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PHOTOSYNTHETIC PHOSPHORYLATION AS AN EXPLANATION FOR INDUCTION PHENOMENA IN PHOTOSYNTHESIS

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1. INTRODUCTION

When green plant material is brought from darkness into light, photosynthesis in general does not immediately start at full rate; in most plants some time is needed for the adaptation from the dark to the light state. During this period photosynthesis may increase in some cases continuously, in others the steady state rate is reached only after one or more oscillations.

AUFDEMGARTEN (1939) and VAN DER VEEN (1949) measured these induction phenomena with a diaferometer [1]), and observed with some plants a particular phenomenon which they called "initial uptake": at the onset of illumination, the rate of CO_2 uptake rises sharply for about 15 seconds, then it decreases more or less sharply, then rises again more slowly and after several minutes eventually reaches the steady state level, often after one or two further and slower oscillations. The initial uptake was quite independent of the rest of the adaptation process and in some respects of the steady state level of photosynthesis: at a temperature of 1 to 2°C photosynthesis was slowed down almost to zero rate, but the initial uptake was hardly smaller than at 20°C. When the leaf was kept in water of about 45°C for some minutes prior to the measurement, photosynthesis was completely inhibited, whereas the initial uptake was not impaired. In this case, a gush of CO_2 was observed at the end of the illumination period which was the reverse of the initial uptake. In normal leaves the dark level of CO_2 release (dark respiration) is reached at once and without any oscillations at the end of illumination. Fig. 1 shows adaptation lines which display these phenomena schematically.

An explanation of these adaptation phenomena may be found in changes of steady state concentrations: in a living tissue which metabolizes under given, constant external conditions, the concentration of each metabolite assumes a certain value, such that the rates of the reactions by which the compound is formed balance the rates of the reactions by which it is transformed. If the external

[1]) The diaferometer is an instrument by which the concentration of CO_2 in the air which has passed through a small photosynthetic chamber is measured continuously by means of the heat conductivity of the air. This property is much more dependent on the concentration of CO_2 than of O_2.

conditions are suddenly changed, some reaction rates will change too; the concentrations then have to assume a new value in order to come into equilibrium with the new reaction rates. As, in general, a reaction

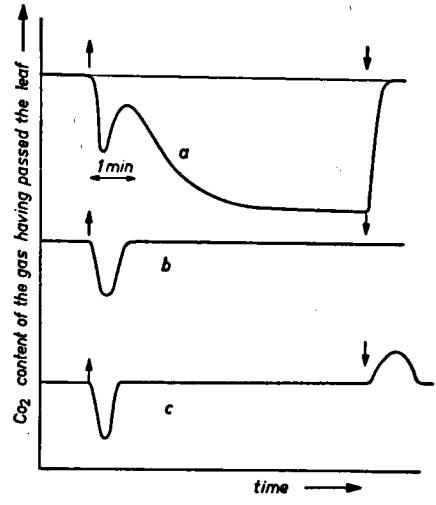

Fig. 1. Adaptation lines showing the initial uptake schematically (from VAN DER VEEN (1949). *a*) normal leaf at room temperature; b) normal leaf at 2°C; c) heat treated leaf, at room temperature; ↑ begin, ↓ end of illumination period.

rate is dependent on the concentration of the reactants, the rates will continue to change so long as the concentrations of the metabolites are not constant.

The work described here was undertaken with the aim of finding the reactions which give rise to the induction phenomena of photosynthesis, and especially to the initial uptake.

2. MATERIALS, METHODS AND RESULTS

With different plants, very different induction curves can be obtained after the transition from dark to light. Whereas some plants show a very marked initial uptake after short dark periods (5 to 30 minutes), other plants do not show it at all: upon illumination, the rate of CO_2 uptake increases gradually or with some very slow oscillations and reaches the steady state value after 5 to 10 minutes. Furthermore one plant displaying for some time a marked initial uptake may suddenly fail to do so. The occurrence of this phenomenon is probably dependent on certain culture conditions or on the nutritional state of the plant. AUFDEMGARTEN (1939) describes such an effect: the green alga *Stichococcus bacillaris* shows a strong initial uptake when cultivated in Eiler solution but fails to do so in Kolkwitz solution.

