometimes deep incisions. In each cell there are two contractile vaçuoles and some small, unstained granules. There is no eye-spot.

The whole colony is enveloped in a transparent, unstained mucilage, which is invisible without previous staining. This is why the mucilage can easily be overlooked. Scherffel is of

the opinion that Klebahn found *Naegeliella*, only he did not notice the mucilaginous envelope. In my work gentian violet, methyl blue and iodin green were used for the staining of the mucilage. Every single cell is covered by a thin layer of

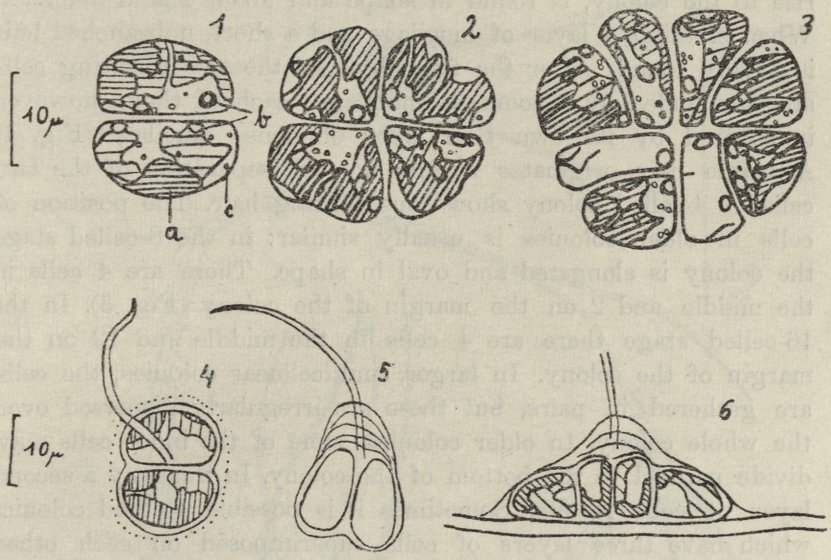

Fig. 1), 2), 3), 2- 4- and 8-celled colonies, unstained. a) chromatophore, b) contractile vacuole, c) small unstained granules.
Fig. 4) 2-celled colony; the mucilage is stained.
Fig. 5), 6) 2- and 8-celled colonies, after staining of the mucilage, side view: the stratification of the mucilage is distingly visible.

mucilage, which is particularly dense. This mucilage preserves the shape of the cell, even after this has decayed. The dense mucilaginous layer is stained very intensively by the dyes mentioned. All the cells in the colonies are surrounded by a common layer of mucilage, which is not so dense as the layer round each single cell. This common mucilage is thin on the bottom and on the sides of the colony, but is much thicker on the top of the colony, particularly in the young ones. I was successful in observing the structure of the mucilage in only a few cases: it shows a delicate stratification (Fig. 5, 6). Staining of the mucilage makes visible mucilaginous, usually branched hairs, which originate in the colony. In tap water and without staining is almost impossible to notice the hairs; they appear a light grey colour when

the colony is placed in distilled water. It is possible, that the hydrogen-ion concentration (pH) of the medium is here of importance.

The growth of the colony is on the whole quite similar to that described by Correns and Godward. The cell, which gives rise to the colony, is round in shape and about 9 μ in diameter. When staining, a layer of mucilage and a short, unbranched hair is usually found. After the first division the two resulting cells are surrounded by a common mucilage; each of them, however, is covered by its own thin layer of dense mucilage (Fig. 4). A single hair originates at the line of separation of the two cells. A 4-celled colony shows one forking hair. The position of cells in older colonies is usually similar: in the 8-celled stage the colony is elongated and oval in shape. There are 4 cells in the middle and 2 on the margin of the colony (Fig. 3). In the 16-celled stage there are 4 cells in the middle and 12 on the margin of the colony. In larger, multicelluear colonies, the cells are gathered in pairs, but these are irregularly dispersed over the whole colony. In older colonies some of the inner cells may divide parallel to the bottom of the colony. In this way a second layer of cells results. Sometimes it is possible to find colonies which have three layers of cells superimposed on each other. The hairs in colonies older than the 4-celled stage, are no longer regular. A colony in the 8-celled stage, or somewhat older, possesses a few hairs about 600 μ in length; some of them are forked or branched, and some are not. Occasionelly only one thick branched hair is found on an old colony. The single hairs are usually of the same diameter (about 2 μ); they are thicker at their base and gradually taper towards the end. Thick hairs are sometimes seen to be composed of several hollow tubes, which may be twisted or run parallel to one another. Branching is found at any point in the whole length of the hair. The branches may be of the same or of different length and thickness. Sometimes one thick hair may divide at one point into several delicate tubes. Old colonies have usually several hairs (Fig. 9); the thick ones originate in the centre of the colony while the thin ones are found on its margins.

According to Correns the mucilage-hairs are formed as described below: the zoospore becomes attached to the substratum and surrounded by mucilage. The inner and denser part of this

mucilage forms a short thin hair. After the first division the inner mucilage of two resulting cells forms two new hairs. These grow into the existing hair and tear it at the top. In this way

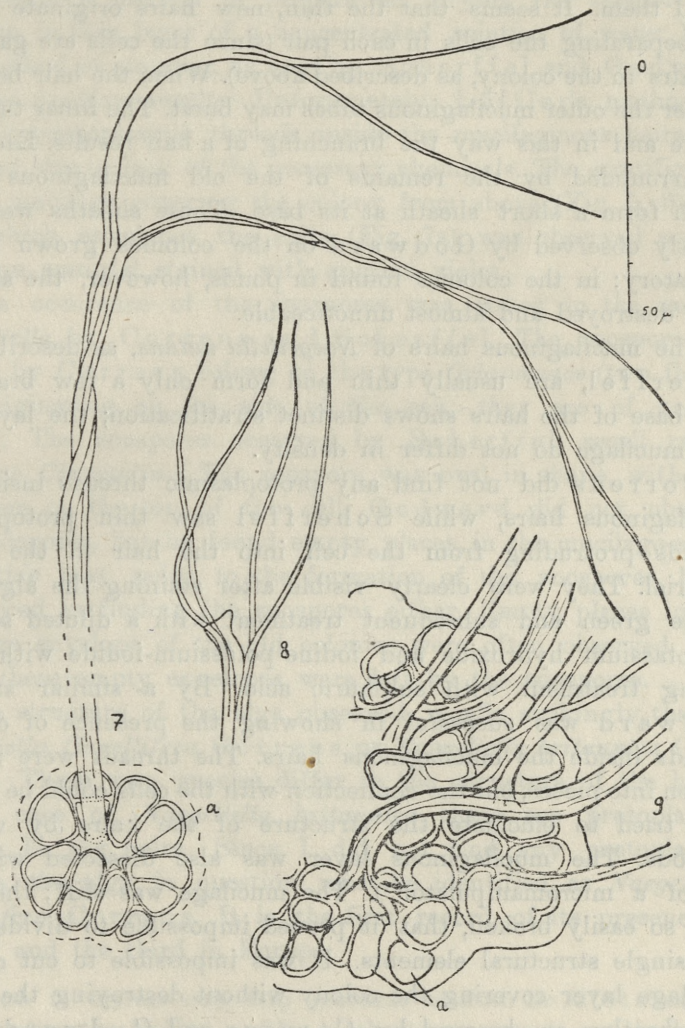

Fig. 7) young colony with a branching hair. a) the stratification of the mucilage.

Fig 8) the point of branching of a hair; a loose sheath is visible.

Fig. 9) a part of an old colony; the bases of the hairs and empty places (a) in the mucilage are visible.

the old hair forms a sort of sheath round the newly-grown ones. This process goes on as the cells divide, so that a hair in an old colony is a bunch of gelatinous tubes; the newly formed ones are fastened together by the older ones, which form sheaths round them. It seems that the thin, new hairs originate at the line separating the cells in each pair (since the cells are gathered in pairs in the colony, as described above). When the hair becomes thicker the outer mucilaginous tubes may burst. The inner tubes separate and in this way the branching of a hair results. Each hair is surrounded by the remains of the old mucilaginous tubes, which form a short sheath at its base. These sheaths were frequently observed by Godward on the colonies grown in the laboratory; in the colonies found in ponds, however, the sheaths were destroyed and almost unnoticeable.

The mucilaginous hairs of *Naegeliella natans*, as described by Scherffel, are usually thin and form only a few branches. The base of the hairs shows distinct stratification; the layers of this mucilage do not differ in density.

Correns did not find any protoplasmic threads inside the mucilaginous hairs, while Scherffel saw thin protoplasmic threads protruding from the cell into the hair in the living material. They were clearly visible after staining the alga with iodine green and subsequent treatment with a diluted solution of potassium hydroxide and iodine-potassium-iodide with a following treatment with sulphuric acid. By a similar staining Godward was succesful in showing the presence of delicate threads inside the mucilaginous hairs. The threads were usually broken into pieces, but the connection with the cells could be traced.

I tried to elucidate the structure of the hairs by various methods. The mucilaginous layer was also dissected with the aid of a micromanipulator [1]). The mucilage was stiff; the hairs were so easily broken, that it proved impossible to divide them into single structural elements. It was impossible to cut off the mucilage layer covering the colony without destroying the hairs. The sheaths, as observed by Correns and Godward, were not found in my case. Only once I saw a sort of loose sheath

[1]) I wish to thank Dr A. Pigoń for his ready help in the performance of this work.

around the hair at the point where it branched (Fig. 8). It must be added that large number of colonies, grown both under natural conditions and cultivated in the laboratory, were investigated. No structure of the mucilage and mucilaginous hairs was shown by Bresslau's method of preparation (drying the specimen in question in thin layer of a concentrated solution of water blue). The method of staining, as used by Scherffel and Godward gave no positive results. Unfortunately, Löffler's method for staining protoplasmic threads inside the mucilaginous hairs was not used, due to lack of the necessary chemicals. The stratification of the mucilage covering the colony from above (Fig. 5, 6) and that which envelopes the cells (Fig. 7a), was observed several times on material stained with gentian violet.

The occurence of the zoospores was stated in the case of *Naegeliella* by Correns and Scherffel. The zoospores observed by Correns belong to the type *Ochromonas* (two flagellae, originating on the side of the cell; they are of uneven length). The zoospores observed by Scherffel recall rather the type *Chromulina*. The zoospore was oval in shape, with one flagellum on the top of the cell. Godward did not observe any zoospores, but he found empty places in the mucilage-envelope; this may point to the formation of the zoospores. I did not succed in finding the zoospores either. Empty places in the mucilage envelope of the old colonies were often observed. Probably, these empty envelopes were left by the zoospores.

The structure of the alga observed recalls strikingly that of *Naegeliella flagellifera* Correns or *Naegeliella britannica* Gorward. These two species differ in the structure of the hairs: in the case of *Naegeliella britannica* there are protoplasmic threads in the hairs. Since I did not find any protoplasmic threads, the alga in question must be identified as *Naegeliella flagellifera* Correns. It is the first record of its presence in Poland and the third in Europe.

I wish to express may deep gratitude to Prof. Dr K. Starmach for suggesting the problem and for much encouragement; my thanks are also due to Prof. Dr J. Wołoszyńska for the kind advice and criticism.

From the Fishery Department of the Animal Breeding Institite of the Jagiellonian University in Kraków and from the Fishery Experimental Station of the Jagiellonian University in Mydlniki. Poland.

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Studia nad kopalnym rodzajem **Tsuga** *Carr. w trzeciorzędzie europejskim. — Studies on the Genus* **Tsuga** *Carr. in the Tertiary of Europe.*

Mémoire

de M. **W. SZAFER** m. t.

présenté dans la séance du 25 Mars 1949

(Plate 1).

Contents

I. Introduction

In the year 1938 I brought to notice the fact that in the deposits from the Middle Pliocene at Krościenko (West Carpathian Mountains) had been found some remains of the genus *Tsuga*, such as scales of cones, needles and pollen grains; they were reported under the name *Tsuga* cf. *canadensis Carr.* (Szafer 1938).

In the course of further exploration of the two localities in the vicinity of Krościenko (Dziadowe Kąty and Grywałd) containing pliocene flora, abundant and well preserved material of fossil *Tsuga* was found and described in my later work (Szafer 1946-47) as two different species: *Tsuga europaea* (Menzel) *n. comb.* and *Tsuga caroliniana* Engelm. *foss.*. During the years 1947 and 1948 I continued to wash out the fossil plants from Krościenko and once more enriched my material of the pliocene *Tsuga*. Since my description of the fossil remains was published only in Polish in 1947, and since in that year, owing to the kindness of Dr Frans Verdoorn and Dr. Fl. A. Lilienfeld, I received from the U.S.A. sufficient comparative material of *Tsuga caroliniana* Engelm., the lack of which I had badly felt before, I therefore thought it would be interesting to undertake a revision of the whole my material both living and fossil and on that basis to give an outline of our present knowledge of the genus *Tsuga* in the Tertiary of Europe.

II. Material and methods of investigation

The material of the fossil remains belonging to the genus *Tsuga* which formed the basis for these work consisted of a great number of cones (146), needles, shoots and buds. With respect to their amount this is the wealthiest material of these genus in the European Tertiary, especially as far as cones with the seeds inside are concerned, which are of the greatest importance from the systematic point of view.

My task consisted of analysing the material, composed of a mixture of cones, needles, shoots, buds and pollen from the point of view of the morphological characters, and in trying to separate its components. In other words I had to decide whether all the material represented the remains of one species only, or was a mixture of two or more species which lived beside one another. Already a perfunctory review of the characters of the fossil remains belonging to the genus *Tsuga* had furnished grounds for the supposition that in the Pliocene of Krościenko we had to deal not with one but with two different systematic units which should be distinguished on the basis of a comparison with the now-existing species of the genus *Tsuga* by means of statistic methods.

Among the species of *Tsuga* that are living now only two might be taken into consideration as probably related to the fossil *Tsuga* known heretofore from the European Tertiary; these are: *Tsuga canadensis* Carr. and *Tsuga caroliniana* Engelm., both of which occur in North America. It is possible that in future other fossil remains of *Tsuga* related to the species which are living now in Central or Eastern Asia may be found, but at present we have no positive indications in that line. (Compare page 44).

In spite of the existence of detailed descriptions of both the above species of the American *Tsuga* which are easily found in the principal dendrological works (Beissner 1909, Rehder 1934, Schenck 1939 and others), our knowledge of their morphological and anatomical characters is limited by the fact that the spontaneous variation of these characters is still very little known. It is true that not only do the respective data cited by the authors differ very often, but also the material that was used by them was most often gathered by chance and proved to be quite insufficient from the point of view of the demands of biometry.

It is of great value to know in every case the natural variation of each morphological and anatomical character which is important from the taxonomical point of view, but in the case of comparison of species which differ from one another mostly in their quantitative properties the knowledge of the variation of their characters is absolutely necessary. As far as our two species of *Tsuga* (*T. canadensis* and *T. caroliniana*) are concerned it is easy to show that without a sufficient investigation of the natural variation of their quantitative characters any comparison between them and the fossil remains of *Tsuga* would be hopeless.

From the 17 characters which were chosen as comparable both for the living and the fossil specimens of *Tsuga* only two were of purely qualitative nature, and 15 were more or less different with respect to quantity. It is clear that under such circumstances I was obliged to undertake a series of biometrical measurements of both the American species of *Tsuga* which I wanted to compare with the very rich but mixed fossil material.

The following morphological and anatomical characters were taken into consideration: 1) length of cones, 2) breadth of cones, 3) length of fertile scales, 4) breadth of fertile scales, 5) length of

seeds with wings, 6) length of seeds without wings, 7) shape of wing, 8) length of leaf-blade, 9) breadth of leaf-blade, 10) length of leaf-petiole 11) shape of blade, 12) dentation of blade, 13) development of hypoderma in the blade, 14) number of rows of stomata on leaf-blade, 15) characters of shoots, 16) shape of buds, 17) morphology of pollengrains.

Each of the above-mentioned characters was investigated separately, first those of *Tsuga canadensis* and *T. caroliniana* and then the pliocene fossil material from Krościenko was compared with them.

