Studies on the activity and ultrastructure of the photosynthetic apparatus in the earliest stages of primary bean leaves development

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INTRODUCTION

Some investigators have been trying to link the photosynthetic process occurring upon illumination of dark-grown seedlings with the quantity of accumulated chlorophyll and with the degree of the lamellar system development. Smith (1954) found that during the first few minutes of illumination of dark-grown plants production of oxygen does not occur. According to Tolbert and Gailey (1955) incorporation of C^{14}O_{2} into photosynthetic metabolites begins some hours after chlorophyll synthesis. Rhodes and Yemm (1966) have also found a delay in the development of photosynthetic activity upon illumination of young barley seedlings. According to Rhodes and Yemm the CO_{2} assimilation does not occur at the phase of initial protochlorophyllide transformation and at the lag phase of chlorophyll accumulation. Its appearance seems to be connected with the formation of double lamellae in chloroplasts.

On the basis of the experiments of Irving (1910) and of Briggs (1920) it may be concluded that "inactive" chlorophyll is accumulated not only after illumination of etiolated plants but also at the initial stage of leaf growth in favourable conditions.

Our previous experiments (Więckowski 1966) have shown that chlorophyll which is accumulated in bean leaves at emergence stage is photosynthetically very active. Heterotrophic green leaves, if any, should exist at the earliest stages of their ontogeny.

We tried to elucidate this problem in the experiments described below. Furthermore the aim of these studies was to give some details on the dynamics of chlorophyll accumulation and development of photosynthetic activity at the earliest stages of bean leaves growth. Changes of ultrastructure of plastids at the earliest stages of bean leaves ontogeny are also described.

MATERIAL AND METHODS

Plant material. Experiments were carried out on the primary leaves of Phaseolus vulgaris var. 'Bronowicka'.

Plant cultivation. Seeds were soaked for 24 hours in darkness at 24°C. The coat and one cotyledon were removed from the soaked seeds. The other cotyledon
together with the remaining part of the embryo was put into a Petri dish on watered cotton and remained in a light thermostat (light intensity measured in the range 375—700 nm was about 24 000 ergs·cm⁻²·s⁻¹, continuous irradiation, 29°C; for other details see Więckowski 1959). In these conditions embryonic primary leaves were illuminated from the earliest stages of their growth. A greater number of seeds germinated and after 24 hours the selection of seedlings was made. Chlorophyll accumulation, development of photosynthetic activity and the changes of chloroplast ultrastructure were studied during the first four days of growth of the selected seedlings.

Chlorophyll determination. Chlorophylls were quantitatively extracted with 80 per cent acetone from a large number of leaves (100—300). The concentrations of chlorophyll a and chlorophyll b were determined spectrophotometrically (Uvispek Hilger spectrophotometer) in 80% acetone. (For details see previous papers, Więckowski 1959, 1966).

For preparation of leaf homogenate leaves were ground in a mortar with several milliliters of phosphate buffer (pH 7). Tissue homogenate was centrifuged at 1000 g for 15 minutes. The supernatant was used for the determination of the absorption spectrum in the red region. Leaf homogenates were prepared in weak green light and absorption spectra were measured immediately after their preparation by the opal glass method (Shibata 1959).

Estimation of photosynthesis and respiration. Gas exchange was measured manometrically (see Więckowski 1966). About 300 leaflets were put into every vessel on a piece of gauze. The rate of respiration was measured in darkness and photosynthesis in light (Incandescent lamps) of approximately 24 000 erg·cm⁻²·s⁻¹, both at 25°C.

Electron microscopy studies. Small pieces of leaf (about 4 mm²) were fixed for 4 hours in 2% OsO₄ buffered to pH 7.2 by phosphate buffer. Fixation was carried out at ice melting temperature. Subsequently the material was dehydrated in alcohol of increasing concentrations and embedded in Epon 812 (Luft 1961). A Porter-Blum ultramicrotome equipped with a diamond knife was used for section preparation. Sections were stained with uranyl acetate and lead citrate (Reynolds 1963). The JEM 5Y electron microscope was used for observations and preparation of photographs.