The unicellular alga *Scenedesmus* shows no initial uptake at all under the conditions of the diaferometer and has a very short induction

time; photosynthesis reaches its full value within 30 seconds. When the suspension of algae is heated to 47° C prior to the measurement, photosynthesis is almost completely stopped, but sometimes a faint initial uptake can be observed.

Most of the work described here has been done with leaves of *Dahlia* (a horticultural variety) and of *Datura arborescens* grown in the garden. The *Datura* tree was covered with a collapsible hot house during the cold months.

The changes of the concentrations of the intermediate products of photosynthesis during the transition from the dark to the light state were studied by means of CO_2 labelled with the radioactive isotope C^{14}. The set up was similar to the one used by CALVIN and MASSINI (1952) for the measurement of metabolite concentrations.

12 rectangular pieces of *Dahlia* leaves, 10 by 50 mm, were arranged in a flat rectangular perspex vessel in a plane parellel to the double walled windows of the vessel. Each piece was mounted in a frame which could very quickly be pulled out of the vessel.

Between the parallel window panes water of constant temperature circulated. The vessel was included in a closed system consisting of a gas circulating pump of the rubber tubing type, a reservoir vessel of 3 l volume containing a mixture of air with 1 % radioactive CO_2 (about 0.2 mc/mmol), a flask for the generation of the CO_2 from radioactive $BaCO_3$ and two traps filled with a 1-n solution of NaOH to trap the CO_2 not used during the experiment. The perspex vessel was illuminated through its two windows by two banks of sodium vapour discharge lamps providing a light intensity of 4500 $\mu W/cm^2$. The cooling water was kept at 20°C.

The leaf pieces were cut from freshly harvested leaves and mounted in the frames of the illumination vessel. Then they were allowed to assimilate for 15 minutes the radioactive CO_2 generated previously and mixed with the air in the reservoir vessel. During this time the intermediate products of photosynthesis were so thoroughly labelled with C^{14} that they acquired a constant activity. At this moment the perspex vessel was darkened for 10 minutes, then illuminated again. During the last minutes of the first illumination and of the dark period, and during the first minutes of the second illumination period the leaf pieces were successively withdrawn from the vessel and immersed in chilled alcohol containing 1 % acetic acid, then ground and extracted successively with 80 % alcohol, 30 % alcohol and finally with water. The extracts were concentrated in vacuo and the soluble compounds separated by means of two dimensional paper chromatography (BENSON, *et al.*, 1950) using n-butanol-acetic acid-water 4:1:5 as first solvent and phenol, saturated with phosphate buffer of pH 7.5 (LEVY and CHUNG, 1953) as second solvent. The radioactive spots were detected by means of autoradiography on X-ray film and counted directly on the paper with a large area end window Geiger-Müller tube.

As the intermediate products had acquired a constant activity during the first illumination period, the radioactivity contained in a compound on the chromatogram was a direct measure of its concentration at the moment the cells were killed. In this experiment the concentration of the phosphorylated intermediate products of the photosynthetic cycle (carbohydrate phosphates and phosphoglyceric acid, (see BASSHAM *et al.*, 1954) were very low during illumination, dropped to $^1/_3$ in the dark and reached the light value again within 1 minute upon re-illumination. The steady state concentration of all the phosphate esters together in the light was about 0.1 μmoles/g

fresh weight. In two similar experiments made with the unicellular alga *Scenedesmus* this value was about 1 μmole/cc wet packed algae. The concentration of malic acid was in the light also about 0.1 μmoles/g. It dropped to $^1/_2$ this value in the dark but recovered within 15 seconds of light.

In order to check whether the CO_2 fixed during the initial uptake is incorporated into a stable compound, the rate of incorporation of radioactive C in light was measured without prior saturation of the photosynthetic reservoirs with C^{14}. It turned out that the photosynthesis of normal leaves was so high compared to the initial uptake, that the latter could hardly be expected to rise above the background of photosynthesis in the time curve of fixation of C^{14} in light. An experiment was therefore performed with 3 pieces of *Datura* leaf treated at 46° C for two minutes, in order to depress photosynthesis without affecting the initial uptake.