III. Statistical investigations

A. The cones.

Tsuga canadensis has ovate cones according to Rehder, and elliptical according to Schenck. Their length amounts to 15—20 mm according to the former author, while according to the latter it attains 15—18 mm. The cones of *Tsuga caroliniana* are ovate-elongate according to Rehder and cylindrically elongate according to Schenck. The former investigator estimates their length to be 20—35 mm, and the latter 20—32 mm. However the scale of variation of the length of cones of both species is really much wider.

I was able to examine the variation of cones of *T. canadensis* which came from 7 trees cultivated in the following places: Warszawa, Lwów, Kórnik near Poznań, Czechy near Miechów, Kamionna near Wiślica, Białowieża (the Park) and Geneva. The length and breadth of 300 normal cones (in the closed state) were measured; cones undeveloped or misshapen during their growth were excluded. I had at my disposal 208 ripe cones of *T. caroliniana* from the U.S.A. collected by Dr F. Verdoorn from 5 separate trees. My fossil material was composed of 146 well preserved cones.

1 and 2. *Length and breadth of cones.*

The results of the measurements of cones is shown in Table I and II.

From Table I it results that the cones of *T. canadensis* and *T. caroliniana* taken separately represent one two-topped and very

TABLE I

Length of cones of *Tsuga canadensis* in mm.

Amount	13	14	15	16	17	18	19	20	21	22	23	24	25	M.
300	2	2	26	38	48	46	46	41	28	12	8	2	1	**18·33**

Lenght of cones of *Tsuga caroliniana* in mm.

Amount	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	M.
208	1	1	1	5	4	10	14	13	13	19	17	28	18	21	18	11	5	5	4	**34·36**

Length of fossil cones of the genus *Tsuga* in mm.

Amount	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	M.
146	4	6	9	10	9	8	13	15	14	6	4	5	3	6	6	6	5	6	2	1	0	3	1	0	1	3	**23.63**

TABLE II

Breadth of cones in mm.

	Amount	6	7	8	9	10	11	12	13	14	15	16	17	18	19	M.
Tsuga canadensis	300	1	19	99	132	44	4	1	—	-	—	—	—	—	—	**8·71**
Tsuga caroliniana	168	—	—	-	--	—	1	10	24	26	39	27	30	10	1	**15·00**
Fossil *Tsuga*	111	3	4	8	8	16	22	16	14	9	5	3	2	1	–	**11·34**

extensive curve with one summit (*T. canadensis*) at about 18 mm, and the other summit at about 35 mm (*T. caroliniana*).

If our supposition are true that the fossil *Tsuga* from Krościenko represents a mixture of two species which are closely related to both American species, the curve of variation of fossil cones ought to be intermediate.

The fossil cones of Krościenko investigated using 146 (resp. 111) well-preserved specimens present at first sight an evident mixture of two forms: all smaller cones correspond to *T. canadensis* in their size, all large ones, elongate-ovate in shape and distinctly sharpened at the apex, approach *T. caroliniana*. Their mutual curve of variation possesses a wide range with two sections distinctly marked. The cones lying in the central section of the scale belong to both species.

The peculiarities of the variation of the breadth of cones, will be treated in chapter IV.

3 and 4. *Length and breadth of fertile scales.*

Schenck reports that there are about 30 seed-scales in one cone of *T. canadensis* and that they are almost circular in shape at their upper edge; about the scales of *T. caroliniana* he says that they are tongue-shaped, 18 mm long and 8 mm broad. Rehder describes the shape of the scales of *T. canadensis* as roundish-abovate and oblong-ovate, rounded and thin in *T. caroliniana*, without citing any dimensions for either or them.

In order to get a correct idea of the morphological characters of the seed-scales of *T. canadensis* I took to pieces 5 of its cones of medium size; each of these cones came from a different locality (Kamionna near Opatów, Czechy near Miechów, Białowieża, Lwów, and Warszawa). The number of seed-scales in one cone varied from 23 to 34 (29 on the average). The scales at the base of cones are sterile and variable in shape and dimensions: some are strictly circular or a little longer than broad, others are flattened-circular (i. e. broader than long), others ovate; their dimensions vary also: they are 2·2—8 mm. long and 2·3—7 mm broad. The apical sterile scales in the number of 4—7 in each cone are always longer than the basal scales and are of a narrow, elongate, boat-like shape. Their length varies from 3·2 to 8 mm, and their breadth from 1 to 5 mm.

Considering the great variation in the sterile seed-scales both apical and basal, and the difficulty in measuring the apical scales we excluded them from comparison with the fossil scales and restricted ourselves to the fertile middle scales.

Analogical measurements were conducted for *T. caroliniana* on 5 cones derived from 5 different trees.

From among the fossil cones of the *Tsuga* from Krościenko 4 cones of the type »*canadensis*« and 3 cones of the type »*caroliniana*« of an average size were chosen and taken to pieces into separate scales, the sterile apical and basal scales being excluded from measurement.

The investigated fossil cones of the type »*canadensis*« had altogether 26—30 scales with about 18 fertile ones on the ave-

rage; the fossil cones of the type »*caroliniana*« had 28—32 scales in all, with 20 fertile ones on the average.

The Tables III and IV show the results of our measurements;

TABLE III

Length of middle ovuliferous scales in mm.

	number of cones	number of scales	6	7	8	9	10	11	12	13	14	15	16	17	18	M.
the living *T. canadensis*	5	88	5	11	17	26	15	10	4	—	–	—	—	—	–	**8·97**
the fossil *T.* »*canadensis*«	4	76	2	10	10	16	17	12	5	3	2	—	—	—	—	**9·55**
the living *T. caroliniana*	5	92	—	—	—	1	2	4	8	8	5	7	24	18	5	**14·44**
the fossil *T.* »*caroliniana*«	3	61	—	–	3	4	2	4	9	11	9	8	6	2	3	**13·19**

TABLE IV

Breadth of middle ovuliferous scales in mm.

	number of cones	number of scales	4	5	6	7	8	9	10	11	M.
the living *T. canadensis*	5	88	1	7	20	29	27	1	—	—	**6·97**
the fossil *T.* »*canadensis*«	4	59	1	5	15	24	11	2	—	—	**6·78**
the living *T. caroliniana*	5	91	—	—	3	15	22	38	12	1	**8·74**
the fossil *T.* »*caroliniana*«	3	51	—	—	5	13	17	11	3	2	**8·00**

The proper shape of the scales being expressed best in the proportion between their length and breadth I give here this ratio. For *T. canadensis* (living) it is 1:1·28, for *T.* »*canadensis*« (fossil) it is 1:1·37, for *T. caroliniana* (living) 1:1·65, and for *T.* »*caroliniana*« (fossil) 1:1·75. The shape of the fertile scales is usually broadly-elliptical or (in about 11%) strictly circular in both the living and the fossil *T. canadensis*, while in *T. caroliniana* the fertile ovuliferous scales are longer and of elliptically-tongue-like shape, distinctly auriculated at the base.

It is clear that the mixture of the scales from both fossil types of cones must represent an intermediate form of the curve.

The bracts do not seem to show any peculiar characters which would help to distinguish the species from each other, especially in the fossil state, and therefore I do not describe them in this place.

5, 6 and 7. *The seeds.*

According to Schenck (1939) a seed of *T. canadensis* is 2 mm long without the wing, and 8 mm long with the wing; the wings are 3 mm broad; on the lower side of the seed there are 4—8 small resin glandules.

The seeds of *T. caroliniana* are (according to this author) 3·5 mm long without the wing, and 14 mm long with the wing; on the lower side of the seed there are numerous microscopically small resin glandules.

I investigated the variation of seeds of *T. canadensis* using 4 samples each of which contained 25 specimens coming from different stations (Toronto in Canada, Białowieża, Lwów, and Czechy near Miechów). The dimensions of *T. caroliniana* are based upon measurements of 68 seeds extracted from 5 cones. The results are given in the tables below:

TABLE V

Length of seeds without wings in mm.

	Amount	1·8	2·0	2·2	2·4	2·6	2·8	3·0	3·2	3·4	3·6	3·8	4	4·2	4·4	4·5	M.
T. canadensis (living)	100	—	—	—	—	—	5	9	15	21	14	17	9	5	3	2	**3·59**
T. caroliniana (living)	68	1	8	5	8	18	13	14	6	6	5	1	1	1	1	—	**2·75**

As we see from the above, the scale of variation in length of seeds of *T. canadensis* is wide, and its mean length reaches a little over 3·5 mm, while the mean length of the seed with wing corresponds to that given by Schenck (8 mm). The seeds of *T. caroliniana* without wings are, according to my measurements, distinctly smaller than those of *T. canadensis*, contrary to

the data given by Schenck. The wings of *T. canadensis* are distinctly broader at their base, while those of *T. caroliniana* are almost linear along their whole length.

The material of seeds of both our fossil forms is scanty. We failed to extract undamaged seeds with wings from cones taken to pieces, and we had to restrict ourselves to measurements of their impressions on the surface of scales where they were visible. We succeed in measuring only 6 seeds without and 9 seeds with wings of the type »*canadensis*». The length of seeds oscillates here between 3—4·5 mm, and the length of seeds with wings from 6·6 to 10·0 mm, which corresponds to the living *T. canadensis*. Besides, 2 separate winged seeds of *T.* »*canadensis*« have been found; one is demaged, the other is entire (cf. Szafer 1947, Table IV, fig. 4); it is 7·7 mm long and 3·1 mm broad, and corresponds precisely to Schenk's description.

From the fossil cones of the type *T.* »*caroliniana*« I measured the impressions of the winged seeds in 10 cases but unfortunately the exact dimentions could not be obtained on account of the seeds being damaged.

B. The leaves.

The morphology of leaves resp. needles of *T. canadensis* and *T. caroliniana* shows comparatively small but distinct differences which ares shown in the following table after Schenck:

TABLE VI

Length of seeds with wings in mm.

	Amount	5·6	6·1	6·6	7·1	7·6	8·1	8·6	9·1	9·6	10·1	10·6	11·1	11·6	12·1	12·6	13·1	13·6	14·1	14·6	15·1	15·6	M.
T. canadensis (living)	100	2	5	13	18	14	13	13	9	4	6	3	—	—	—	—	—	—	—	—	—	—	**7·99**
T. caroliniana (living)	57	—	—	—	—	—	2	6	4	4	7	6	5	4	6	8	6	2	2	2	2	—	**11·32**

TABLE VII

T. canadensis	*T. caroliniana*
Length of blade: 6–18 mm.	Length of blade: up to 17 mm.
Breadth of blade: diminishes although inconsiderably, from the base towards the apex.	The breadth of linear blade up to 2 mm.
Length of petiole: —	Length of petiole: —
Apex of blade: rounded, rarely weakly depressed.	Apex of blade: rounded or bluntly sharpened, rarely weakly depressed.
Margin of blade: especially in its upper half delicately and sharply dentate.	Margin of blade: entire
Upper surface of blade: delicately and flatly channeled.	Upper surface of blade: channeled.
Lower surface of blade: provided with a distinct and strongly protruding midrib.	Lower surface of blade possesses a very thin midrib.
Stomata: in 2 bands, 5—6 distinct rows of stomata in each; the marginal bands without stomata are broad.	Stomata: in two bands, undistinctly divided from each other by a thin midrib; each band has 6—9 rows of stomata; marginal bands without stomata are broader than the midrib.

Beissner (1909) reports that *T. caroliniana* differs from *T. canadensis* by its longer (16—23 mm) and broader (2 mm) needles and by the presence of a hypodermal tissue which is located under the epidermis on the margins and under the ribs. Rehder (1934) reports that the leaves of *T. canadensis* as well as those of *T. caroliniana* are 8–18 mm long.

Desiring to obtain more precise data concerning the variation of the morphological characters of the leaves of *T. canadensis*, I investigated an abundant material composed of samples of 100 needles each, coming from different stations of fruiting trees: Ocean Co. Mayne in Canada (1 tree), Lwów (3 trees), Kórnik (1 tree), Królikarnia (1 tree).

The variation of leaf-character in *T. caroliniana* was investigated using one sample (100 needles) from a natural station in North Carolina (Pinnacle M.), and 5 samples from 5 trees of this species, the latter material received owing to the kindness of

Dr. F. Verdoorn from the USA. The length of the blade without the petiole, the breadth of the blade and the length of the petiole were measured. The results of the measurements are shown in the tables below.

8 *and* 9. *Length and breadth of blade*

TABLE VIII

Length of blade (without petiole) in mm.

Amount	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	M.
T. canadensis 1.000	3	30	29	57	96	116	165	143	123	108	66	37	17	6	2	—	**9·60**
T. caroliniana 1.000	1	2	2	8	25	55	98	106	117	166	156	121	98	31	11	3	**11·83**
the fossil *Tsuga* 104	1	7	8	13	22	17	11	10	5	4	2	2	1	2	—	—	**8·03**

TABLE IX

Breadth of blade in mm.

Amount	0·5	0·6	0·7	0·8	0·9	1·0	1·1	1·2	1·3	1·4	1·5	1·6	1·7	1·8	1·9	2·0	2·1	2·2	2·3	2·4	2·5	M.
T. canadensis 1.000	—	—	—	1	1	7	27	29	37	74	108	148	168	141	82	79	48	44	11	3	2	**1·71**
T. caroliniana 1.000	1	1	1	1	2	14	11	60	137	211	327	128	72	21	5	8	—	—	—	—	–	**1·45**
Tsuga (fossil) 259	—	—	1	1	2	7	13	17	28	32	36	39	35	21	13	12	6	1	2	—	—	**1·53**

10. *Length of the petiole.*

A comparison of the above morphological characters of the needles, that is the length and breadth of the blades and the length of their petioles, shows that the needles of *T. canadensis* compared with those of *T. caroliniana* are a little broader but shorter and possess a slightly shorter petiole. Their comparison with the mixture of fossil needles may be considered possible only as far as breadth of blade and length of the petiole are

concerned. In this respect they distinctly approach *T. caroliniana*. But instead if we consider the length of blade the result obtained by us (Table VIII) is certainly inaccurate for the fossil needles, and too low in its mean.

TABLE X

Length of petiole in mm.

Amount	0·5	0·6	0·7	0·8	0·9	1·0	1·1	1·2	1·3	1·4	1·5	1·6	1·7	M.
T. canadensis 1.000	14	29	36	100	224	308	163	80	32	12	2	—	—	**0·98**
T. caroliniana 614	2	5	9	17	40	118	129	141	91	46	14	2	—	**1·13**
Tsuga (fossil) 266	1	2	8	22	41	50	56	38	30	14	12	3	2	**1·09**

This lower value for the mean length of blades of the fossil needles should be explained — in my opinion — by the fact that the longer needles have been preserved in a fossil state more rarely in their entirety than the short ones.

11. *Shape of needles.*

The needles of *T. canadensis* are either linear or cuneate i. e. broader at their base. The needles of *T. caroliniana* are linear. Very small needles of both species may be more or less elliptical-lanceolate, that is strongly narrowed towards the apex as well as towards the petiole. The shape of the fossil needles is linear as a rule, more rarely they narrow towards the base, their shape being dependant neither of the thickness of the blade nor of the presence or absence of the dentation of the margin of the blade. Quite exceptionally there appear among the small needles others that narrow towards both ends which are narrow-elliptical-lanceolate in their shape. But cuneate leaf-blades, i. e. broadening distinctly towards the petiole, have never been met with, not even in one case. This shape of blade appears today in *T. canadensis* beside the linear shape. I emphasize this fact here because on page 184 (22) Part II of my paper, while describing the pliocene flora of Krościenko (Szafer 1947), I have already drawn attention to this fact but mentioned that two needles had been found the

shape of which resembled a wedge. A repeated investigation of these needles showed that in both these cases the blades were slightly rolled up at the apex and therefore made the impression of being cuneate.

12. *Dentation of the margin of blade.*

T. canadensis has denticulate blades with small teeth while those of *T. caroliniana* have an entire margin.

The assorting of fossil needles on the basis of indentation of their margin is difficult because the delicate teeth are often destroyed and therefore the needles must be investigated under a considerable magnification and only the best preserved needles are of use.