RESULTS

1. Changes in the rates of chlorophyll accumulation and photosynthetic activity

Under the growth conditions applied the rate of respiration (Fig. 1) per single leaf increased from 0.02 to about 0.22 µl O₂/5 minutes exponentially between 50 and 110 hours of experimentation. The rate of respiration per unit of leaf area rose to the value of 0.22 µl O₂ per 5 minutes and per sq. centimeter after 70 hours, and then declined slowly.
Detectable quantities of chlorophylls were observed about 39 hours after soaking of seeds (Table 1). In the following 20 hours the chlorophyll content increased very slowly and then the increase became rapid attaining the approximate value of 0.08 mg per single leaf after about 110 hours (Fig. 2). The chlorophyll concentration per unit of leaf area increased linearly between 60 and 110 hours of experimentation (Fig. 2).

The beginning of the photosynthetic process occurred after about 45 hours (Fig. 3, Table 1). The rate of true photosynthesis remained slow up to 10 hours and was followed by a period of very rapid increase. The rate of photosynthesis per single leaf increased exponentially and linearly per unit of leaf area (Fig. 3). It is interesting to note that we could not reveal any photosynthetic activity during the first few hours of chlorophyll accumulation.

In a previous paper (Więckowski 1966) it has been shown that the assimilatory number is higher soon after leaf emergence than 24 hours later. The same phenomenon was observed at the earliest stages of leaf development (Fig. 4). The greatest value
(about 35) was found at the beginning of the rapid chlorophyll accumulation phase. This value fell approximately to 7 at the end of the experiment. In the initial phase of chlorophyll accumulation the assimilatory number was found to be either very low or very high (it is not marked in Fig. 4).

![Graph showing photosynthesis rate over time](image)

**Fig. 3. Changes of the rate of true photosynthesis per one leaf (full line) and per square centimeter (broken line) in the earliest stages of leaf development. Photosynthesis was measured at about 24000 erg·cm⁻²·s⁻¹. Other details as in Fig. 1.**

<table>
<thead>
<tr>
<th>Time (from seed soaking)</th>
<th>Respiration (μl O₂/5 min/100 leaves)</th>
<th>Photosynthesis (mg/100 leaves)</th>
<th>Chlorophyll a + b (mg/100 leaves)</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.5</td>
<td>0.797</td>
<td>0.000</td>
<td>0.00114</td>
</tr>
<tr>
<td>45</td>
<td>1.146</td>
<td>0.050</td>
<td>0.00102</td>
</tr>
<tr>
<td>48</td>
<td>1.944</td>
<td>0.093</td>
<td>0.00140</td>
</tr>
</tbody>
</table>

**Table 1. Rates of respiration, true photosynthesis and chlorophyll accumulation during the initial phase of leaf greening.**

2. Changes of the absorption spectrum of chlorophyll *in vivo*

The analysis of the red region of the absorption spectrum of leaf homogenate (Fig. 5) indicates that in the earliest stages of leaf development, the red absorption peak shifts its position from about 678.5 nm to about 680 nm with growth. This result suggests that the relative quantity of the short-wavelength form of chlorophyll *a* declines with leaf development.
3. Changes in the ultrastructure of plastids

Primary bean leaves are initiated at the time of seed formation. Their development is interrupted in ripe seeds and begins to continue at the moment of seed germination. Therefore some cell organelles in primary leaves should be differentiated before seeds germinate.

The ultrastructure was investigated at five phases of leaf development: A. before seed soaking, B. after 24 hours of seed soaking, C. after 48 hours — leaves were exposed to light during the last 24 hours, D. after 60 hours, E. after 100 hours from the moment of seed soaking — at that time a high photosynthetic rate is observed.

Phase A (Fig. 6). At that phase almost all organelles appearing at further stages of leaf growth are present in a high degree of differentiation. Amoeboidal plastids occurring in groundplasm are greater than mitochondria and are easily distinguished. Vesicles of various size and shape, membrane fragments and small osmiophilic particles are distributed irregularly in the plastid matrix. Less electron-dense areas in which small lamellae are seen occur also in the matrix.

Phase B (Fig. 7). The general picture of the plastids structure of leaflets after 24 hours of seed soaking is similar to that described above. They are still amoeboidal in shape and filled with starch grains of various size. Very often fragments of membrane, laminae or vesicles occur beside starch grains. Particles like cytoplasmic ribosomes, if any, occur rather rarely and therefore stroma is more transformable for electrons than groundplasm.

