Biochimica et Biophysica Acta, 593 (1980) 85—102

BBA 47929

METABOLITE LEVELS DURING INDUCTION IN THE CHLOROPLAST AND EXTRACHLOROPLAST COMPARTMENTS OF SPINACH PROTOPLASTS

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(Received March 5th, 1980)

Key words: Protoplast; Chloroplast metabolism; Induction; CO_2 fixation; Calvin cycle; Photosynthesis; (Spinach)

Summary

The light activation of photosynthesis has been investigated in spinach palisade cell protoplasts.

(1) After a short induction period, maximal rates of photosynthesis are achieved.

(2) [^{14}C]Bicarbonate initially labels anionic compounds in the chloroplast and then in the extrachloroplast compartments. These pools saturate within 2—4 min and radioactivity accumulates mainly in sucrose in the extrachloroplast compartment, in starch and in cationic compounds.

(3) Enzymic determinations were made of metabolite levels during the induction period in the chloroplast and extrachloroplast compartments. There is no general build-up of intermediates. Perturbations of individual intermediates occurred, consistent with the activation of specific enzymes.

(4) It is suggested that fructose-1,6-bisphosphatase and ribulose-1,5-bisphosphate carboxylase may limit flux in the Calvin cycle during induction.

(5) The onset of sucrose synthesis is not accompanied by accumulation of intermediates in the cytosol. It is suggested that sucrose phosphate synthase or sucrose phosphate phosphatase is activated.

(6) Measurements of metabolites in whole leaves during a 24 h illumination cycle confirmed that substrates are not depleted during the dark period, and

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mes, 4-morpholinoethanesulphonic acid.

that the onset of photosynthesis is not accompanied by a rise in intermediate levels.

(7) It is concluded that the causes of the induction lag in protoplasts can differ from those in isolated chloroplasts.

Introduction

When leaves or isolated chloroplasts are illuminated, there is a characteristic lag before the onset of rapid photosynthesis [1]. Osterhout and Haas [2] long ago suggested that induction might be attributed to the need for build-up of intermediates, or for the light activation of catalysts.

Subsequent work has clearly demonstrated that in isolated chloroplasts the induction lag is due to a need to build up the pool sizes of the autocatalytic intermediates of the Calvin cycle [1]. During the induction period, intermediate levels increase [3]. Intermediates which can enter the chloroplast will shorten the lag; phosphate will extend the lag by exchanging intermediates away from the chloroplast [1]. Although it is clear that the rate-limiting and potentially regulatory enzymes of the Calvin cycle [4] increase their activity when chloroplasts are illuminated [5—7], this does not appear to be the main factor involved in causing the induction lag in isolated chloroplasts. In isolated wheat chloroplasts the extent of the activation observed was small and occurred long before full rates of photosynthesis were achieved; on the other hand, adding low concentrations of 3-phosphoglycerate abolished the lag period without leading to a more rapid or greater activation of enzymes [8].

Studies of the induction period have provided much information regarding the control of photosynthesis. It has become clear [1] that chloroplast metabolism is markedly influenced by the external levels of the substrates of the phosphate translocator [9]. The question therefore arises as to whether the isolated chloroplast may show a modified response to illumination because the surrounding environment is different to that found in the cell. It might be predicted from work with isolated chloroplast that, in vivo, the need for autocatalytic build-up of intermediates would be diminished if appreciable substrate levels were found in the cytosol in the dark.

Recently, methods for rapid preparation of chloroplast- and extrachloroplast-enriched fractions from protoplasts have been developed [10,11]. Protoplasts are disrupted by centrifugation through a fine-mesh net. Intact chloroplasts are released, which continue downwards through a layer of silicon oil, whilst the other components of the disrupted protoplasts remain above the oil. This method has been extended [11] to allow measurement of the absolute metabolite levels in the chloroplast and extrachloroplast compartments by means of enzymic substrate analysis.

In the present report we have measured the metabolite levels in the chloroplast and extrachloroplast compartments of spinach palisade protoplasts during the induction of photosynthesis in order to investigate the method of light-regulation of the Calvin cycle and the accompanying effects on the cytosol.

Protoplasts are also an artificial system. Cell interactions are lost. Changes may result from the trauma of the isolation procedures. To assess the relevance

of results obtained with protoplasts to the whole leaf, we have studied the general characteristics of protoplasts photosynthesis and have made measurements of metabolite levels in whole-leaf tissue.

Materials and Methods

The preparation of protoplasts from leaves of spinach (*Spinacia oleracea* U.S. Hybrid 424, Ferry-Morse Seed Comp., Mountain View, CA), silicon oil centrifugation of intact protoplasts, fractionation of protoplasts by centrifugation through a net held in a microfuge tube, collection, neutralization and storage of samples were carried out essentially as described earlier [11]. Protoplasts (50 μg/ml) in a 100 μl volume in 0.42 M sorbitol/20 mM Hepes (pH 7.6)/1.24 mM Mes/7.5 mM $CaCl_2$/7.5 mM $NaHCO_3$ were placed above a 17 μm mesh net held in place in a 400 μl microfuge tube by an inserted length of tubing. Below the net was a 120 μl airspace, 70 μl silicon oil (19 vols. AR 200 : 1 vol. AR 20) and 20 μl 10% (v/v) $HClO_4$. A lid on the tube contained 5 μl 0.3 M HCl in a well which was connected to the interior by a fine pore. Upon centrifugation, the protoplasts were forced through the net and disrupted, and released intact chloroplasts which continued down through the silicon oil into the $HClO_4$. The 5 μl HCl entered the supernatant and lowered the pH to 3—3.5. After 9 s centrifugation, an aliquot of the supernatant was immediately removed and brought to 10% (v/v) $HClO_4$. In parallel experiments, intact protoplasts and medium were separated by directly pipetting the suspension over 70 μl silicon oil (75 vols. AR 20 : 180 vols. AR 200) and centrifuging for 5 s. Cross-contamination between the chloroplast and extrachloroplast fractions was monitored by the distribution of phospho*enol*pyruvate carboxylase and ribulose 1,5-bisphosphate. For the former, an average value of 2.5% cross contamination was taken from 15 experiments. Ribulose 1,5-bisphosphate was determined in each individual sample. All the results in the present article have been corrected for the metabolites in the medium and for cross contamination, as described earlier [11].