24 fossil needles out of 80 possessed more or less distinct dentation of the margin in their upper part or at least at the apex; about 20 had some (2—3) very indistinctly developed teeth near the apex, and the rest of the needles had no teeth. It was possible to ascertain at the same time that the dentate needles possessed thinner blades while those with an entire margin had thicker blades.

While investigating this character I had the impression that the indentation of fossil needles stands in an evident correlation to the thickness of their blade, but instead it has no connection either with the shape or the dimensions of the blade.

The mere presence or lack of indentation cannot serve as a basis for distinguishing fossil needles of the type *T.* »*canadensis*« from the type *T.* »*caroliniana*«. It seems that in the fossil type *T.* »*canadensis*«, much as in the living species, there appeared thin needles with distinct as well as with slight denticulation, and perhaps also without dentation, while all those of *T.* »*caroliniana*« had an entire margin. This supposition seems the more probable that this character (i. e. the dentation of the margin of the blade) is today also a variable character in some species of the genus *Tsuga.* The blades of *T. chinensis* Pritz. are denticulate only in young specimens of this species and on fertile shoots, while the dentation of *T. Jeffrey* Henry is altogether a variable and slightly pronounced character (Schenck 1939).

13. *The hypoderma.*

I have mentioned above that according to Beissner (1919) the needles of *T. canadensis* have no hypoderma under the

epidermis but the needles of *T. caroliniana* have 1- to 3-layered hypoderma. In order to investigate this character cross-sections were made first of both the living species of *Tsuga*, and next of the fossil needles.

In order to present in figures the degree of development of the hypoderma the following 5 classes of variation of this character have been established: 0 = a total lack of the hypoderma; 1 = weakly developed hypoderma, one-layered with intervals, i. e. only in both corners and at the midrib; 2 = one-layered, continuous on the upper part of the blade; 3 = two-layered at least in the corners; 4 = three-layered at least in the corners. The above scheme of classes is not strict and therefore the variation of this character could be conceived only generally.

TABLE XI

Hypoderma in leaves.

	Amount	0	1	2	3	4
T. canadensis	94	66	21	7	—	—
T. »canadensis« fossil	5	2	2	1	—	—
T. caroliniana	114	—	2	12	35	65
T. »caroliniana« fossil	23	—	—	1	9	13

As is seen from the above table the needles of *T. canadensis* may possess a hypoderma although they lack it in the majority of cases (almost 70%). In *T. caroliniana* the hypoderma appears always but as a very variable character.

Although only a small quantity of fossil needles has been investigated their number seems to be sufficient for making the statement that both fossil types do not differ distinctly, with respect to the character discussed, from the two living species with which they are being compared.

14. *Stomata*

In their anatomical characters the stomata of the fossil *Tsuga*-genus correspond to Florin's (1931) description and pictures. We had to investigate only the number of rows of stomata

which are gathered in 2 bands on the lower side of the blade. *T. canadensis* was in this respect investigated on the basis of 6 samples (50 needles in each) taken of six trees from different localities (Ocean Co. Mayne in Canada, Białowieża, Warszawa, Lwów, Kórnik and Kraków). An analogical material of *T. caroliniana* was derived from 6 different specimens of trees from the USA.

TABLE XII

Number of rows of stomata in one band.

Amount of needles	Amount of bands	3	4	5	6	7	8	9	10	11	12	M.
Tsuga canadensis 300	600	4	40	152	225	124	40	13	2	—	6	**6·01**
Tsuga caroliniana 300	600	—	6	58	164	221	92	41	9	5	4	**6·91**
fossil *Tsuga* 65	130	1	8	20	29	35	19	8	6	4	—	**6·79**

To the above Table I wish to add that the number of rows of stomata in one band (at the same time the breadth of the band) evidently depends in *T. canadensis* on the breadth of the midrib; in the samples of needles with a thick and much protruding midrib the bands of stomata are narrower (the average number of rows is 5—6), while in the samples with a thin and less protruding midrib the bands of stomata are wider (the average number of rows of stomata is 7—8); the needles with a thin rib and a higher number of rows of stomata were taken from specimens from Lwów and Kraków (from the Botanical Gardens). The latter forms approach *T. caroliniana* and thus practically abolish the difference to which Schenck points as existing between these two species.

With regard to the number of rows of stomata in the bands on the lower sides of the needles the fossil needles of the genus *Tsuga* differ from *T. canadensis* only in so far as the summit of the curve of variation of this character of the fossil needles is shifted towards higher values, and that its range goes further in the same direction. It is clear that this corroborates the statement that the fossil needles belong to *T.* »*canadensis*« and *T.* »*caroliniana*« taken together.

C. The shoots, buds and pollengrains.

15. and 16. *Shoots and buds.*

The young shoots of *T. canadensis* (Schenck 1939) are thin and pilose; they are covered with two kinds of hair: short and bristly, or long and threadlike; the buds are ovate, blunt or pointed, 1·5 mm long and with short hair. The scales of the buds remain at the base of the shoots up till the sixth year. The yong shoots of *T. caroliniana* are slightly pilose with short and delicate hair; the buds are ovate, more or less pointed, delicately pilose, with scales fitting tightly.

Several fossil fragments of young shoots have been found with distinct cushion-like needles-bases, sometimes also with buds. Unfortunately they are most often without the epidermis and for this reason they lack any trace of hair, which would enable us to distinguish the fossil shoots of the type *T.* »*canadensis*» from the type *T.* »*caroliniana*«. Only 3 specimens have preserved their bristly hair on shoots with occasional thread-like hairs in it. These 3 shoots when compared with similar shoots of *T. canadensis* do not differ from them in any respect.

17. *The pollen.*

The pollen of the genus *Tsuga* was the subject of several publications (Baas 1932, Jimbo 1933, Wodehouse 1933, 1935, Kirchheimer 1934 and 1935, Rudolph 1935, Erdtman 1943 and others) in which different views were expressed on its importance for the determination of fossil species. In my opinion, however no important taxonomic role can be ascribed to these characters until the pollen of all existent species of the genus *Tsuga* coming from natural stations has been thoroughly investigated. Therefore without discussing the problem introduced by the above-mentioned authors I only report here in brief my own results.

Beside *T. canadensis* I had also the opportunity to investigate one sample (100 measurements) of pollen of *T. caroliniana.* I found 47 μ as minimum, 100 μ as maximum and 70 μ as the average size of the grains, and from 2—8 μ (mean: 5 μ) as the breadth of their marginal fringe.

The pollen of *Tsuga* is rather rare in the Pliocene of Krościenko. Most of it has been found in the microscopic preparations which were made of fossil cones stuck all over with loam. The

ten pollen grains which have been measured possessed the characteristic sculpture of the exine and had the following diameters: 65 μ, 70 μ, 75 μ, 77 μ, 78 μ, 80 μ, and 83 μ. The breadth of the marginal fringe varied within the following limits: (the specimens are enumerated in the same order): 7—10 μ, 7—11 μ, 8—9 μ, the fringe of the fourth specimen was not measured, 10—11 μ, 7—10 μ, 7—8 μ, 6—10 μ, 7—10 μ, and 7 μ.

Our fossil pollen corresponds more or less to the pollen of *T. canadensis*, but it differs from it by a broader marginal fringe which in the living species is distinctly narrower (it amounts to 4—5 μ); in this respect it approaches more nearly *T. caroliniana* and seems to agree with the pliocene pollen of *Tsuga* from Willershausen in the Harz Mountains (Kirchheimer 1934) and also with the pliocene pollen *T. moenana Kirchh.* (1935) from a locality called Gross-Steinheim on the river Main.

IV. Discussion of some results obtained by the analysis of systematic characters

Some results of the comparison of the fossil *Tsuga* from the Pliocene of Krościenko with two closely-related living species of *Tsuga* need critical discussion. This may be expressed in the following points:

1. The material of the living *T. canadensis* and *T. caroliniana*, although fairly abundand, was not collected in nature in a manner satisfactory for the requirements of biometry which demands that it should be gathered from a great number of separate trees scattered all over the natural range of the species. Ours was not collected in this way but rather had character of an occasional collection from trees often cultivated even in gardens. As a result it did not lead to a comprehension of a full and natural variation of the characters described here but only gave us a general idea of the vartation of these characters.

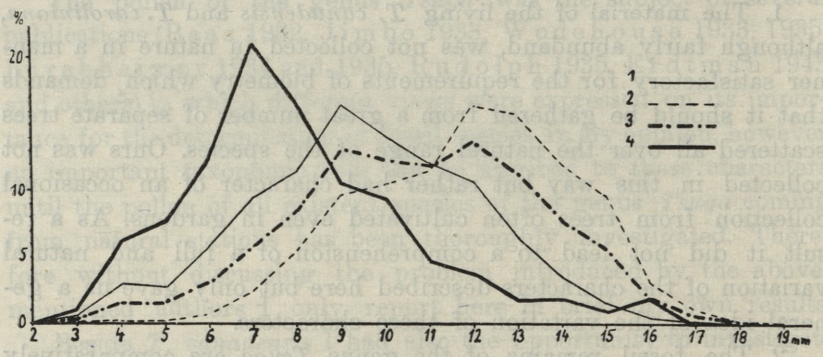
2. The fossil remains of the genus *Tsuga* are comparatively rather abundant; they are no doubt derived from numerous specimens of trees and from a long geological period because they have been collected at different strata of the very thick pliocene deposits. This circumstance raises the value of the fossil material, but its disadvantageous circumstance is no doubt the mechanical selection of cones and needles owing to which cones and needles

of small size are preserved whole more numerously than large cones and long needles. So it results that the length of fossil cones and needles in the variation shown by their curves (Graph. 1 and 2)

Graph. 1.

Length of cones in mm. 1 = *Tsuga canadensis*, 2 = *T. caroliniana*, 3 = Combined curve of both these species, 4 = Fossile mixed *Tsuga*.

are no doupt over-represented in the lover and medium classes, and therefore they are deficient through a relative lack of large-



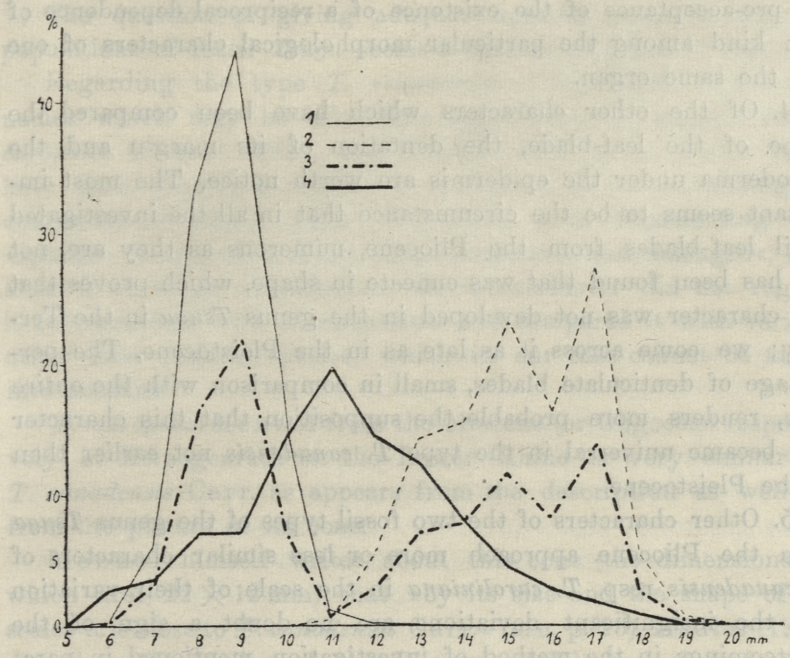
Graph. 2.

Lenght of blade in mm. 1 = *Tsuga canadensis*, 2 = *T. caroliniana*, 3 = Combined curve of both these species. 4 = Fossil mixed *Tsuga*.

size classes. This remark does not apply to the breadth of cones and needles. It was possible to measure this character even on specimens with damaged apices. Therefore the measurements of

the breadth of cones and needles are of great importance for their comparison with the cones and needles of the living species of *Tsuga*.

3. The breadth of fossil cones does not give a picture of its variation that might be compared with the joint curve of va-



Graph. 3.

Breadth of cones in mm. 1 = *Tsuga canadensis*. 2 = *T. caroliniana*. 3 = Combined curve of both these species. 4 = Fossil mixed *Tsuga*.

riation of this character in *T. canadensis* and *T. caroliniana* (Graph. 3) but it distinctly shows an intermediate summit in the curve of variation of this character, and its range is as wide as of both these species. This striking fact proves that the fossil populations of *T. »canadensis«* and *T. »caroliniana«* in the Pliocene were very close to each other in respect to the breadth of cone, unlike their living populations which distinctly differ in this character.

At the same time the cones of both fossil species differed widely in other characters, much like those of the living *T. cana-*

densis and *T. caroliniana*. This circumstance corroborates once more the fact, well known also from other sources, that the variation of particular characters may take place independantly of one another. This does not deny the possibility of correlative intermingling of certain characters, but it nevertheless demands caution against the pre-acceptance of the existence of a reciprocal dependence of that kind among the particular morphological characters of one and the same organ.

4. Of the other characters which have been compared the shape of the leaf-blade, the dentation of its margin and the hypoderma under the epidermis are worth notice. The most important seems to be the circumstance that in all the investigated fossil leaf-blades from the Pliocene, numerous as they are, not one has been found that was cuneate in shape, which proves that this character was not developed in the genus *Tsuga* in the Tertiary; we come across it as late as in the Pleistocene. The percentage of denticulate blades, small in comparison with the entire ones, renders more probable the supposition that this character also became universal in the type *T. canadensis* not earlier than in the Pleistocene.

5. Other characters of the two fossil types of the genus *Tsuga* from the Pliocene approach more or less similar characters of *T. canadensis* resp. *T. caroliniana* in the scale of their variation and the insignificant deviations are no doubt a sign of the shortcomings in the method of investigation mentioned in paragraph 1.

V. The systematic value and the nomenclature of the fossil forms of *Tsuga*

From the foregoing analysis of the morphological and anatomical characters of our fossil remains of the genus *Tsuga* it is clear that at Krościenko we had to deal with two species of *Tsuga* which are closely related to *T. canadensis* resp. *T. caroliniana*.

According to the morphology of cones (size and shape of: cones, ovuliferous scales, seeds with and without wings), the morphology and anatomy of needles, young shoots and buds, our fossil type *T.* »*canadensis*« closely approaches the species *T. canadensis* Carr.. Nevertheless it must not be regarded as quite identical with this mostly because the blade of the leaf of the

fossil form is never cuneate in shape, as it is so frequently in the *T. canadensis.*

The other fossil type, *T.* »*caroliniana*«, resembles *T. caroliniana* Engelm. so closely in every respect that it can be regarded as specifically identical.

The question of giving adequate specific names to our two populations of fossil *Tsuga* seems a simpler task than it really is.

Regarding the type *T.* »*canadensis*«, there exist so far two names which may be taken into consideration, namely: *Tsuga europaea* Menzel (1913) and *T. moenana* Kirchh. (1935). Both have been described chiefly on the basis of the morphological characters of single cones. The fossil cone documenting the »species« *T. moenana* is »a little mishhapen and damaged«, and after a thorough examination Kirchheimer did not regard it as connected with *T. canadensis* and compared it with various other East Asiatic species, which in our case cannot be taken into account.

T. europaea Menzel from the Miocene (or Oligocene respectively) of Herzogenrath on the Lower Rhine is very similar to *T. canadensis* Carr. as appears from the description as well as from the picture of its cone.

Menzel himself writes about this cone (the dimensions of which are: 22 × 12 mm) that »by its size and the shape of its scales it is close to *T. canadensis* Carr.« (o. c. p. 23). Mädler (1939) describes 15 needles of the Pliocene at Klärbecken, under the name *Tsuga europaea* Menzel and says that »they correspond to the needles of the present *T. canadensis* Carr.«. They are 5—19 mm long and 1—2·5 mm broad. Unfortunately he says nothing about their dentation and since he has not examined their anatomy either, we do not know if they have any sub-epidermal hypoderma. In their form they are very similar to our pliocene needles and probably even identical with them. Neither have any of them a truncate-lanceolate shape.