![Fig. 4](image)

Fig. 4. Changes in the true photosynthetic intensity per one mg of chlorophyll $a+b$ in the earliest stages of leaf development. Other details as in Fig. 1

![Fig. 5](image)

Fig. 5. Changes in the position of the main red absorption peak of chlorophyll $a$ in vivo during the earliest time of leaf development. Time was counted from the moment of seeds soaking
Phase C (Fig. 8). Plastids are still amoeboidal in shape. Starch grains are found as often as in the previous phase but they fill relatively smaller parts of the plastids. Vesicles may occur separately or are organized into primary layers. Double lamellae occur between many adjacent vesicles. Primary layers are usually not oriented in a certain direction but they are arranged at various angles. Spaces optically less electron-dense, particles like cytoplasmic ribosomes and osmiophilic globules are also seen in the stroma.

Phase D (Fig. 9). In the plastids still amoeboidal in shape vesicles are often dispersed into oriented primary layers. Single vesicles, particles like cytoplasmic ribosomes and osmiophilic globules occur in stroma.

Phase E (Fig. 10). The structure of chloroplasts is similar to the one previously reported (Więckowski 1967a) as phase II. Primary layers look like bundles of lamellae differentiated on grana regions and stroma regions. On the section about eight thylakoids are seen in every granum initial. Starch grains occur rather rarely at that stage of chloroplast development. Osmiophilic globules and particles like ribosomes occur in stroma.

DISCUSSION

Our present experiments show that in very young bean leaflets no photosynthetic process exists during the first few hours of chlorophyll accumulation. Presumably this phase has been described by Irving (1910) and Briggs (1920). It also occurs after irradiation of some dark-grown seedlings (Smith 1954, Tolbert and Gailey 1955, Rhodes and Yemm 1966). Some authors believe that the appearance of photosynthetic activity and its further development is connected with the development of some enzyme systems. In fact activity of many photosynthetic enzymes changes markedly during the leaf ontogeny, e.g. Chen et al. (1967) have found that the activity of kinase 5-phosphoribulose increases during the first six hours of illumination of maize leaves. The increase of carboxylase 1,5 diphosphoribulose activity begins after three minutes of illumination and continues during the next three to six hours and subsequently slowly declines. The activity of isomerase 5phosphoribulose does not changes during the first few hours. In many cases changes of photosynthetic enzymes activities are not parallel to the changes of the rate of chlorophyll biosynthesis (Margulies 1965).

In further stages of leaf growth the photosynthetic rate is presumably also limited by the activity of some enzymes, since the activities of carboxylase 1,5 diphosphoribulose (Smillie 1962; Hageman and Arnon 1955; Margulies 1964; Hall et al. 1959) and dehydrogenase 3-phosphoglyceride aldehyde (Hageman and Arnon 1955; Margulies 1965) were found to be changeable.

Changes in the activity of enzymes are undoubtedly responsible for different photosynthetic rats at various stages of leaf development. But the rate of CO₂ assimilation is believed not to be solely dependent on this activity.

It was interesting to establish that the main red absorption peak of leaf homogenate is shifted by about 2 nm towards the longer value of wavelength with the
Fig. 6. Ultrastructure of leaf cells before seeds soaking (phase A — see text). $p$ — proplastids; $m$ — mitochondria; $r$ — cytoplasmic ribosomes; $sf$ — spherosomes (?). $\times$ 30,000
Fig. 7. Ultrastructure of leaf cells in phase B (after 24 hours of seeds soaking). *p* — proplastids; *a* — starch grain; *er* — endoplasmic reticulum; *r* — ribosomes; *m* — mitochondria; *sf* — spherosomes (?). × 15000
Fig. 8. Ultrastructure of leaf cells in phase C (48 hours after seeds soaking). p — plastids with same thylakoids; a — starch grain; m — mitochondrion; r — ribosomes. × 30,000
Fig. 9. Ultrastructure of leaf cells in phase D (60 hours after seeds soaking). \( p \) — plastids; \( m \) — mitochondrion; \( j \) — nucleus with nucleolus. \( \times 29400 \)
Fig. 10. Ultrastructure of leaf cells in phase E (100 hours after seeds soaking).\( p \) — chloroplasts  
\( m \) — mitochondrion; \( \times 30000 \)
leaf growth. It was found also in the entire leaf. This fact indicates that the long wavelength form of chlorophyll a is accumulated in relatively greater amounts during the period of leaf expansion. At the same time the rate of photosynthesis expressed in μl O₂ per 5 minutes and per 1 mg of chlorophyll decreases very markedly. Presumably the accumulation of Ca 682 in a relatively greater quantity causes the decline of the photosynthetic rate. It is supposed that the long wavelength form of chlorophyll a is less active in photosynthetic process or that the accumulation of pigments connected with photosystem I is not a limiting factor of CO₂ assimilation.