The pieces were mounted in a flat glass vessel incorporated in a closed gas circulating system in such a way that they could be illuminated in a constant temperature bath, and at a given moment could be flooded with a cold mixture of ethanol-formic acid-water 10:1:9. In the gas system a reservoir containing $C^{14}O_2$ and a trap filled with a l-n solution of NaOH as well as a circulating pump were incorporated. The first sample was killed after one minute dark in an atmosphere containing $C^{14}O_2$; the second was killed in the light after one minute dark with $C^{14}O_2$ and 10 seconds light; the third after one minute dark, 10 seconds light and one minute dark. The samples were cooled to 0°C immediately after killing, extracted with the killing mixture and centrifuged in the cold. The radioactivity of the extracts was then measured by evaporating an aliquot on an aluminum disc and counting the disc with a Geiger–Müller tube.

TABLE I

Radioactivity fixed by leaf pieces of *Datura*, treated at 46°

	treatment	cpm in extract
1	1 min dark	390
2	1 min dark – 10 sec light	710
3	1 min dark – 10 sec light – 1 min dark	780

The results of this experiment are listed in table I. On another piece from the same leaf, treated in the same manner at 46° C, the induction curve was determined in the diaferometer. This piece consumed 0.15 mm^3 CO_2 during the initial uptake, which corresponds to 1400 counts per minute for the specific activity of the CO_2 used. It is clear that the CO_2 consumed during the initial uptake was not incorporated into a stable, extractable compound.

The dependence of the initial uptake and of steady state photosynthesis on the light intensity was measured in the diaferometer with *Datura* leaf pieces. Fig. 2 shows that the initial uptake is saturated at a light intensity which is much lower than the saturating intensity of photosynthesis.

The action spectrum of the initial uptake of heat treated leaves of *Impatiens* was determined roughly by isolating different spectral regions

by means of Schott filters. Blue and green was slightly less active than red and yellow. This is in qualitative agreement with the action spectrum of photosynthesis.

The dependence of the initial uptake on the concentration of CO_2 was investigated by means of the diaferometer with leaves of *Dahlia* in air containing 0.03 to 20 % CO_2. Fig. 3 shows that the amount of CO_2 consumed during the initial uptake increases to a much greater extent with increasing CO_2 concentration than photosynthesis does.

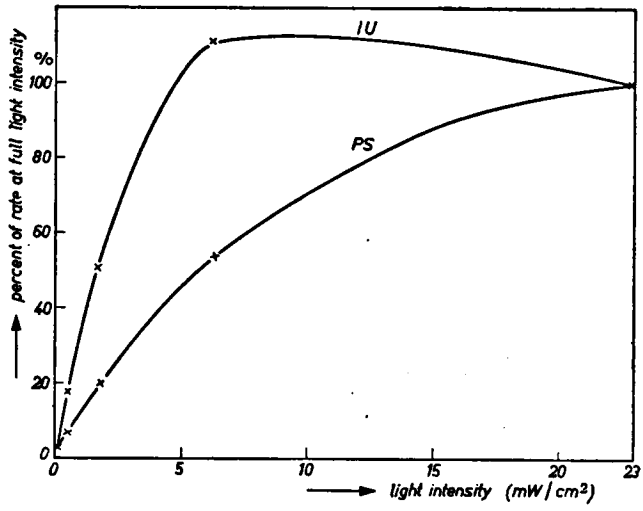

Fig. 2. Light saturation curve of initial uptake and photosynthesis, *Datura*. I U = maximum rate of initial uptake after 10 min. dark; PS = steady state rate of photosynthesis; temperature = 20°C; 3 % CO_2 in air.

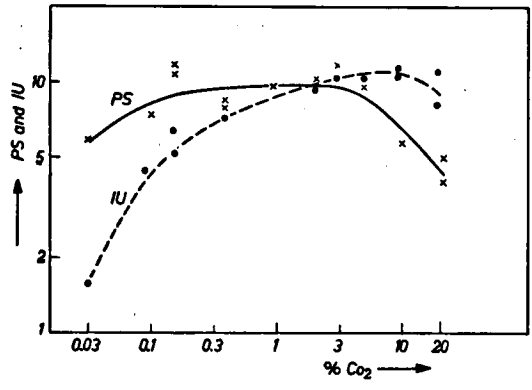

Fig. 3. CO_2-saturation curve of initial uptake and photosynthesis, *Dahlia*. I U = amount of CO_2 consumed during initial uptake per leaf piece after 10 min. dark, relative units; PS = steady state rate of photosynthesis, relative units; temperature = 20°C; light intensity = 23 mW/cm².