In view of the facts that: 1⁰ our type *T.* »*canadensis*« though approaching *T. canadensis* is not quite identical with this species, 2⁰ a species of the genus *Tsuga* identical with our type *T.* »*canadensis*« has been described with considerable probability though only fragmentarily, I consider it most suitable to call our form *T. europaea* (Menzel) *nov. comb.*

As to our fossil type *T.* »*caroliniana*« it seems natural to designate it by the name which I have used already in 1947 (Szafer 1947, page 172) i. e. *Tsuga caroliniana* Engelm. *fossilis* But it would be also possible to choose for it a name in which it could be emphasized that the fossil population of *Tsuga caroliniana* was not quite identical with the population of this species now existent in the U. S. A.. Perhaps in this case the name *Tsuga* pre-*caroliniana* Engelm. might be used, but only under the condition that the prefix »pre« used in this combination has a special meaning: it is not used here as a new specific name (»*pre-caroliniana*«) but as an indication of the fact that the pliocene population of *Tsuga caroliniana*, although very similar to that now-existing, was not quite identical with it. By using the prefix »pre« for the fossil *T. caroliniana* in this new sense I do not want to press other paleobotanists to accept it; my intension is rather to draw their attention to the circumstance that such an application of the prefix »pre«, as is proposed here, seems to suit the purpose better and is more justified in the phylogenetic sense, than using it excessively, as often is the case nowadays, in the designation of the newly described fossil plants. Unfortunately this often happens even in cases where there are no sufficient grounds for belief that an existent species is derived from an extinct one which is specified by the same name with the prefix »*pre*«.

VI. Other fossil representatives of the genus *Tsuga* in Europe and their relation to the living species.

Fossil remains of the genus *Tsuga* are fairly wide-spread in the European Pliocene though they have not been found anywhere in such large quantities as at Krościenko.

Stojanoff and Stefanoff (1929) have described needles from the Pliocene of the environs of Sofia (Kurilo) under the name *Tsuga aff. canadensis* Murr. They are 5—16 mm long, 1·2—1·5 mm broad, linear and straight, without indentation, with a petiole 1—1·5 mm long. Stefanoff and Jordanoff (1935) described from the same Bulgarien Pliocene one branch with needles, one cone (ill-preserved), and one separate seed under the name *Tsuga europaea* Menzel (aff. *T. canadensis* Carr.); the authors see a reason — in my opinion a wrong one — for using

such a name because the wing of the fossil seed is somewhat different and larger than that of *T. canadensis* (cf. page 31). Besides, the authors admit that the *Tsuga* from Kurilo may be identical either with *Tsuga canadensis* Carr. or with *T. caroliniana* Engelm.. In my opinion it corresponds most probably to *T. caroliniana fossilis.* Pop found at Borsec (1936) the pollen of *Tsuga* in two forms, one of which is supposed to correspond to the pollen of *T. canadensis*; the two needles — if at all belonging to the genus *Tsuga* — points rather to the type *T. »caroliniana«*

I have already mentioned the genus *Tsuga* from the German Pliocene (see above, especially Kirchheimer 1934 and 1935).

In Asia there are data concerning the appearance of *Tsuga* in the Upper Pliocene deposits northwards from Tobolsk (a village called Nefedowa — Sukatschew 1933); from the Japanese Pliocene *Tsuga Sieboldii* Carr. and *Tsuga diversifolia* Mast. (Miki 1938) have been recorded.

From the Pleistocene, namely from the European Interglacial Period, the pollen of the genus *Tsuga* as well as its needles and twigs were found in Poland near Raków, about 100 km to the north-east of Kraków (Kozłowska 1923). There were found three whole needles and a damaged one, all of them without petioles; their dimensions were as follows: 4·3 × 0·9, 5·2 × 0·8, 5·1 × 0·9 and — × 1·4 mm. Their distinctly cuneate shape and the indentation of the margin points to their identity with *Tsuga canadensis* Carr.

From what has been said above it results that the genus *Tsuga* has its representatives in Europe not only in the Tertiary but also in the Pleistocene interglacial periods.

Leaving aside *T. moenana* Kirchh., about which it is difficult to say anything certain as long as its description is founded on one damaged cone, it seems to me most probable that the number of species of *Tsuga* in the European Pliocene was limited, and that it was perhaps represented by only two species abundant in the Pliocene of Krościenko, *Tsuga europaea* Menzel and *T. caroliniana* Engelm. *fossilis.* The presence of *T. diversifolia* in the European Tertiary, although its occurence in Europe was deduced by other invesigators on the ground of the morphology of pollen grains, is in my opinion very problematic.

VII. The geological age.

Both fossil species of *Tsuga* in the European Pliocene belong to the group of plants which represent the Arcto-Tertiary element in the sense of Engler. From their true circumboreal or partially circumboreal home in the Tertiary (Miocene?) they wandered in very closely related populations to the eastern Atlantic part of North America on the one side, and on the other to the western wing of the continent of Eurasia. While both American species were able to resist the climatic fluctuations of the whole Pleistocene in their southern refuges, their European relatives were not able to stand them and died out. *T. caroliniana* was probably the first that died out in Europe, and it was followed later on (before the last Glacial Period) by *T. canadensis*.

The geographic range of *Tsuga caroliniana* Engelm. in America has now the character of a relic. *T. caroliniana* appears there (according to Schenck) in strictly limited points in the Appalachian Mountains (in West Virginia and in the northern part of Carolina) at the height of above 1000 m. It develops well in habitats more arid than are required for *T. canadensis*, and the rather xeromorphic anatomical structure of its needles is probably connected with this circumstance.

Tsuga canadensis Carr. is a tree of a wide distribution in the Atlantic part of North America (in the United States and in Canada), in the mountains as well as at their foot; I do not quote here any details about its distribution because these are described in detail in numerous dendrological works (cf. especially Schenck 1939).

VIII. Some remarks on evolution in the genus *Tsuga*.

Since there is rarely an occasion for discussing phylogenetic problems on the basis of an abundant material of closely related plants, both fossil and living, and since our material of the genus *Tsuga* especially encourages such a discussion, let me make here the following few remarks on this matter.

Among the numerous endeavours to grasp the problem of phylogenetic speciation from the point of view of micro-evolution the trials undertaken by Zimmermann (1930, 1938, 1941) are very simple and therefore easily applicable in our case. According to him the real unit from which all permanent (here-

ditary) changes or new »species« originate is the so-called »ontogeny« of species composed of the populations of hereditary forms. At a given moment each species of the plant is composed of a definite number of genetically different biotypes which characterize its ontogeny. When the species changes in its biotypic composition its »ontogeny« changes also. If we imagine these successive changes as a spiral line, each of its coils corresponds to one »phylogeny«. The gradual change of one »ontogeny« into another is performed in such a way that within the range of the given »ontogeny« there appears a certain new character as a new mutation (line). If we assume that this new mutation propagates more quickly than others, then, after some time, the curve of the whole »ontogeny« is distinctly shifted in one direction and with time the old »ontogeny« is transformed into a new one. We then pass to its new coil on the phylogenetic spiral. Such changes in the genetic composition of the population are protected in nature by way of selection (in Darwin's sense). The new race resp. ecotype of the species is favoured by agents of selection of various kinds that operate in nature. Time alone is of no essential value in this process because the changes in the »ontogenies« of different species may take place during different spaces of time, in some species quickly, in others slowly.

If we apply now the above reasoning to fossil and living »ontogenies« of the genus *Tsuga* from the affinity of *T. canadensis* and *T. caroliniana* we obtain the following probable picture of their evolution.

In the Pliocene there lived two closely related but already separate »ontogenies« of *Tsuga*: one approached the now existent *T. canadensis* and has been called *T. europaea* Menzel; the other was close to *T. caroliniana* and has been given the name of *T. caroliniana* Engelm. *fossilis*. The Pliocene ontogeny of *T. europaea* possessed only linear needles but at some time nearer the Pleistocene there appeared in it a new line of cuneate leaves, which was evidently favoured later on by some selective agents, because we meet it frequently in the renovated ontogeny of *Tsuga* from the Interglacial period (Mindel-Riss, Raków), in Central Poland; the same modernized ontogeny of *Tsuga canadensis*, or one very near it in this respect, is dominant till now in North America.

The evolution of the population of the type *T. caroliniana* was quite different. As far as one may judge from the facts described above the population of this species did not undergo any discernable changes, at least from the Middle Pliocene to the Holocene. In connection with this stabilisation of characters its vitality diminished greatly, and as a result we see that *T. caroliniana* in the Atlantic part of N. America is today an example of a species becoming extinct, while in the European Pliocene it was probably equal to *T. europaea* both in its geographical range and its vitality. The inability to renew its tertiary »ontogeny« by producing new genotypes during the Pleistocene has led the present *T. caroliniana* to the stage of diminished vitality.

The above reasoning is based on the fact that in the Pliocene *Tsuga europaea* approached more closely *Tsuga caroliniana fossilis* in its morphology as well as probably in its ecology than the living *Tsuga canadensis* now does in relation to the *T. caroliniana*. It was probably in the Pliocene that the hypothetical primitive *Tsuga* from Miocene which we have mentioned above, was divided into two separate »ontogenies«. In the course of time and the climatic events of the upper Pliocene and the Pleistocene the gap between these two closely ralated populations grew larger and larger. At the present time there are only two species in existence, of which the younger one (*Tsuga canadensis*) has preserved a considerable geographic range and an unweakened vitality, while the older one, (*Tsuga caroliniana*) has been pushed down to the state of a species that is dying out.

As a final remark to the picture of the relations between the recent species of *Tsuga* and their fossil ancestors I wish to express the hope that in future modern paleobotany may consider more comparative biometrical methods in the investigations of the systematic characters of fossil plants, because it is only in this way that this branch of science can get rid of the ballast of names of »new« species which are introduced into it without sufficient reasons. In this way paleobotany can help to solve certain problems concerning the evolution of the contemporary species of plants. It is clear that in relation to the present flora such investigations conducted on the flora of the Tertiary, especially of the middle Pliocene, can be of greatest

value, because in this geological period the evolution seems to have been very rapid. (Axelrod 1948).

IX. Summary

The exceptionally rich fossil material of *Tsuga* which has been found in the deposits of Middle Pliocene at Krościenko (West Carpathian Mountains) presented to the author the opportunity for investigating different morphological and anatomical characters of both the fossil and the living closely related species of *Tsuga* i. e. *T. canadensis* and *T. caroliniana* by means of statistical methods.

From the 17 characters compared some correspond to the data cited by other authors, others differ from them not insignificantly. Among these the breadth of cones and the presence of hypoderma not only in the leaves of *T. caroliniana* but also in those of *T. canadensis* seem to possess a true systematic value. On the other hand the number of rows of stomata gatherd in two bands on the lower side of the blade which was reported to possess systematic importance, the breadth of the midrib of the leaves, and the dimensions of pollen-grains are of minor importance.

The fossil material of *Tsuga* from the Middle Pliocene at Krościenko is composed of two different species one of which stands very near the existent *Tsuga canadensis*, and the other may be regarded as almost identical with *Tsuga caroliniana.*

After a discussion of the difficulties which we meet while giving proper specific names to the fossil species of *Tsuga*, the author proposes to call the fossil type *T.* »*canadensis*« — *Tsuga europaea* (Menzel) *nov. comb.*, and the fossil type *T.* »*caroliniana*« — *Tsuga caroliniana* Engelm. *fossilis.* Perhaps it would be possible to call the latter species *T.* pre-*caroliniana* Engelm., but under the condition that the prefix »pre« does not mean a new specific name.

In the last chapter the author indicates the probable way of evolution in the genus *Tsuga.* This began with a primitive type of *Tsuga* (in the Miocene?) in which many characters were joint which later on underwent the process of differentiation. In the Pliocene this process advanced, but it was only in the Middle Pliocene that there originated the two separate populations:

Tsuga europaea and *Tsuga caroliniana fossilis*. By way of appearance of new characters in the population of *T. europaea* there developed in it the species *T. canadensis* which has been stated for the Pleistocene of Europe. The population *T. caroliniana*, on the other hand, did not undergo any changes and in consequence it has lost its vitality and is now a species that is daying out and possesses only a very limited geographical range.

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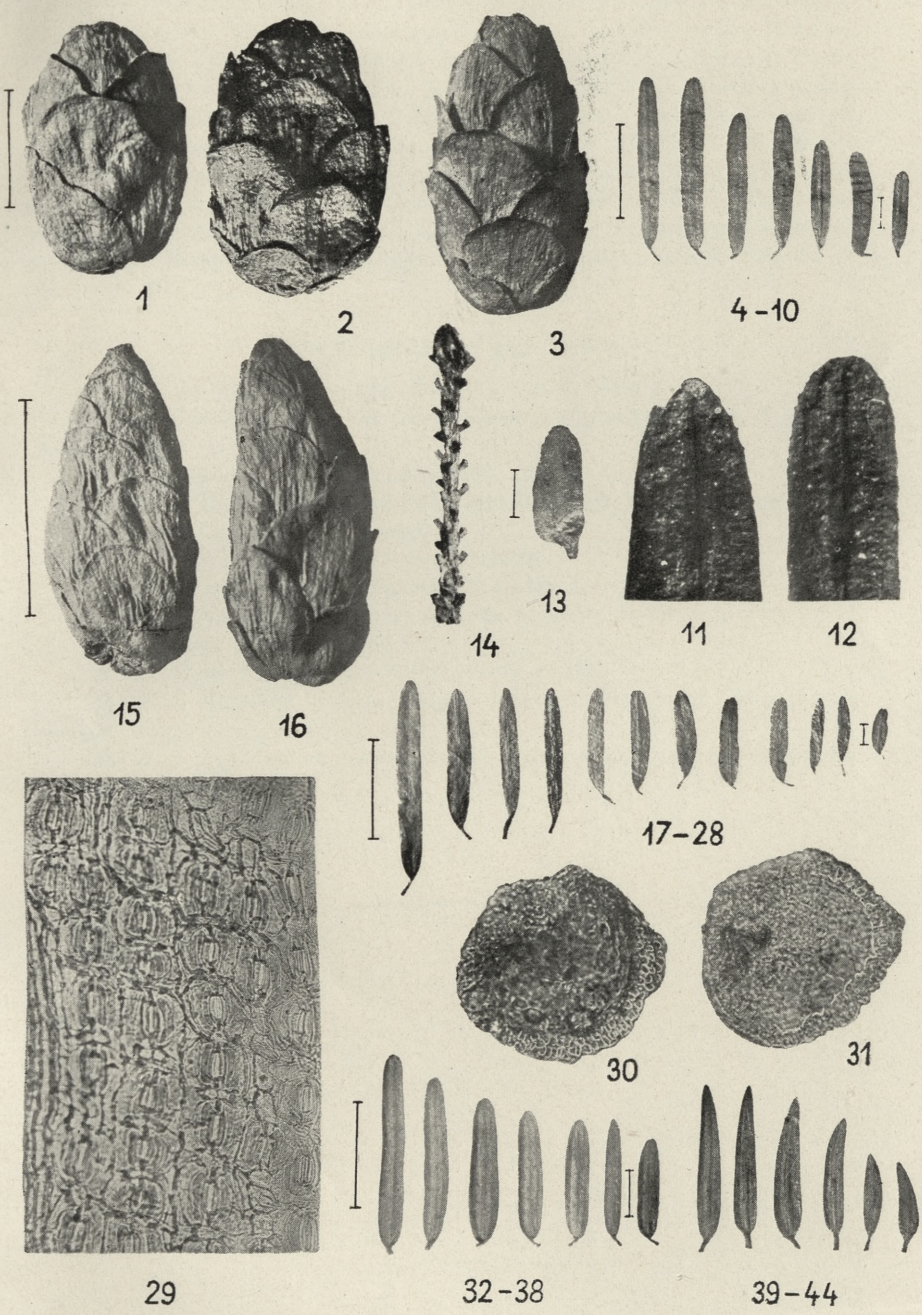

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Explanation of the Table

1 and 2 = *Tsuga europaea* (Menzel) *nov. comb*; cones.