The first interpretation seems to be in agreement with the results of Krasnowsky and his group (Krasnowsky and Kosobutskaya 1952; Krasnowsky, Kosobutskaya and Vojnowskaya 1953; Litwin and Krasnowsky 1957). On the basis of numerous experiments these authors concluded that after illumination of etiolated plants Ca 674 is formed as a photosynthetically active monomer. In later phases of greening Ca 677–678 is formed as a polymer inactive in photosynthesis.

The second interpretation derives from the assumption that all forms of chlorophyll participate in carbon dioxide fixation (for literature review see: Smith and French 1963; Wieckowski 1967 b, and others).

On the basis of the present and previous (Wieckowski 1966) results and of the results of many other authors it is proposed to distinguish six stages in leaf ontogeny in which the rate of photosynthesis per 1 mg of chlorophyll is different. Many facts will become more understandable if we assume that the length of these steps may depend on the plant species and growth conditions.

Stage 1 (Fig. 11). In this phase neither photosynthetic activity nor chlorophyll content are detected. It usually occurs in embryonic leaf primordia containing plastid initials instead of typical proplastids.

Stage 2. At this step a trace of photosynthetically inactive chlorophyll is accumulated or some enzymes participating in photosynthesis are inactive. In the case of primary bean leaves it extends over a few hours. Presumably step 2 has been described by Irving (1910) and Briggs (1920) and it also occurs after irradiation of some etiolated seedlings (Smith 1954; Tolbert and Gailey 1955; Rhodes and Yemm 1966).

Stage 3. The rate of photosynthesis per 1 mg of chlorophyll increases very markedly. We cannot give any evidence for the existence of this stage but it must exist. At that time the already existing chlorophyll molecules become active in carbon
dioxide fixation, or more probably, the biosynthesis of active ones starts. It is also possible that no accumulation of a certain chlorophyll form but development of some enzyme systems is responsible for the appearance of photosynthetic activity.

Stage 4. The rate of photosynthesis per 1 mg of chlorophyll decreases. In our experiments this stage occurred about 24 hours after the appearance of primary leaves (Więckowski 1966) or between 60 and 110 hours of experimentation at the earliest stages of leaf development. This phase has also been established by Blaauw-Jensen et al. (1950) who found a decline of photosynthetic rate per unit of chlorophyll after some hours of illumination of etiolated oat seedlings. There are also some earlier records of its existence (Willstätter and Stoll 1918; Singh and Lal 1935; Gabrielsen 1948 and others).

In the next stage (stage 5) an approximately linear dependence (or slight declining) of the photosynthetic rate on the chlorophyll quantity is noted. In the previously described experiments (Więckowski 1966) this period started about 24 hours following appearance of leaves and continued up to the end of the experiment. Presumably it has been described by Šesták and Čatsky (1962) and Šesták (1963a, b, 1966). The fifth phase is not restricted to the period of leaf growth.

Stage 6. In the last stage of leaf ontogeny the rate of photosynthesis per unit of chlorophyll decreases. We have not been dealing with it in our present studies. But it is a known fact that in senescent leaves the drop in the rate of photosynthesis starts earlier than the decrease in chlorophyll content.

Plastids in embryonic leaves of matured bean seeds differ from those of apical meristems, where they occur as particles of about 0,5 μ in diameter, surrounded with a double membrane having homogenated stroma (Leyon 1956; v. Wettstein 1957; Mühlethalter and Frey-Wyssling 1959; Hohl 1960 and others). In leaflets of bean seeds they are of considerable dimensions and contain starch grains like the proplastids in other plant species (Granick 1955; Frey-Wyssling and Mühlethalter 1965). In bean during the initial developmental stages proplastids play the function of amyloplasts which presumably participate in the carbohydrate metabolism of a young cell. As our electronograms show, starch grains increase even during the initial phase of seed germination; their diameters decrease at further developmental stages. The number of vesicles gradually increases in stroma and formation of granum initials takes place. The first formed primary layers are not oriented in plastids; they usually surround the starch grains. The further development of the lamellar system proceeds in a similar way to that described previously (Więckowski 1967 a).