The influence of a series of inhibitors on photosynthesis and on the initial uptake is shown in Table II.

Leaf pieces of *Datura*, *Dahlia* or *Impatiens* were allowed to float on the solution of the inhibitor for 2 to 8 hours. A control piece was placed on distilled water for the same time. The induction curve was

TABLE II

Influence of inhibitors on photosynthesis and initial uptake. For explanation see text.

Inhibitor	conc.	time of inhibition hours	plant	% inhibition	
				PS	IU
o-phenanthroline	10^{-3} – m	2	*Datura*	50	50
hydroxylamine	10^{-2} – m	2	*Dahlia*	55	50
phenyl-urethane	10^{-3} – m	2	*Dahlia*	60	40
2,4 – dinitrophenol . . .	10^{-3} – m	2	*Dahlia*	60	40
N-p-chlorphenyl-N'N'-dimethylurea	10^{-5} – m	5	*Impatiens*	60	45
iodoacetamide	10^{-3} – m	2	*Dahlia*	50	0
	10^{-2} – m	2	*Dahlia*	95	0
	10^{-3} – m	5	*Dahlia*	100	30
	10^{-3} – m	5	*Datura*	50	0
	5.10^{-3} – m	2	tomato	60	0
aminophenyl-dichloro-arsine	10^{-3} – m	5	*Dahlia*	35	60
K-p-chloro-mercuri-benzoate	5.10^{-4} – m	5	*Dahlia*	25	15
malonate	up to 0.1 – m	6–8	*Datura*	little effect	
NaF.	up to 10^{-2} – m	3	*Dahlia*	little effect	

then measured with the diaferometer. Infiltration of the pieces in vacuo resulted in the disappearance of the initial uptake even with water, probably because the clogging of the internal gas spaces of the leaf caused a much slower diffusion of the CO_2 and therefore a more sluggish response of the gas phase to reactions taking place in the liquid phase of the leaf.

Most inhibitors of photosynthesis inhibit the initial uptake also, with the exception of iodoacetamide which affects it much less.

Malonate was tried because the first experiment with labelled CO_2 suggested the possibility that the CO_2 consumed during the initial uptake was fixed into malic acid. Malonate is known to inhibit this reaction (DAS, 1937); it was found however that this compound has little effect on both the initial uptake and on photosynthesis.

DISCUSSION

If we wish to consider the possible reactions which could give rise to the initial uptake we will have to look first into the photosynthetic cycle proper, the conversion of carbon dioxide to carbohydrates.

According to BASSHAM *et al.* (1954) the only step of the cycle where CO_2 is consumed is the carboxylation of ribulose diphosphate. The reaction product is split into two molecules of phosphoglyceric acid. The carboxylation is catalyzed by the enzyme carboxydismutase and

is independent of light (QUAYLE *et al.*, 1954 and WEISSBACH *et al.*, 1954). The light energy absorbed by chlorophyll is converted into the chemical energy necessary for the reduction of phosphoglyceric acid to triose phosphate. Part of the energy, however, is converted into the free energy of hydrolysis of the "high-energy phosphates" such as adenosine di- and triphosphate (ADP and ATP), which is partly used to keep the photosynthetic cycle turning. (A survey of the role of high-energy phosphates in photosynthesis is given by ARNON, 1956.)

If the CO_2 consumed during the initial uptake is incorporated into ribulose-diphosphate, then light must influence this reaction indirectly, either by supplying the substrates or by removing the products of the carboxylation reaction. Both assumptions are rendered improbable in the light of the experiments described above. The reaction by which the products are removed is the reduction of phosphoglyceric acid. This reaction is inhibited strongly by iodoacetamide (MEYERHOF and KIESSLING, 1935), whereas the initial uptake is not affected at a concentration where photosynthesis is strongly inhibited (see Table II). If the substrates for the carboxylation are supplied by a light dependent reaction, then the product of carboxylation, phosphoglyceric acid, should accumulate during the first minute after illumination. This was not the case according to the first experiment described where the intermediates were saturated with C^{14} and their concentration measured during the adaptation period. Only traces of phosphoglyceric acid could be detected during the whole experiment.