3 = Intermediate form of cone between *T. europaea* and *T. caroliniana* Engelm. *foss.*

4—10 = Leaves of *Tsuga europaea.*

11—12 = Upper part of dentated blades of leaves of *Tsuga europaea.*

13 = Seed of *Tsuga europaea.*

14 = Shoot with bud of *Tsuga europaea.*

15 and 16 = *Tsuga caroliniana* Engelm *foss.*; cones.

17—28 = Variation of leaves of *T. caroliniana* Engelm. *foss.*

29 = *Tsuga europaea*; stomata.

30 and 31 — Pollengrains of the fossile *Tsuga.*

32—38 = Variation of linear shaped leaves of the recent *Tsuga canadensis* Carr.

39—44 = Variation of cuneat shaped leaves of the recent *Tsuga canadensis* Carr.

Studia nad brodawkami korzeniowymi u roślin motylkowych. — Studies on the root nodules of Leguminous Plants.

Mémoire

de M^me^ **A. NOWOTNY-MIECZYŃSKA**

présente le 7 Mars 1949 par M. T. Lityński m. c.

Content

Problem

In 1939 Kubo discovered that in the root nodules of the soya there is a red pigment, which according to him is a homoproteinous compound analogous to the haemoglobin in the blood of vertebrates, performing in the nodules the function of carrier and transferrer of oxygen. In 1945 Virtanen established the presence of pigment of the same type in the root nodules of the pea; and at the present time we know that all leguminous plants inoculated with an active strain of *Rhizobium* contain in their root nodules a homoproteinous pigment which, as Keilin

and Wang (1945) have shown, is not merely a compound »similar« to haemoglobin (nor yet an oxide-reductive catalytic in which Fe alters its value) as Burris and Haas (1945) maintained but true haemoglobin, indentical with the haemoglobin of the blood of vertebrates. Root nodules, so far as present knowledge goes, constitute the only case of the occurence of true haemoglobin in plants, although other haematin compounds such as cytochrome, peroxidase and catalase are widely distributed in the vegetable world.

As is known, two pigments, a red and a green, occur periodically in root nodules: the former in the initial period of plant vegetation, the latter in the final period. Investigations of the red pigment have been carried out principally by Keilin and Wang. The pigment isolated by them by expression from the root nodules of the soya forms with molecular oxygen a reversible compound of oxyhaemoglobin, whose two-striped absorptive spectrum has its maxima at wave-lengths of a-574 mμ and b-540 mμ. The pigment isolated by Keilin forms an impermanent compound with CO, which, like the oxycarbonated haemoglobin of the blood of vertebrates, in an atmosphere devoid of CO dissolves into its components. Under the influence of potassium ferrocyanide Keilin's pigment changes its colour from red to brown. Microspectroscopic examinations of this combination have given a spectrum typical of the methaemoglobin of the blood of vertebrates.

What function is performed by the haemoglobin in the nodules of leguminous plants? Virtanen, when investigating the red pigment in the root nodules of the pea, found that it occurs there principally in the form of methaemoglobin, and on this basis he constructed his theory of the fixation of nitrogen by *Rhizobium*, which he expressed in the following formula: N_2-Methaemg. ($Fe^{\cdots}$) $\rightleftarrows NH_2OH$-Haemgl. ($Fe^{\cdot\cdot}$). According to Virtanen the activity of haemoglobin in the nodules consists of: 1) facilitation of the respiratory activities of bacteria, and 2) oxygenation of N, accompanied by change in the electric charge of the haemoglobin, or in other words its self-oxygenation and transformation into methaemoglobin. The whole process of the assimilation of nitrogen would thus consist in cyclic transformation of the value of the iron contained in the red pigment of the root nodules of leguminous plants.

However, the latest researches of Keilin (1947) have shown that Virtanen's argumentation is based on a false premise, inasmuch as the red pigment in the root nodules of leguminous plants, whether grown in normal conditions or in shade, contains no methaemoglobin at all. Virtanen found this compound, Keilin says, because he was dealing with pigment isolated from the nodules by extraction, during which the haemoglobin was oxygenated under the influence of the quinones formed during the actual process of extraction. According to Keilin, active root nodules exhibit only a mixture of haemoglobin and oxyhaemoglobin. On the other hand the facts that the red pigment is formed only, when leguminous plants are inoculated with an active strain of bacteria of the genus *Rhizobium*, that it is localized only within cells containing symbiotic organisms, and that the process of assimilation of nitrogen is actually checked in the presence of CO, prove that the presence of haemoglobin is closely connected with the whole phenomenon of the assimilation of nitrogen by bacteria. Keilin contents himself with stating these facts, and for the time being puts forward no hypothesis to explain the function of haemoglobin in the fixation of nitrogen, waiting for the fresh results from the continuation of his researches which he promises in his latest reports.

What is the green pigment in root nodules? So far it is principally Virtanen (1947) who has occupied himself with this question. Judging from his most recent publications he has not yet succeeded in solving this problem. According to him the green pigment in the nodules is a mixture of chromoproteins soluble in water with an iron content of 0·28%. He asserts that in the formation of this pigment the decisive part is played by ascorbic acid. His experiments carried out *in vitro* have shown that the haemoglobin and methaemoglobin of the nodules rapidly change from red to green at room temperature if the solution contains ascorbic acid and peroxide of hydrogen. Perhaps, he suggests, at the time of the plant's ripening some substance disappears from the nodules which had previously prevented the change of red pigment into green. This substance might be oxal-acetic acid or some other compound active in the nodules. The question of the formation of green pigment in root nodules and of the part it plays is accordingly still unsettled.

The aim of the present work was:

1) to trace the course of growth of the root nodules of leguminous plants from their first appearance until their final desintegration.

2) to find out the morphology of the nodules of peas and lupines grown on various types of soil and inoculated, e. g. in the case of the lupine, always with the same strain of *Rhizobium lupini.*

3) to investigate the growth of root nodules of peas and lupines in connexion with the growth of their vegetable hosts.

4) to investigate the changes taking place in the nodules under the influence of restriction of the supply of carbohydrates from the green parts of the plant to its roots by: a) shading the plant, b) pruning the parts of the plant above ground, or c) stripping the leaves from the plant-stems.

5) to make an introductory investigation of the effect of solutions of maltose on the changes of pigmentation in root nodules.

Methods

The plants chosen for experimentation were: the (Victoria) pea, the yellow sweet lupine, and the serradilla. The experiments were carried out in Mitcherlich pots in 5, or in 3 types of soil, namely: 1. clean sand several times washed in water and containing only traces of nitrogen. 2. sandy soil (coarse sand containing a large proportion of skeletal parts). 3. typical loess. 4. moderately heavy alluvial-soil. 5. chernozyom soil (from Hrubieszów).

These samples were taken in spring at a depth of about 35 cm.

The basic fertilizers used for the pea, lupine and serradilla were the following: 0·35 gr K_2HPO_4, 0·25 gr KH_2PO_4, 0·30 gr $MgSO_4$ and 0·80 gr K_2SO_4. The washed sand received in addition 10 mg $FeCl_3$, 8 mg H_3BO_3, 0·2 mg $CuSO_4$, 2 mg $MnSO_4$ and 10 mg N in potassium nitrate to cover the needs of the plants in respect of that element in their first stage of growth. The pea received besides 7 gr $CaCO_3$ per pot, and the lupine and serradilla 2 gr each of the same. Considering the high pH of the alluvial soil it was given no lime fertilizer either in the experiment with the pea or with the lupine. The plant seeds

were inoculated with cultures of *Rhizobium* bacteria: for the pea *Rh. leguminosarum* No. 1, for the lupine *Rh. lupini* strain. Cz, and for the serradilla *Rh. lupini* 5 (obtained from the Department of Agricultural Microbiology in the Puławy Institute). In each pot (containing 10 kg of sand) grew 5 plants of lupine and 5 of pea.

For the observations of the growth of the root nodules 20 plants were taken out every 10 days, and immediately after their roots had been carefully washed their shape was sketched and the distribution and shade of the pigment in the nodules were noted. For the observations of the nodules a small pocket microscope of 60-fold magnification was used.

Investigations into the yield of the peas, measured by their dry mass, and into the nitrogen content of the plants were carried out 4 times during the vegetation period. Similar investigations in respect of the lupines were carried out 3 times in the vegetation period. Both lupines and peas were sown on May 12. The peas, gathered in the final stage of vegetation (on July 31) had their fruit quite ripe, but the seeds of the lupines gathered on August 3 were not yet quite ripe.

Development of root nodules of leguminous plants

Peas

As we have indicated above, one of the aims of our work was to trace the development of the root nodule of the pea from its first appearance to its disappearance, with special attention to the changes of pigmentation in the nodules as they develop. Parallel with these investigations, observations were made and recorded of the growth of the host-plant itself. It was found that the life-period of peas, i. e. the time from their sowing until the ripening of their seeds, amounted in our experiment to about 11 weeks, while the growth-period of a nodule on their roots was nearly 6 weeks.

Nodules of peas (Fig. 1) are pear- (or acorn-) shaped and occur, particularly in the first period of vegetation, only singly, on the upper part of the top root of the host, or thereabouts. The colouring of the young nodules is pale rose, but as the nodule grows it becomes gradually darker. The pigment fills

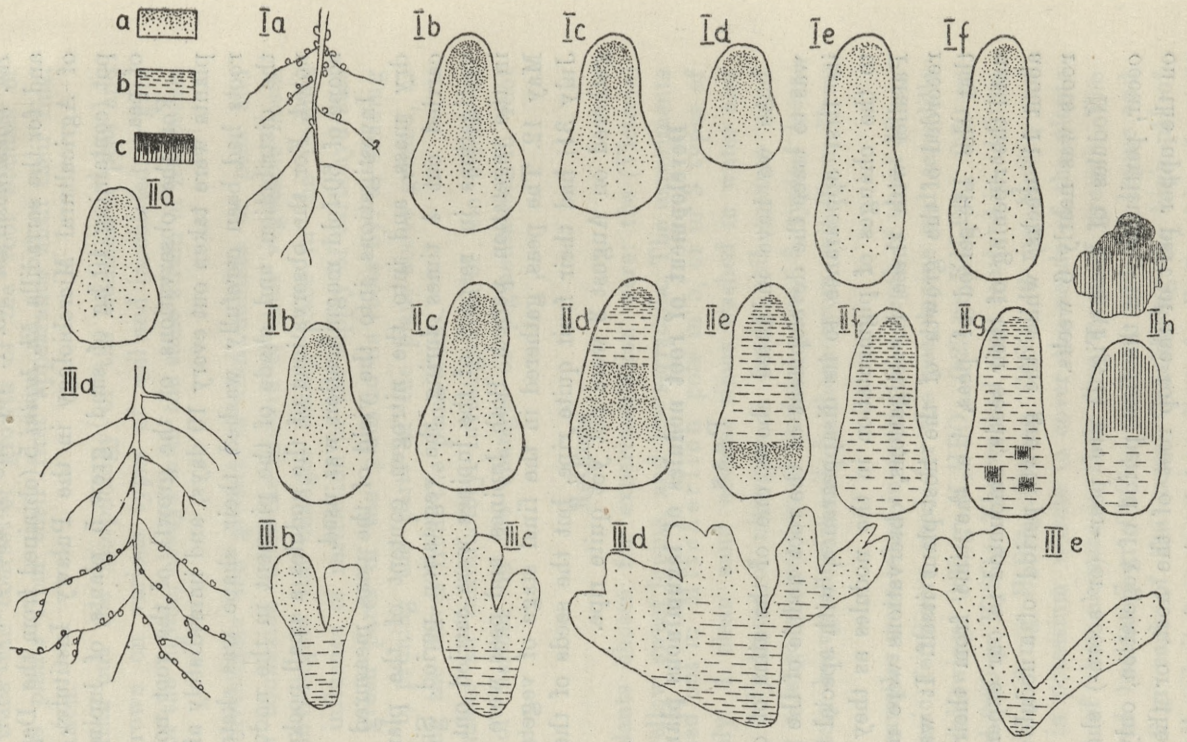

Fig. 1. Root nodules of peas.

Ia — the primary nodules, Ib, — growing on pure sand, Ic — on sandry-soil, Id — on alluvial soil, Ic — on loess soil, If — on chernozyom soil, II, — nodules in different periods of plant growth, IIa — early period, IIb — before flowering, IIc — flowering period, IId, e — end of flowering, IIf, g — pods formation, IIh — the decay of nodules, III — secondary nodules, IIIa — secondary nodulation, IIIb, c — on sandy soil, IIId — on loess or chernozyom soil, IIIe — on alluvial soil, a — red pigment, b — green pigment, c — the brown colour.

approximately half the nodule and the intensity of its colour is greatest at the top of the nodule. In the period preceding florescence the rose colour changes to blood-red and now takes up about $\frac{3}{4}$ of the capacity of the nodule. In the period between the 37th and 51st days of the life of the nodule, approximately in the 6th week of the plant's growth, i. e. during florescence, the intensity of the red colour attains its maximum, the weight of the dry mass of the nodules increases at this time about 100%, and simultaneously the quantity of symbiotically assimilated nitrogen doubles (Table I, III). A fortnight later the majority of pea-nodules are already dead and are easily detached from the roots, especially while the plants are being washed. The florescence of the host is the turning-point in the development of the nodule: at this period another pigment makes its appearance in it, and is green. It does not mix with the red pigment, but slowly and gradually drives it from the upper part of the nodule to the bottom. Presently the green pigment occupies the whole volume of the nodule, the greatest intensity being at its narrowest point. This is the period in which the nodules begin to wither: they become covered with increasingly numerous bronze-coloured spots, and at the same time shrink, lose their substance, and finally fall off the roots. Nevertheless the assimilatory activity of the symbiotic bacteria does not cease: if the substratum in which the leguminous plant is growing is still rich in nutritive elements, new nodules constantly appear, but now only on the secondary roots, on the freshest rootlets. Moreover, they are no longer single nodules, but smaller or larger groups, of a greenish-rose colour, turning green, then bronze, and finally dying. These nodules we have called »secondary«. It is possible that on other varieties of pea the distribution of the nodules will be different. It should be pointed out that these latter nodules have only greenish-rose pigmentation (Fig. 1); no quite rose-coloured nodules have been found among the »secondary« ones.

Lupines

The nodulation of the lupine is different in character from that of the pea. The bacteria attacking the root tissue of the plant penetrate between the epidermis of the axial cylinder and the endodermis of the primary bark, and as a result excrescences

are formed which encircle the plant's roots. If a cross-section is taken through the nodule, »nests« of bacterioid tissue of varying dimensions are clearly visible, separated from one another by walls (Fig. 2).

The growth phases of the nodule of the lupine are in principle similar to those which we have observed in pea-nodules. In the cross-section of a young lupine nodule we see rose-colouring, which darkens as the plant grows. The annularly united nodules multiply along the tap-root and slowly thicken. At the period of florescence of the host the nodules already show a blood-red colouration. When the host-plants fade, green pigment appears in the nodules, advancing from the primary skin of the root towards the centre of the nodule in the direction of the axial cylinder and, again without combining with the red pigment, gradually pressing forward until at the period of the formation of pods by the host it occupies the whole capacity of the »nests« of the nodule. Shortly afterwards, much as we have already observed in the case of pea-nodules, the green pigment within the nodules changes to brown, and the whole nodule shrinks, wastes away, loses its pulpiness and finally undergoes decomposition. At the period of the host-plant's withering, the root nodule of the lupine may easily and without damage be lifted off the root, and then it can be seen how the axial cylinder (Fig. 2) of the root has been deformed under the pressure of the nodule, which encircled the axial cylinder like a hoop. Immediately after the removal of such a nodule clear blood-red points (Fig. 2) can be seen on the cylinder.

No formation of »secondary« nodules was observed. On the contrary, it was found that the period of growth of the lupine nodule is approximately three weeks longer than that of the pea nodule. At the time when the »early« nodules of the pea had already withered those of the lupine were in full growth, having only reached the stage of red colouration.