In conformity with the results of many authors (Hodge et al. 1956, v. Wettstein 1959 and others) we have not found prolamellar bodies in developing bean chloroplasts. Under the conditions of the experiment the transformation of proplastids into chloroplasts may be compared with the transformation of amyloplasts into chloroplasts already described by many investigators (e. g. Berger and Bergmann 1967). In earlier stages only single membranes occur in plastids, sometimes they form spherical structures similar to those described by Hohl (1960) in meristematic cells of Datura stramonium.
Stroma also undergoes some differentiation. Particles looking like cytoplasmic ribosomes do not occur at the earliest stages. Their absence in hypocotyl and roots of *Phaseolus lunatus* was also observed by Klein and Ben-Shaul (1966) during the first few hours of seed soaking. According to Klein and Ben-Shaul the formation of ribosomes precedes the development of the lamellar system with which a very active protein synthesis is connected. Our results also lead to the conclusion that in bean leaflets the development of the lamellar system follows ribosome formation.

Now the question arises if there are any visible changes in the plastids ultrastructure at the time when the photosynthetic process starts. In one set of experiments it was found that very low photosynthetic ability existed after 15—24 hours of illumination. At the same time double lamellae formation took place. Therefore our present results seem to support the view that the photosynthetic ability appears at the beginning of double lamellae formation (Rhodes and Yemm 1966).

It seems that the formation of double lamellae follows the stage of inactive chlorophyll accumulation. If we accept Weier and Benson’s (1966) scheme of localization of chlorophyll molecules in the membranes of the chloroplast lamella it may be concluded that the first forming pigment molecules (inactive in photosynthesis) are presumably those which are localized inside the thylakoid membranes and the photosynthetically active ones are presumably those which are packed in the middle part of the “partition” — between two lamellae belonging to two neighbouring thylakoids.

**SUMMARY**

Chlorophyll concentration, photosynthetic activity and the ultrastructure of chloroplasts were investigated during the first one hundred hours of primary bean leaves differentiation. The coat and one cotyledon were removed from every seed for illumination of leaves at the earliest stages of their development. The seedlings grew on watered cotton in Petri dishes in a light thermostat (29°C, continuous irradiation of about 24 000 erg·cm⁻²·s⁻¹ light intensity).

It results from the experiments that chlorophyll biosynthesis started after about 36—39 hours of seeds soaking and the photosynthetic ability appeared some hours later. The rate of photosynthesis per one mg of chlorophyll *a+b* decreased markedly during the time of experimentation. At the same time a 2-nm shift of the main red absorption peak of chlorophyll *in vivo* towards the longer value of wavelength took place.

Electron microscopic studies have show that the appearance of photosynthetic ability coincides in time with the beginning of double lamellae formation in plastids. In earlier developmental stages the proplastids contain big starch grains and proplastids development may be compared with the transformation of amyloplasts into chloroplasts.

On the basis of our present results and those of many other authors it is proposed to distinguish six phases in the leaf ontogeny in which the rate of photosynthesis per unit of chlorophyll is different or shows different directions of changes.
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Badania nad aktywnością i ultrastrukturą aparatu asymilacyjnego w najwcześniejszych fazach rozwojowych liści fasoli

STRESZCZENIE

W opisanych doświadczeniach zbadano zmiany w stężeniu chlorofilu, intensywności fotosyntezy i ultrastrukturze plastydów zachodzące w pierwszym okresie wzrostu młodocianych liści fasoli. Siewki rozwijały się z nasion pozbawionych łupiny i jednego liścienia, w termostacie świetlnym (temperatura 29°C, nieprzerwane oświetlenie o intensywności około 24 000 erg cm⁻² s⁻¹) w szalikach Petriego wyłożonych wilgotnąwatą.

Stwierdzono, że syntezą chlorofilu rozpoczyna się po około 36—39 godzinach licząc od momentu namoczenia nasion. Natomiast zdolności do fotosyntezy pojawiają się kilka godzin później. Natężenie fotosyntezy w przeliczeniu na mg chlorofilu \(a+b\) spada w miarę zaziełeniania się liści. W tym samym czasie główne maksimum absorpcji w czerwieni chlorofilu \(in vivo\) zostaje przesunięte o około 2 nm w kierunku dłuższego zakresu fal.

Na podstawie naszych badań i badań innych autorów zaproponowano wyróżnienie w rozwoju liści sześciu faz, w których natężenie fotosyntezy w przeliczeniu na mg chlorofilu jest inne lub wykazuje inny kierunek zmian.

Analiza zdjęć z mikroskopu elektronowego pokazała, że pojawienie się zdolności do fotosyntezy pokrywa się w czasie z początkiem powstawania podwójnych lamell w systemie lamellarnym.

W dyskusji starano się opisać czynniki, które mogą być odpowiedzialne za różnicę aktywność aparatu fotosyntetycznego w różnych fazach wzrostu liści.