Phosphoglyceric acid which is a stable and alcohol soluble compound should also have been present in the extract of a leaf which has consumed radioactive CO_2 in the light, according to the experiment summarized in table I. This experiment points to the possibility that the CO_2 is not incorporated into an organic compound at all but is converted into bicarbonate ions dissolved in the cell sap. According to the "Henderson-Hasselbach equation"

$$\log \frac{(HCO_3^-)}{(CO_2)} = pH - pK$$

(see UMBREIT *et al.*, 1954), the concentration of bicarbonate ion is directly connected with the pH so long as the concentration of dissolved CO_2 remains constant owing to the equilibrium with the CO_2 in the gas phase, and a shift of the pH toward alkalinity causes a conversion of CO_2 to bicarbonate ion. This is followed by an uptake of CO_2 from the gas phase according to the equation

$$OH^- + CO_2 \rightleftarrows HCO_3^-.$$

The extent of the uptake is roughly proportional to the concentration of the CO_2 in the gas at a given pH. At the high concentration used in the diaferometer experiments a very small change in the pH is sufficient for an appreciable conversion of CO_2 into HCO_3^-, as can be shown by calculation: if the pH of 1 cc solution which is in equilibrium with a gas phase containing 3 % CO_2, changes from 7.0 to 7.1, then 1 μmole CO_2 passes from the gas to the solution. In comparison,

the amount of CO_2 consumed during the initial uptake is of the order of 0.2 μmoles/g leaves, fresh weight. Fig. 3 shows that the initial uptake is much more dependent on the concentration of CO_2 than photosynthesis is. This is in agreement with this hypothesis.

CHRIST (1955) measured electrical potentials between the roots and an illuminated leaf of *Pelargonium zonale*. At the moment of illumination the potential difference rises sharply, then decreases again and reaches a steady state value after about 40 min. in a manner similar to the curves obtained with the diaferometer. Although it is very difficult to interpret the physical meaning of those potential differences in such a complicated electrochemical chain it is conceivable that the changes in potential difference reflect a change in the pH of the cell content at the transition from dark to light.

Some aspects of the initial uptake described by VAN DER VEEN (1949) and represented in Fig. 1, b and c suggest that it may occur independently of photosynthesis as a whole. A survey of the action of inhibitors (Table III) shows that under the three partial processes

TABLE III

Survey of the action of different inhibitors

inhibitor	PL a)	PSP b)	CF c)	IU d)	PS e)
o-phenanthroline	+	+	+	+	+
2,4-dinitrophenol	(+)	+	+	+	+
KCN	—	+	+	+ f)	
K-p-chloromercuriben-zoate	—	+	+	(+)	(+) g)
iodoacetamide	—	—	+	—	+

a, b and c according to ARNON *et al.* (1956)
a = photolysis of water
b = photosynthetic phosphorylation
c = CO_2 fixation
d and e measurements with the diaferometer
d initial uptake
e steady state rate of photosynthesis
f according to AUFDEMGARTEN (1939)
g the inhibition by p-chloromercuribenzoate was comparatively small, probably because of slow entrance of the inhibitor into the intact leaf.

which together form photosynthesis, the photosynthetic phosphorylation rather than the photolysis of water or the CO_2 fixation contains the cause of the initial uptake. It is very probable that photosynthetic organisms form ATP and ADP from ADP and AMP respectively and inorganic phosphate (P_i) (ARNON,1956 and SCHWINCK, 1956). If this reaction proceeds at a pH of 7, about 0.7 moles of OH^--ion is formed per mole reactants (ALBERTY e.a. (1951)):

$$ADP^{2-} + HPO_4^{2-} \rightleftarrows ATP^{3-} + OH^-$$

In the living cell the OH^--ions react partly with the buffering system which consists of proteins, amino acids, phosphates etc. If the amount of the phosphate which takes part in the reaction is an appreciable part

of the whole buffering system, then a small increase of the pH, and hence an uptake of CO_2 from the gas, will result in spite of the buffering action. It is difficult to estimate the buffering capacity of the plant cells in vivo, also because the reaction may take place only in part of the cell volume or only in part of the cells of the tissue. But there is some evidence that the amount of energy-rich phosphates formed in light, especially during the first moments of illumination, is not negligible compared to the rest of the buffering system.