Serradilla

The serradilla was grown on pure quartz sand in small earthen-ware pots each holding $1\frac{1}{2}$ kilogrammes of sand, with 10 plants to a pot. The addition of fertilizers to the sand was

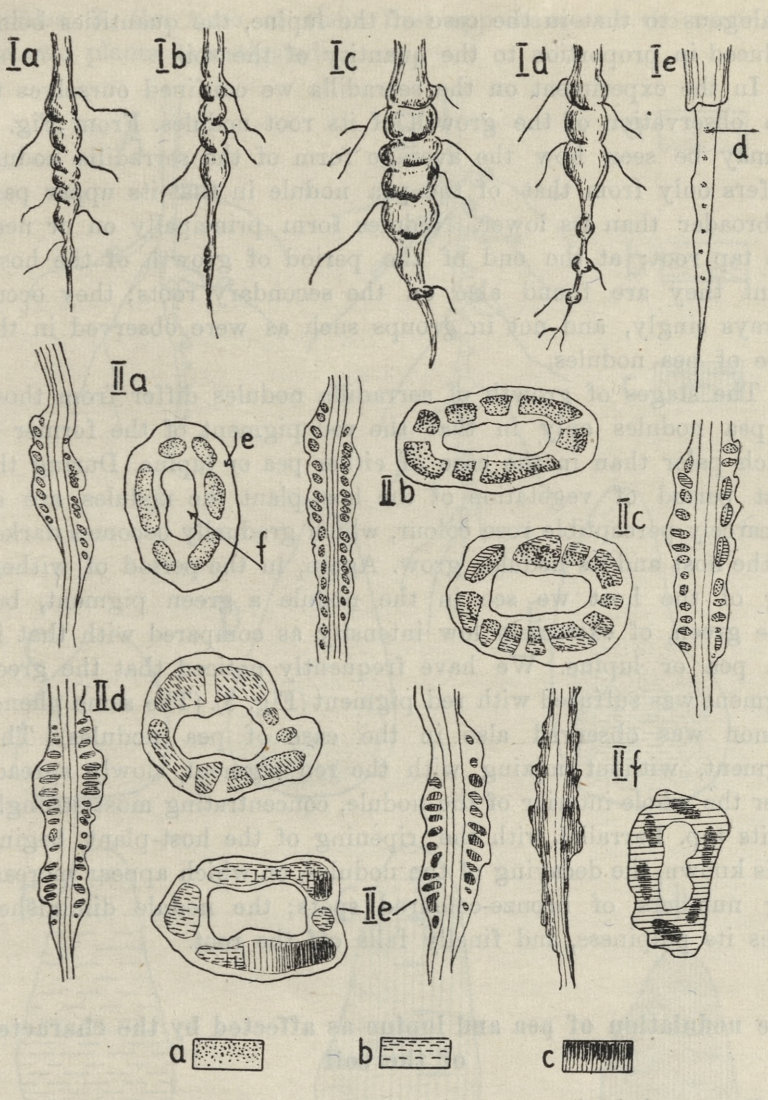

Fig 2. The root nodules of yellow (sweet) lupine.
Ia — growing on pure sand or sandy soil, Ib — on alluvial soil, Ic — on loess soil, Id — on chernozyom, Ie — the root liberated from the nodules, II — the vertical and horizontal sections of root of yellow lupine in different periods of growth. IIa — before flowering, IIb — the flowering period, IIc — the end of flowering period, IId — the pods formation, IIe — the maturity, IIf — the decayed nodules. a — red pigment, b — green pigment, c — the brown pigment, d — haemoglobin spots, e — epidermis, f — endodermis.

analogous to that in the case of the lupine, the quantities being reduced in proportion to the quantity of the soil.

In the experiment on the serradilla we confined ourselves to the observation of the growth of its root nodules. From Fig. 3 it may be seen how the average form of the serradilla nodule differs only from that of the pea nodule in that its upper part is broader than its lower. Nodules form principally on or near the tap root; at the end of the period of growth of the host-plant they are found also on the secondary roots; they occur always singly, and not in groups such as were observed in the case of pea nodules.

The stages of growth of serradilla nodules differ from those of pea nodules only in that the red pigment of the former is much paler than in the case of either pea or lupine. During the first period of vegetation of the host-plant the nodules are of a scarcely-perceptible rose colour, which gradually becomes darker as the host and its nodules grow. Again, in the period of withering of the host we see in the nodule a green pigment, but pale green, of extremely low intensity as compared with that in the pea or lupine. We have frequently noticed that the green pigment was suffused with red pigment (Fig. 3). (The same phenomenon was observed also in the case of pea nodules). This pigment, without mixing with the red pigment, slowly spreads over the whole interior of the nodule, concentrating most strongly at its top. Parrallel with the ripening of the host-plant begins, as is known, the decaying of the nodules, on which appear increasing numbers of bronze-coloured spots; the nodule diminishes, loses its pulpiness, and finally falls off the root.

The nodulation of pea and lupine as affected by the character of the soil

Peas and lupine were grown on 5 different types of soil: 1) on washed sand, 2) on light sandy soil, 3) on loess, 4) on chernozyom soil and, 5) on alluvial soil.

When observing the development of the nodulation of the pea and lupine we found certain differences dependent on the type of soil on which the host-plant was growing, in relation to: 1. the intensity of the red pigmentation, 2. the size of the

nodules, 3. the time of their first appearance on the roots of the two plants investigated.

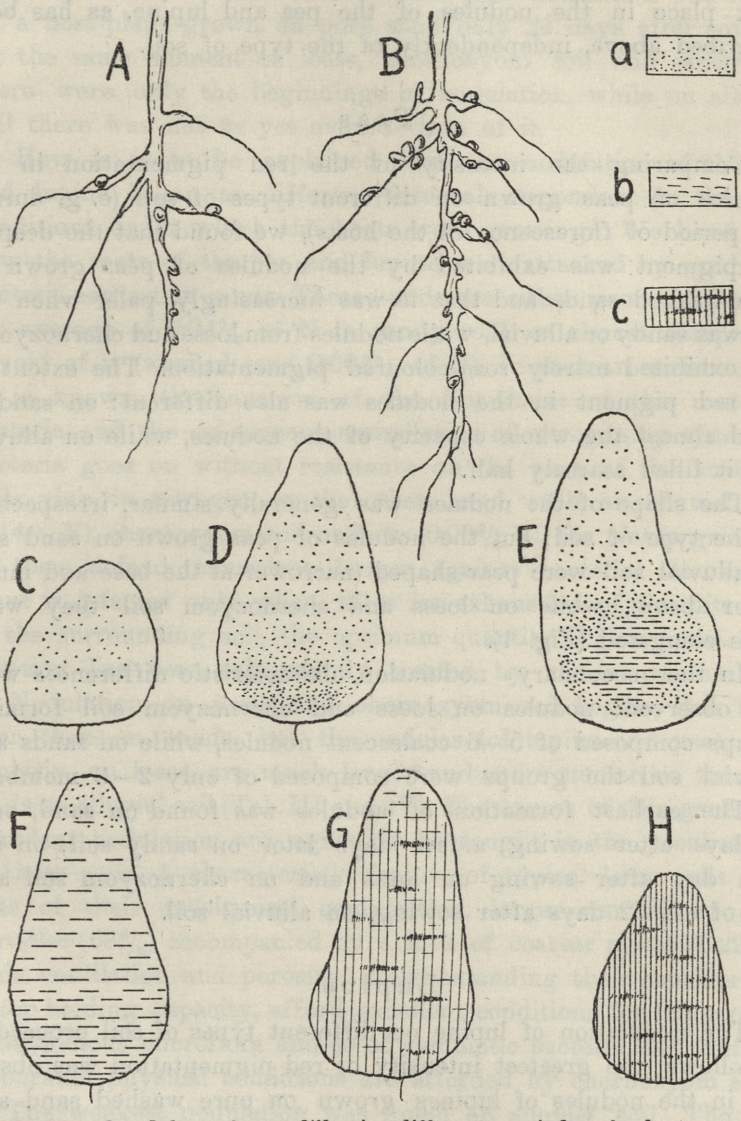

Fig. 3. Root nodules of seradilla in different periods of plant growth. A — early period of plant growth; B — end of flowering, C — cross section of nodule before flowering, D — in the flowering period, E — at the end of flowering, F — at the time of pods formation, G — at the maturity, H — nodule in decay. a — red pigment, b — green pigment, c — the brown colour.

The pigmentation of the nodules, i. e. the reinforcement of the intensity of the red colouring and its change into green, took place in the nodules of the pea and lupine, as has been described above, independently of the type of soil.

Peas

Comparing the intensity of the red pigmentation in the nodules of peas grown on different types of soil (e. g. during the period of florescence of the hosts), we found that the deepest red pigment was exhibited by the nodules of peas grown on pure washed sand, and that it was increasingly paler when the soil was sandy or alluvial, while nodules from loess and chernozyom-soil exhibited merely rose-coloured pigmentation. The extent of the red pigment in the nodules was also different: on sand it filled almost the whole capacity of the nodules, while on alluvial soil it filled scarcely half.

The shape of the nodules was generally similar, irrespective of the type of soil; but the nodules of peas grown on sand and on alluvial soil were pear-shaped (narrower at the base and much wider above), while on loess and chernozyom soil they were more elongated (Fig. 1).

In the »secondary« nodulation characteristic differences were also observed: nodules on loess and chernozyom soil forming groups composed of 5—6 coalescent nodules, while on sands and alluvial soil the groups were composed of only 2—3 members.

The earliest formation of nodules was found on sand, only 17 days after sowing; a few days later on sandy soil; on the 28th day after sowing on loess and on chernozyom soil and last of all, 32 days after sowing, on alluvial soil.

Lupine

The nodulation of lupine on different types of soil proceeded as follows: the greatest intensity of red pigmentation was observed in the nodules of lupines grown on pure washed sand and on loess, on the three other types of soil the colouration inside the nodules being rose. The number and breadth of the nodule »rings«, on the other hand, was greatest in the case of lupines grown on loess, and then successively on chernozyom soil and

on sands, while the weakest nodulation was exhibited by lupines growing on alluvial soil. The earliest clear nodulation was found on a host-plant grown on pure sand only 24 days after sowing. At the same moment on loess, chernozyom soil and sandy soil there were only the beginnings of nodulation, while on alluvial soil there was not as yet even a trace of it.

How is it to be explained that the nodulation of the pea and lupine began at different intervals dependent on the soil-conditions under which the hosts were growing? We have seen how the roots of the pea and lupine were attacked by symbiotic bacteria earliest on sands. These sands are either poor in nitrogen (an average of 0·04% of N in sandy soil) or almost completely devoid of it (washed sand 0·003% of N). In such an environment, as is known, the invasion of the fine hairs of the roots by bacteria and the subsequent assimilation of nitrogen by symbiotic bacteria goes on without resistance on the part of the host. In soils rich in nitrogen, on the other hand, such as alluvial soil (0·14% N), chernozyom soil or loess (0·09% N), the plants growing on them defend themselves longer against bacterial attack; the plant is infected only when, after its exhaustion of the nitrogen in the surrounding soil, the optimum quantity C:N is upset. This question has been exhaustively treated by various researchers.

Nodulation on loess and chernozyom soil begins, it is true, later than on sands, but the nodules (of lupine and pea), particularly on loess, are much larger and more numerous than on sands or alluvial soil (Tab. III and IV). The causes of this generally abundant nodulation are, again, to be sought in the peculiarities of these soils. A characteristic feature of typical loess soils consists of their mechanical composition, larger number of dust particles (63%) accompanied by a lack of coarser pieces (0·29%); their ventilation and porosity, notwithstanding their considerable water-holding capacity, afford excellent conditions for the growth of oxygenous microflora and so of symbiotic bacteria also. Similar favourable physical conditions are afforded by chernozyom soils.

The weakest nodulation was found on alluvial soil. The dry mass of the root nodules of pea and lupine is least on such soils. The alluvial soil on which the peas and lupines were grown was of medium heavy type. These soils, with their high percentage (about 50%) of loamy constituents and their comparatively

small percentage (29%) of dusty constituents, are in general badly ventilated and permeable with difficulty, and therefore do not always offer suitable conditions for the favourable growth of microflora, while further their high percentage of nitrogen (0·14%) and their high pH constitute factors which, in the conditions of our experiment, hampered and delayed nodulation both in the pea and lupine and influenced for the worse the general growth of these plants, and particularly of the lupine.

How is it to be explained that the nodules of leguminous plants grown in various soil conditions exhibited varying quantities of red pigment? It seems to us that the causes of this phenomenon, again, are to be sought in the varying peculiarities of the substratum on which the plants in our experiments were grown. For we see that the factors which encouraged or hampered the growth of root nodules on peas produced similar effects in the case of lupines.

As may be seen from Table I peas (from 1 pot) grown on sand assimilated in the course of the first 5 weeks of vegetation about 327 mg. of nitrogen, or about as much as peas grown on loess (292 mg), and considerably more (100 mg N) than peas grown on alluvial soil. At the same moment the dry mass of pea nodules from 10 plants grown on sand weighed only 0·28 gr (Tab. III), and nodules from loess 0·41 gr, or about 40% more; but the nodules from sand at this time exhibited a blood-red colouring, while those from loess were only rose-coloured. Comparing the total quantity of nitrogen (Tabl. I) assimilated during the course of the whole vegetation period by peas grown on sand (1380 mg N per pot) and by peas grown on loess (1450 mg N), we see that these values are approximately equal, or in other words that on loess a large mass of nodules with weak intensity of red pigmentation performed the same function of assimilating nitrogen as a smaller mass of nodules on sand, with a stronger intensity of red pigment.

In the case of peas grown on alluvial soil a smaller dry mass of nodules was found with colouration somewhat stronger than on loess, but localized at the head of the nodule. The paler coloured, smallest and least numerous nodules (Tables I and III) of the peas grown on alluvial soil gave the smallest amount of nitrogen (per pot).

In lupines the nodulation presented a somewhat different appearance: the root nodules of lupines grown on loess had as strong an intensity of red pigment as the nodules of lupines on sand. They dry mass (Table II) of the root nodules of lupines grown on sand, in the 3 first periods of vegetation of the hosts investigated by us, was greater than that of the nodules from loess; yet in the final stage of vegetation of the hosts (after 84 days) the root nodules of lupines from sand were already withering. Nodules from loess still exhibited an increase of dry mass in comparison with the preceding period (70 days). From Table II we see that the quantity of nitrogen assimilated during the whole vegetation period by those plants which were grown in these different soil conditions was approximately the same.

The nodulation of lupines on alluvial soil was the weakest in all 3 periods of growth of the hosts. The dry mass of the nodules was the lowest and the intensity of the red colouration was considerably weaker than that in nodules from loess or sand. Plants grown on alluvial soil also assimilated the smallest quantity of free nitrogen.

We know from the literature of the subject that the intensity of nodulation is (among many other factors) a function of the number of bacteria which have attacked the root tissue of a leguminous plant. The excellence of the co-operation between bacterium and host is measured by the quantity of nitrogen assimilated by them together. One of the conditions affecting both the numerical development and the activity of the bacteria is an environment favourable to their development. In our experiment a favourable environment for the bacteria of the lupine and pea consisted of loess and sand reinforced by a well-balanced feed of minerals. Under such conditions the symbiotic microorganisms produced either a large mass of nodules with a low intensity of red pigment, or a smaller mass rich in this pigment. These two differing types of nodulatory embellishment assimilated approximately the same quantity of nitrogen during the whole period of vegetation of the host-plants. From the above considerations it would appear that the activity of symbiotic bacteria is proportionate to the qnantity of red pigment in the root nodules. The smaller or larger production of the pigment by the plant is connected in its turn with the conditions of its substratum.

Nodulation of the roots of lupine and pea as it affects the assimilation of nitrogen

In Tables I and II we have presented the nitrogen production of the lupine and pea in different phases of their growth; in Tables III and IV are the numerical results depicting the actual growth of root nodules on these plants. It is apparent that the quantity of nitrogen symbiotically assimilated by the plants is proportionate to the quantity of dry mass of their root nodules in the various stages of growth of these plants, alike in the case of peas and in that of lupines.