Unfortunately, most of the measurements of incorporation of phosphate which have been reported have been made with *Chorella*, an organism which does not display an initial uptake at all, and even gives off a gush of CO_2 at the onset of illumination. However, it should be pointed out that in spite of a fast phosphorylation in the light, an initial uptake will not occur if the pH in situ is such that no OH^-ions are formed during phosphorylation, or that the CO_2-carbonate system has no buffering capacity, or if the cell content is so strongly buffered that the pH shift is extremely small. It seems therefore not unreasonable to use the evidence with regard to light phosphorylation in *Chorella* in this connection.

KANDLER (1950) found a quick decrease of the P_i-content of Chlorella in the first seconds of illumination; the decrease was of the order of 0.5 to 1 μmoles/cc wet packed algae. WASSINK, WINTERMANS and TJIA (1951) illuminated a suspension of *Chlorella* in the absence or presence of CO_2 and also measured the decrease of the P_i of the cells after one to six hours. They found a strong decrease of P_i in the light when photosynthesis was inhibited by lack of CO_2, to an extent of up to 30 μ moles/cc wet packed algae per hour. As photosynthesis as a whole is inhibited in the first moments of illumination, it is possible that at this moment the formation of energy-rich phosphate too proceeds at an increased rate.

STREHLER (1953) measured the formation of ATP in a suspension of *Chlorella* incubated anaerobically in the dark, upon illumination or upon admission of O_2 in the dark. He found that the production of ATP by illumination was comparatively little inhibited by low temperatures. This is in agreement with the slight inhibition of the initial uptake by low temperatures (see Fig. 1, b).

SCHWINCK (1956) found ATP also in leaves of *Elodea densa*, but could not detect it in extracts of *Spinacia* or *Viola* by means of paper chromatography because some substances present in the extract hindered a good separation.

WESSELS (1957) measured the light-induced phosphorylation in a suspension of chloroplasts of spinach. He found a formation of ATP of up to 0.25 μ moles per minute in the chloroplasts isolated from 1 g leaves.

An incorporation of P_i into other compounds than adenosine nucleotides must also influence the pH of the cell content. HOLZER (1951) found that a great part of the easily hydrolysable phosphates

of *Chlorella* consists of metaphosphates. WINTERMANS (1954) detected an accumulation of polyphosphates in *Chlorella* during illumination. The incorporation of P_1 into polymeric phosphoric acids must be accompanied by an increase of the pH analogous to the formation of ATP. On the other hand, phosphorylation of e.g. glucose causes a decrease of the pH at pH 7. Glucose-1-phosphoric acid has a second pK of 6.5, compared to 7.2 for orthophosphoric acid (ASHBY *et al.*, 1955), so that, at pH 7, about 0.4 moles H^+ ion are formed per mole reactants for the reaction

Glucose $+ H_3PO_4 \rightleftarrows$ glucose-1-phosphoric acid $+ H_2O$.

As this and similar reactions can only proceed at the expense of ATP, the rise of pH created by the formation of ATP must be reversed as soon as the ATP is used up for different phosphorylations. It is therefore quite possible that the initial uptake should be interpreted as a rapid sequence of a "gulp" and a "gush" of CO_2 superimposed on the slope of the CO_2 fixation curve caused by the increase in photosynthetic rate during the induction period.

The hypothesis presented for the origin of the initial uptake might also furnish an explanation of the inverse induction phenomenon, the so-called gush of CO_2 observed sometimes at the onset of illumination (for a review see RABINOWITCH, 1956). If the reactions using up ATP predominate over its formation, the pH of the cell content should decrease and therefore an evolution of CO_2 should result. However, in view of the observation of BROWN and WHITTINGHAM (1955) that o-phenanthroline does not affect the gush at a concentration where photosynthesis is almost completely inhibited, it is more probable that the gush has no connection with the photosynthetic phosphorylation.

SUMMARY

The initial uptake of CO_2 by photosynthetically active tissue after the transition from dark to light is investigated. It is suggested that this phenomenon is caused by a shift in the pH of the cell medium toward alkalinity which is due to the formation of energy-rich phosphates in the light.

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