Peas

In the period between the 37th and 51st days (Tab. III) in the life of a pea the dry mass of its root nodules per pot increases by about 100%, the nitrogen productiveness of the plants doubles, and the general harvest of the plant increases approximately in the same proportion. At the same time, however, we observe that the red colouration of the root nodules of the plants, irrespective of the soil on which they are growing, be it sand, loess or alluvial soil becomes increasingly intensive (Fig. 1).

In the next vegetation periods of the pea (28 days) a diminution is found in the increase both of the dry mass of the plants and of their nitrogenous substances. Approximately in the 8th week of plant-growth the majority of the root nodules (especially on sand) have greenish-red or wholly green pigmentation, whereas on loess and alluvial soil an insignificant quantity of nodules are still uniformly red. A fortnight later some of the nodules are already in process of disappearing, easily falling off the roots, and so the determination of the quantity of their dry mass was discontinued (so that the significance of the results might not be interfered with). It is apparent, then, that the period between the 37th and 51st day of the plant's life is that of the most energetic development of the plant as a whole. This period, in our experiment, coincided with the florescence of the plants, and in it, according to our observations, the intensity of the red pigment in the nodules reaches its maximum, and the assimilation of free nitrogen is greatest. From the moment when the green pigment gradually begins to take the place of the red, the symbiotic assimilation of free nitrogen diminishes, and it ends when

TABLE I

Pea (»Victoria« peas): dry matter and N content per pot at different periods of growth.

Time of sowing: 11. V Time of harvesting	Washed sand pH = 7·60 % N = 0·00				Loess-soil pH = 7·59 % N = 0·09				Alluvial-soil pH = 7·90 ½ N = 0·14				Remarks:
	dry matter g	increase in p. c. (14 days)	N mg	increase in p. c. (14 days)	dry matter g	increase in p. c. (14 days)	N mg	increase in p. c. (14 days)	dry matter g	increase in p. c. (14 days)	N mg	increase in p. c. (14 days)	
17. VI. (37 days)	9·8 ±0	—	327	—	12·4 ±1·41	—	292	—	10·8 ±0·79	—	227	—	Bud formation
3. VII. (51 days)	26·2 ±1·98	167	744	127	25·7 ±2·75	107	757	159	26·9 ±0	150	574	152	Flowering period
17. VII. (65 days)	43·2 ±7·48	65	1223	65	49·1 ±5·04	91	1447	91	37·4 ±0	39	988	72	Pod formation
31. VII. (79 days)	53·2 ±2·47	23	1383	13	53·0 ±6·1	7	1445	0	39·9 ±2·3	6	1102	11	Ripening

TABLE II

Lupine (sweet, yellow): dry matter and N content per pot at different periods of growth.

Time of sowing: 11. V. Time of harvesting	Washed sand pH = 7·60 % N = 0·00				Loess-soil pH = 6·70 % N = 0·09				Alluvial-soil pH = 7·90 % N = 0·14				Remarks:
	dry matter g	increase in p. c. (14 days)	N mg	increase in p. c. (14 days)	dry matter g	increase in p. c. (14 days)	N mg	increase in p. c. (14 days)	dry matter g	increase in p. c. (14 days)	N mg	increase in p. c. (14 days)	
6. VII. (55 days)	27·4 ±2·66	—	1020	—	23·9 ±3·53	—	847	—	9·6 ±0·84	—	307	—	Bud formation
22. VII. (70 days)	54·4 ±2·57	98	1550	51	48·9 ±6.30	104	1514	85	19 3 ±0	100	625	103	End of flowering
3. VIII. (84 days)	69·4 ±2·55	27	1590	2	61·4 ±3.1	25	1761	16	29·1 ±5·11	50	820	31	Ripening

there is a complete absence of red pigment in the root nodules. During the last fortnight before the ripening of the plants the function of further assimilation of nitrogen is taken over by the »secondary« nodules growing upon the newest secondary roots. These nodules, irrespective of the type of soil, are exclusively pale rose-green in colour. As may be seen from Table I the quantity of nitrogen assimilated during the final vegetation-stage of the plants, by the mediation of the »secondary« nodules, is minute.

Lupines

The growth of the lupine was investigated only in three vegetation-periods: 1) before florescence, 2) after the cessation of florescence, and 3) when the pods were ripening. In the period between the 55th and 70th days of vegetation of the lupine the dry mass of the plants increases by almost 100%, and the same figure denotes the increase in general nitrogen-content on loess and alluvial soil, whereas on sand it is only 50%; the dry mass of the root nodules meanwhile increases, on loess and loam, three-fold, but less on sand: not much more than two-fold.

The florescence period of the lupine falls only in the 9th week of vegetation of the plants (i. e. between the 55th and 70th days of their growth). At this same time we observe the greatest concentration of red pigment in the nodules (most intense on sand and loess, much weaker on alluvial soil) and note the greatest assimilation of nitrogen; in the following period (70th—84th day) the nitrogen-fixation gradually diminishes, and simultaneously the red pigment begins to disappear from the nodules, and the green pigment appears. The retreat of the red pigment before the green takes place in the root nodules of the lupine more slowly than was observed in the case of the root nodules of the pea: it is only about the 80th day of vegetation that the nodules of the lupine (especially on loess and alluvial soil) are entirely green, while on sand they are partially disintegrated. By this time all the »secondary« nodules on peas have already withered.

If then the close connexion between the presence of haemoglobin in the root nodules of leguminous plants and the nitrogen-fixing activity of their symbiotic bacteria has been established beyond all doubt by Keilin and Virtanen, then the above-described observations made on the pea and lupine have added

TABLE III

The root nodules of peas (»Victoria« peas) at different periods of development
Dry matter and ash content of nodules per 10 plants.

Time of sowing: 11. V. / Time of harvesting	Washed sand pH = 7·60 N% = 0·00			Loess-soil pH = 7·59 %N = 0·09			Alluvial-soil pH = 7·90 %N = 0·14			Remarks:
	dry matter g	increase in p. c. (14 days)	ash mg	dry matter g	increase in p. c. (14 days)	ash mg	dry matter g	increase in p. c. (14 days)	ash mg	
27. V. (17 days)	0·03	—	3·43	—	—	—	—	—	—	
17. VI. (37 days) Before flowering	0·28	—	37·80	0·41	—	54·50	0·155	—	12·90	The nodules are red
3. VII. (51 days) Flowering period	0·59	96	70·23	0·85	106	75·90	0·46	196	35·23	The nodules are: green-red

TABLE IV

The root nodules of lupine (sweet, yellow) in different periods of growth.
Dry matter and ash of nodules per 10 plant.

Time of sowing: 11. V. / Tim of harvesting	Washed sand pH = 7·60 %N = 0·00			Loess-soil pH = 7·59 %N = 0·09			Alluvial-soil pH = 7·90 %N = 0·14			Remarks:
	dry matter g	increase in p. c. (14 days)	ash mg	dry matter g	increase in p. c. (14 days)	ash mg	dry matter g	increase in p. c. (14 days)	ash mg	
4. VI. (24 days)	0·18	—	14	—	—	—	—	—	—	
6. VII. (55 days) Bud formation	0·72	300	68	0·40	—	30	0·17	—	15	The nodules are red
22. VII. (70 days) End of flowering	1·68	133	118	1·23	207	136	0·52	206	36	The nodules are green-red
3. VIII. (84 days) Ripening	—	—	—	2·05	66	274	0·84	60	65	The nodules on sand are desintegrated

a further argument in its favour. The *Rhizobium* bacteria assimilate only in the presence of haemoglobin in the root nodules. Between the quantity of this pigment in the nodules (measured in our experiment only by observation of the increasing intensity of the red pigment), and the quantity of nitrogen combined, there is a positive correlation. Simultaneously with the dissappearance of haemoglobin from the root nodules the symbiotic fixing-power of nitrogen gradually declines. From the moment when only green pigment is to be found in the nodules, the assimilation of free nitrogen ceases. The above observations therefore appear to confirm Virtanen's hypothesis (1947) concerning the existence of interdependence between the quantity of nitrogen assimilated and the quantity of haemoglobin in the nodules.

We should like to draw attention to a further detail observed in the course of our experiment: incinerating the root nodules of pea we found that the ash of the nodules of peas grown on sand had a uniformly rusty colour, and with careful incineration the ash preserved the shape of the nodules, on the skeletons of which deep-rust-coloured round spots were visible. The ash of nodules coming from loess soil was of a light-yellow colour. That from alluvial soil was of a deeper yellow.

The pigmentation of root nodules and photosynthesis

When dealing in the preceding sections with the increase of mass and development of pigmentation of root nodules during the course of the whole vegetation period of the pea, lupine and serradilla, we drew attention to the fact that at a certain phase in the growth of these plants (in the case of the pea and serradilla during the period of florescence, and in the case of the lupine somewhat later) the red pigment changes to green all at once (without the appearance of any intermediate colour between them), the pigments being sharply divided from one another in the nodule as though by an invisible partition. This separation is particularly clear in the nodules of the pea and serradilla, where we have frequently observed that the green is, as it were, suspended within the red (fig. 3E, 4,Ic). A bronze colour appears only during the period of the plant's fading, i. e. in the period of the ripening of the pods and the dying away of the nodules. This question we decided to investigate further, by observing

what changes take place in the pigmentation of the nodules under the influence of changes in their nutrition, when: 1) the plants are shaded, 2) the parts of the plant above ground are

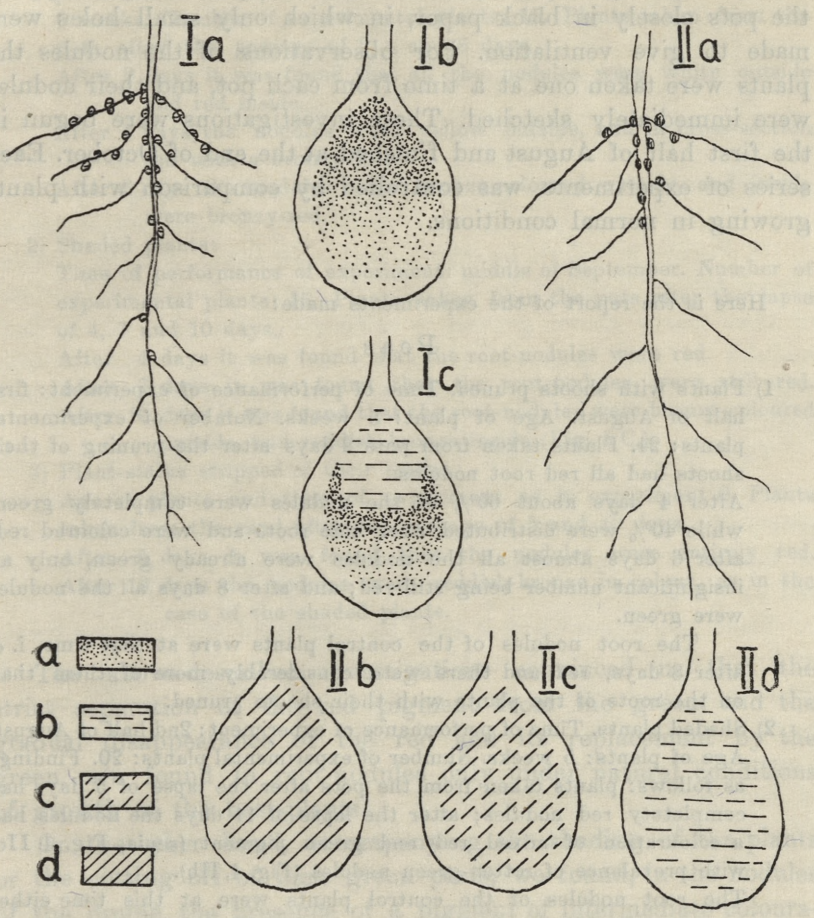

Fig. 4. Effect of shading on the root nodules of pea in the time before flowering

Ia, b, c, — the nodules of pea growing in light, IIa, b, c, d — the nodules of pea after the plant stayed in the dark for 11 days, a — red pigment, b — green pigment, c — the yellow-green colour, d — the reddish colour.

pruned (down to soil level), 3) or the plant-stalks are stripped of leaves.

The experimental pea and lupine plants were grown (5 in a pot) in a greenhouse on clean washed sand. They were inocu-

lated with an active culture of *Rhizobium* and strengthened by the addition of mineral food as described on p. 56 above.

The shading of the plants was accomplished by wrapping the pots closely in black paper, in which only small holes were made to give ventilation. For observations of the nodules the plants were taken one at a time from each pot, and their nodules were immediately sketched. These investigations were begun in the first half of August and finished at the end of October. Each series of experiments was controlled by comparison with plants growing in normal conditions.

Here is the report of the experiments made:

Peas

1) Plants with shoots pruned. Time of performance of experiment: first half of August. Age of plant: 5 weeks. Number of experimental plants: 24. Plants taken from pots 2 days after the pruning of their shoots had all red root nodules.
After 4 days about 60% of the nodules were completely green, while 40% were distributed over side roots and were coloured red; after 6 days almost all the nodules were already green, only an insignificant number being still red; and after 8 days all the nodules were green.
The root nodules of the control plants were at that time, i. e. after 8 days, red and there were considerably more of them than on the roots of the plants with their shoots pruned.
2) Shaded plants. Time of performance of experiment: 2nd half of August. Age of plants: 5 weeks. Number of experimental plants: 20. Findings as follows: plants taken from the pots after the lapse of 5 days had completety red nodules; after the lapse of 11 days the nodules had a colouration of mixed red and green pigment (sepia, Fig. 4, IIc) with prevalence of rotten-green nodules (Fig. 4, IIb).
The root nodules of the control plants were at this time either completelely red or green-red (base of the nodules red, top green) (Fig. 4, Ib, Ic).
The dry mass of nodules of 5 plants shaded for 10 days weighed 0·053 g; from 5 control plants 0·172 g (or 3 times as much).
3) Plant-stalks completely stripped of leaves. Time of performance of experiment: second half of August. Age of plants experimented upon: 5 weeks.
It was found that after the lapse of 10 days the nodules had a green, or more frequently rotten-green, colouration, or else bronze-red. It is apparent that the effect of the removal of the leaves was quite similar to that obtained in experiment 2 (shading of the plants).

Lupines

1) Portion of plant above ground pruned to ground level. Time of performance of experiment: first half of September. Age of plants: 6 weeks. Number of experimental plants: 15. Plants taken from the pots after the passage of 2, 4 and 6 days.
   After 2 days it was found that all the nodules were white outside and red inside.
   After 4 days the nodules were yellow outside, and in cross-section red with a bronzy hue.
   After 6 days the nodules were bronze-coloured outside, and inside were bronzy-red.
2) Shaded plants:
   Time of performance of experiment: middle of September. Number of experimental plants: 15. Plants taken from the pots after the lapse of 4, 7 and 10 days.
   After 4 days it was found that the root-nodules were red.
   After 7 days it was found that the root-nodules were still red.
   After 10 days it was found that the root-nodules were bronze-coloured outside, and reddish-bronze inside (Fig. 5 C.).
3) Plant-stalks stripped of their leaves.
   Age of plants and time of experiment as in experiment 2. Plants taken from the sand after the passage of 5 and 10 days.
   After 5 days it was found that the nodules were entirely red.
   After 10 days the nodules were reddish-bronze in colour, as in the case of the shaded plants.

The above-described investigations convinced us that the strict separation of the red pigment from the green, and the gradual disappearance of the red and its replacement by the green, are found in root-nodules only under natural conditions of growth of the host-plants.

On the other hand, accompanying the shading of the plants or the cutting off of their green parts, we found, in the nodules of the lupine, the presence of a pigment of intermediate colouration between green and red (sepia).

In the nodules of pea (after the plants had been shaded, or stripped of young shoots) we found as a rule a rotten-green colouration.

Differences were, however, observed in the rapidity of the changes which took place: plants which were shaded or deprived of leaves retained the normal pigmentation in their nodules from 2 to 4 days longer than plants whose parts above ground were drastically pruned.

The cause of this was probably the fact that when the young shoots of the plant were cut off the supply of carbohydrates to the roots was also suddenly cut off; but when the plants were shaded the interruption of the supply of carbohydrates to the roots took place slowly and gradually, and the nodules were still drawing upon the stores of carbohydrates in the plant. Only when these were exhausted did they enter that phase of pigmentation which the nodules of the other plants entered immediately after their parts above ground were cut off. These are, however only conjectures requiring confirmation by detailed investigation.

It should, however, be emphasized once again that the nodule-pigmentation observed in the case of plants shaded or deprived of their parts above ground is different from that of the root nodules of such leguminous plants as slowly terminate their vegetation after passing through all the normal phases of their growth.

The nodules of plants shaded by us were still in the full course of nitrogen-fixing activity, whereas in the case of ripening plants they had already completed their assimilation. The colour of these latter was distinctly brown, without any admixture of red or green.

From our experiment it may be concluded that the synthesis of haemoglobin in the root nodules goes on in the presence of *Rhizobium* bacteria so long as there is sufficient carbon in the roots. As soon as this source is exhausted disintegration of the haemoglobin in the nodules follows, products of disintegration of the coloured component of haemoglobin are created, and simultaneously nodulation and the assimilation of free nitrogen are checked.

The influence of sugar on the pigmentation of nodules

In connexion with this problem a further series of tentative experiments were carried out, with the object of finding whether:

1) the addition of soluble carbohydrates restores their original pigment to the nodules of shaded plants, or whether

2) the refreshment with a solution of maltose or glucose of the roots of plants which have been shaded or have had their above-ground parts cut off prevents the formation of pigment intermediate in colour between red and green.

Experiment A): Lupines after 8 weeks growth were shaded for 10 days and then taken out of their dark covering. One

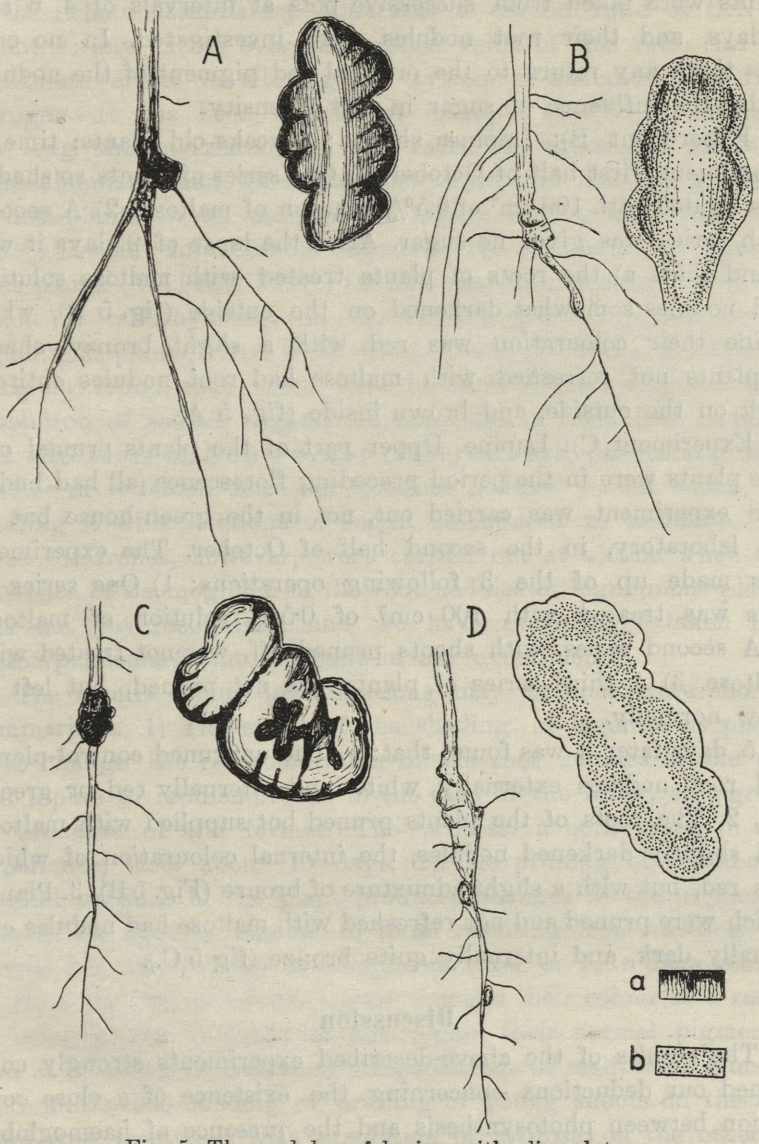

Fig. 5. The nodules of lupine with clipped top.
A — without added maltose, B — supplied with 0·5 % of maltose, C — the nodule of lupine kept in darkness for 8 days, D — the nodule of lupine kept in light. a — the brown colour, b — red pigment.

series of these plants was treated with a 0·5% solution of maltose (100 cm³) and another with a similar solution of glucose. The plants were taken from successive pots at intervals of 4, 6 and 8 days, and their root nodules were investigated. In no case was there any return to the original red pigment of the nodules under the influence of sugar in this intensity.

Experiment B): Lupines shaded; 8-weeks-old plants; time of experiment: first half of October. 1) One series of plants so shaded was treated with 100 cm³ of 0·5% solution of maltose. 2) A second such series was given no sugar. After the lapse of 9 days it was found that: a) the roots of plants treated with maltose solution had nodules somewhat darkened on the outside (fig. 5 B), while inside their colouration was red with a slight bronzy shade. b) plants not refreshed with maltose had root nodules entirely dark on the outside, and brown inside (fig. 5 A).

Experiment C): Lupine. Upper part of the plants pruned off. The plants were in the period preceding florescence (all had buds). The experiment was carried out, not in the green-house but in the laboratory, in the second half of October. The experiment was made up of the 3 following operations: 1) One series of pots was treated with 100 cm³ of 0·5% solution of maltose. 2) A second series, with shoots pruned off, was not treated with maltose. 3) A third series of plants was not pruned, but left to grow normally.

5 days later it was found that: 1. The unpruned control-plants had root nodules externally white and internally red or green-red. 2. The roots of the plants pruned but supplied with maltose had slightly darkened nodules, the internal colouration of which was red, but with a slight admixture of bronze (Fig. 5B). 3. Plants which were pruned and not refreshed with maltose had nodules externally dark, and internally quite bronze (fig 5 C.).

Discussion

The results of the above-described experiments strongly confirmed our deductions concerning the existence of a close connexion between photosynthesis and the presence of haemoglobin in the root nodules of leguminous plants.

Numerous researches in this field have already shown that there exists a dependence between photosynthesis and the capacity

to fix free nitrogen by symbiotic *Rhizobium* bacteria. It has already repeatedly been demonstrated that for the normal functioning of the whole assimilatory apparatus a certain ratio of C:N is essential; any excess over the critical value of this ratio has an immediate effect in altering the system of assimilation of free nitrogen. It has been found, for example, that when the days are long the nodulation on the roots of leguminous plants is more abundant than when they are short, and that the growing of host-plants in an atmosphere highly impregnated with CO_2 (0·5%) has an unfavourable effect on the nodulation of leguminous plants (Wilson 1931). Of experiments concerned with the action of carbohydrates on the development of nodulation in leguminous plants, those of Wilson (1940) should be mentioned; since he found that vetch kept in darkness, but supplied with a solution of soluble sugars, did not cease to form root nodules, and also those of Schweizer (1931) on soya, particularly that species of it which does not produce nodules — but which, by treating it with solutions of sugar, he induced to nodulate. All these researches, however, were carried out at a time when the existence of haemoglobin in the root nodules of leguminous plants was not yet recognized, and we have accordingly taken this factor particularly into account in our experiments.

The results of our investigations may be once more shortly summarized. 1) The effect of the shading of leguminous plants is to change the red colouration of the root nodules of the pea and lupine to reddish-bronze in the case of the latter, and green in the case of the former. The process in both cases, in our experience, lasts about 10 days. 2) The pruning of the above-ground portions of the plant produces changes in the pigmentation of the nodules similar to those resulting from shading the plants but the process is completed three or four days sooner. 3) Root nodules which have once changed their colour as a result of being grown in shade do not regain their normal pigmentation as an effect of treatment with solutions of maltose or glucose. 4) Simultaneous shading or pruning of young shoots on the one hand and treatment with a solution of maltose on the other to a certain extent prevents changes in the red pigment.

Since, however, our experiments were carried out in the autumn, when days are short, and on comparatively scanty mater-

ial, we intend to carry them further next year, investigating changes in the pigmentation of root nodules: 1) with varying length of day, 2) with varying strengths of sugar solution, and 3) at various vegetation periods of leguminous plants.

Reviewing the results of our investigations, we see that Keilin's finding concerning the close relation between the biological functions of *Rhizobium* and the presence of haemoglobin in the root nodules of leguminous plants is supported by further proof. Every change in the production of the pigment, whether produced by shading of the plant or by stripping it of its green portions, leads to rapid checking of the biological fixation of free nitrogen.

The red pigment, when undergoing disintegration, gives coloured compounds whose chemical nature has not been determined. Perhaps they are products of the transformation of haem, which goes on in the root nodules according to the same general process as corresponds in an animal organism to the transformation of haem into brownish-yellow pigments. We should then have a phenomenon, the breaking of the porphyrin ring, and the formation of derivative compounds of the bile system. These are, however, conjectures unconfirmed by experiment. What, however, is the green pigment which was observed in the middle and penultimate vegetation-periods of the leguminous plants investigated by us? Without going into the chemical definition of this combination, and merely on the basis of the observations she has made of the changes in the pigmentation of root nodules accompanying the whole course of development of the leguminous plant, the writer of the present paper suggests that the changes in pigmentation of the nodules are closely connected with the changes of bacterial plasm in *Rhizobium*; at the time when the green pigment drives the red pigment from the root nodule the energy of the fixing-power of free nitrogen gradually diminishes, and the whole process of assimilation of nitrogen terminates when the green pigment fills the whole of the root nodule. The diminution of energy in the fixation of molecular nitrogen is, as is known, a result of the change of form »working« bacteria into that of the less vital bacteria which Wilson (1940) calls »senile«. The genesis of the green pigment in the nodules would thus be connected with the change of active into inactive bacteria.

Is, however the disappearance of haemoglobin and its replacement by green pigment a result of the transformation of active bacteria into inactive, or is it the cause? The former supposition would seem the more probable, and if the writer's supposition is correct, then the form of »working« bacteria would be closely connected with the red pigment, and the form of declining bacteria would be accompanied by the green pigment.

Summary

1. The present work investigates the development and changes of pigmentation in root nodules on the pea, lupine and serradilla from the first appearances of these excrescences on the plants (grown in pots in a greenhouse) until their final disappearance (Figs. 1, 2 and 3 illustrate the development-cycle of root nodules on the above-mentioned plants).

2. The character of the nodulation of peas and lupines on sand, loess, chernozyom and alluvial soil has been investigated. It has been found that the nodulation of these leguminous plants is dependent on their environment. This dependence is reflected in the size of the root nodule and the strength of growth of the red pigment. The nodules of plants grown on sand are distinguished by their blood-red colouration, while nodules from loess and chernozyom soils are considerably larger than those described above, but their pigmentation, particularly in the case of peas, is considerably weaker. The least favourable conditions for the development of nodulation in peas and lupines, under the conditions of the present experiments, was found to be provided by alluvial soil. The nodules were small and the red pigment pale.

3. The presence of nodulation was observed earlier in the case of plants growing on sand, and latest on alluvial soil.

4. In the most important periods of vegetation of these plants the increase of lupine and pea yield was determined, as well as their total nitrogen content; in the period before the flowering of these plants, and again after flowering, the quantity of dry matter of their root nodules (of peas and lupines) was determined.

5. It was found that in the period from the formation of buds to the cessation of flowering the dry matter of the plants and

their production of nitrogen approximately doubled, and the dry matter of their root nodules increased equally rapidly.

6. It was established that the greatest increase of dry matter in the plants and of their nitrogen compounds occurred at the time when the greatest number of their root nodules showed exclusively red colouration. This was at the period of flowering and that preceding flowering. At the time when green pigment began to expel the red pigment from the nodules, the increase both of plant dry matter and of their nitrogen substances began to decelerate.

7. It was found that plants grown on sand or loess assimilated the largest amount of nitrogen, and those on alluvial soil the smallest. The quantity of red pigment in the root nodules and the total dry mass of the nodules was in direct correlation with the symbiotic energy of the nitrogen assimilation (Figs. 1 and 2, Tables I, II, III and IV).

8. The shading of plants, stripping them of leaves, or pruning their parts above ground produced changes in the pigmentation of the nodules and checked further nodulation.

9. The nodulation of shaded peas was three times less than that of peas growing in normal light.

10. The watering of shaded plants, or of plants, whose above-ground portion had been pruned, with a 0·5% solution of maltose prevented (though not completely) the occurrence of the characteristic changes in the pigment of lupine nodules (Fig. 5).

11. The strengthening, by means of a solution of maltose or glucose, of plants whose nodules had previously changed the colour of their pigment in consequence of the shading of the plants, did not restore to the nodules their normal pigmentation.

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BULLETIN INTERNATIONAL
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SÉRIE *B*: SCIENCES NATURELLES

DERNIERS MÉMOIRES PARUS

N° 1—10 B I, 1947.

Dyakowska J. The Pollen Rain on the Sea and on the Coasts of Greenland.
Górski F. Recherches sur l'utilisation des inverses optiques de l'acide racémique par des Aspergilles.
Szymkiewicz D. Études phytogéographiques. II. Terres Magellaniques.
Szymkiewicz D. Études phytogéographiques. III. Afrique du Sud.
Szymkiewicz D. Sur les espèces endémiques des plantes vasculaires.
Szymkiewicz D. La distribution géographique des Crucifères.

N° 1—10 B I, 1948.

Kornaś J. et **Medwecka-Kornaś A.** Les associations végétales sous-marines dans le golfe du Gdańsk *(Baltique Polonaise)*.
Pawłowski B. et **Walas J.** Les associations des plantes vasculaires des Monts de Czywczyn (Planche 5—18).
Szafran B. Mosses from the Pliocene deposits of Krościenko in Poland (Plates 1—4).
Szymkiewicz D. Études phytogéographiques. IV. L'Australie.
Szymkiewicz D. Études phytogéographiques. V. La Nouvelle Zélande.
Szymkiewicz D. Les familles dans les flores.

N° 7—10 B II, 1948.

Datkówna H. The influence of hypotonic solutions on the entodermal cells from the area vitellina of the chick's blastoderm, cultivated *in vitro* (Plates 5—6).
Grodzińska N. Influence of moulting on the ability of colour-discrimination of the Water-snake *Tropidonotus natrix* L.
Grodziński Z. The influence of temperature upon the rate of the heart in the embryos of teleost fishes.
Olbrycht T. Studies on reproductive performance and on an effective method of animal selection.
Pigoń A. On the vacuoles in the amphibian erythrocytes (Plates 7—8).
Latinik-Vetulani I. Nouvelles recherches sur la respiration anoxybiotique des embryons de la Grenouille *Rana temporaria* L. dans les stades initiaux de développement.
Stołyhwo K. Les races anthropologiques comme les directions de développement au sein de l'espèce *Homo*.
Świeżawska K. Influence of an increase in the osmotic pressure upon the white yolk spheres of the hen's egg, containing two or more drops of fat.

TABLE DES MATIÈRES

Janvier—Mars 1949.

Le »*Bulletin International*« de l'Académie Polonaise des Sciences et des Lettres (Classe des Sciences Mathématiques et Naturelles) paraît en deux séries. La première *(A)* est consacrée aux travaux sur les Mathématiques, l'Astronomie, la Physique, la Chimie, la Minéralogie, la Géologie etc. La seconde série *(B)* se divise en deux sous-séries; l'une d'elles »I« contient les mémoires qui se rapportent aux diverses branches de la Botanique (la Systématique, l'Anatomie et la Physiologie des Plantes), l'autre »II« est réservée aux publications qui concernent le vaste domaine des recherches morphologiques et physiologiques sur l'homme et les animaux (Anatomie, Biologie générale, Embryologie, Histologie, Physiologie, Psychologie, Zoologie systématique et expérimentale).

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