To investigate $NaH^{14}CO_3$ incorporation in the first 8 min of illumination, protoplasts, previously in the dark for at least 3 h, were incubated as above with 3.4 Ci/mol $NaH^{14}CO_3$. Four 100 μl aliquots were separately incubated and fractionated to give chloroplast- and extrachloroplast-enriched fractions after 2 min dark at 20°C, and then 0.5, 1, 2, 4 or 8 min white light ($180 \cdot 10^3$ erg/cm^2 per s). The acid extract was centrifuged (2 min, Eppendorf microfuge) and the supernatant was taken as the soluble compounds. The sediment was resuspended and was centrifuged twice in 500 μl 0.3 N HCl, and starch was determined from the ^{14}C radioactivity in the residue [12]. The supernatant, containing the soluble compounds, was adjusted to pH 4—5 with $KHCO_3$ and the anionic, cationic and neutral compounds separated by microscale ion-exchange chromatography. Aliquots of 10—50 μl were washed through 200 μl cation resin (Biorad AG 50W-X8, 200—400 mesh H^+ form) and then through anion resin (Dowex 1-X8 minus 400 mesh, CH_3COO^- form) with 1500 μl distilled water. Cations were eluted with 1500 μl 2 M NH_4OH, anions with 900 μl 10 N formic acid. Of the added radioactivity, 100 ± 10% was recovered.

The presence of radioactivity in sucrose was established by thin-layer chromatography. Neutral compounds were isolated by microscale ion-exchange chromatography as described above, freeze-dried, and separated on cellulose plates (20 × 20 cm, 0.1 mm thick, Merck, Darmstadt) with formic acid/methyl ethyl ketone/tertiary butanol/water (15 : 30 : 40 : 15, v/v) using two successive $3\frac{1}{2}$ h developments [13]. Sucrose, glucose and fructose were identified by co-chromatography with authentic markers. Markers were located by aniline phthalate and by radioactivity. The distribution of radioactivity in samples was determined by plane-scanning; then the cellulose was scraped off and the radioactivity was counted in scintillation fluid. The radioactivity co-chromatogramming with sucrose was incubated with invertase, rechromatogrammed and recovered from the positions corresponding to glucose and fructose.

Samples for enzymic analysis of metabolite levels were prepared from 16 individually incubated and fractionated 100 μl aliquots of protoplasts kept on ice in the dark for at least 2 h before the experiment. All aliquots were kept at 20° C for 2 min and then fractionated, or illuminated ($180 \cdot 10^3$ erg/cm^2 per s) for 0.5, 2 or 4 min at 20°C before fractionation. Samples were neutralized and assayed for substrates as in Wirtz et al. [11]. UDPglucose was assayed as in [14]. Fructose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate were separated using method I of reference [14] for triose phosphates and fructose 1,6-bisphosphate but adding aldolase before triose phosphate isomerase.

For measurement of metabolite levels in intact leaf tissue, samples were prepared after 10 h continuous illumination with tungsten lamps. The plants were then darkened and samples made after 15 min and 14 h darkness. The darkness was complete. The plants were then reilluminated and samples prepared 15 min later. The temperature was 25°C in the light and 15°C in the dark. Samples were prepared as follows. At each time point three samples were prepared. Three plants were selected, each at similar growth stage (4—5 weeks), and each possessing a pair of matched leaves. One leaf was used in the evening and one in the morning. For each leaf, samples were taken from one side in the light and from the other side in the dark. Each sample consisted of four discs (diameter 9.5 mm; total 0.56 mg chlorophyll). The discs were cut out quickly and dropped into liquid N_2 in a pestle and mortar. For samples made in the light, illumination was continuous and the liquid N_2 was also illuminated. For dark samples, discs were cut in total darkness. A very weak light allowed location of the mortar and pestle, but this light did not illuminate the liquid N_2. The leaf discs were protected from the light by tipping them from dark paper directly into the liquid N_2. As the liquid N_2 evaporated, the samples were homogenized to a powder (still in the light or dark). As soon as the liquid N_2 had disappeared, 2 ml 20% (v/v) $HClO_4$ precooled to −5°C, was pipetted onto the powdered leaf material and the resulting solid mixture vigourously homogenized. For dark samples the mortar and pestle were surrounded by a clean paper sheet so that it could be seen that no plant material was lost. Samples were left for 30 min at −5°C, and then centrifuged, neutralized and assayed for metabolites as already described [11]. Parallel samples of leaf discs were homogenized in 80% (v/v) acetone for chlorophyll determination.

Results and Discussion

General characteristics of protoplast photosynthesis

The general characteristics of spinach palisade protoplasts were examined to ascertain that the rates and patterns of photosynthesis were physiological and to establish how rapidly full rates of photosynthesis were achieved, total intermediate pools were saturated, and end-product synthesis from newly fixed carbon was begun.

After starting illumination, O_2 evolution (not shown) and $^{14}CO_2$ fixation (Fig. 1A) commenced immediately but there was a lag of up to 2 min before maximal rates were attained. High rates of photosynthesis could be maintained for at least 40 min. Only a minor fraction of the fixed carbon was found in the medium, probably representing breakage of protoplasts. Photosynthesis from

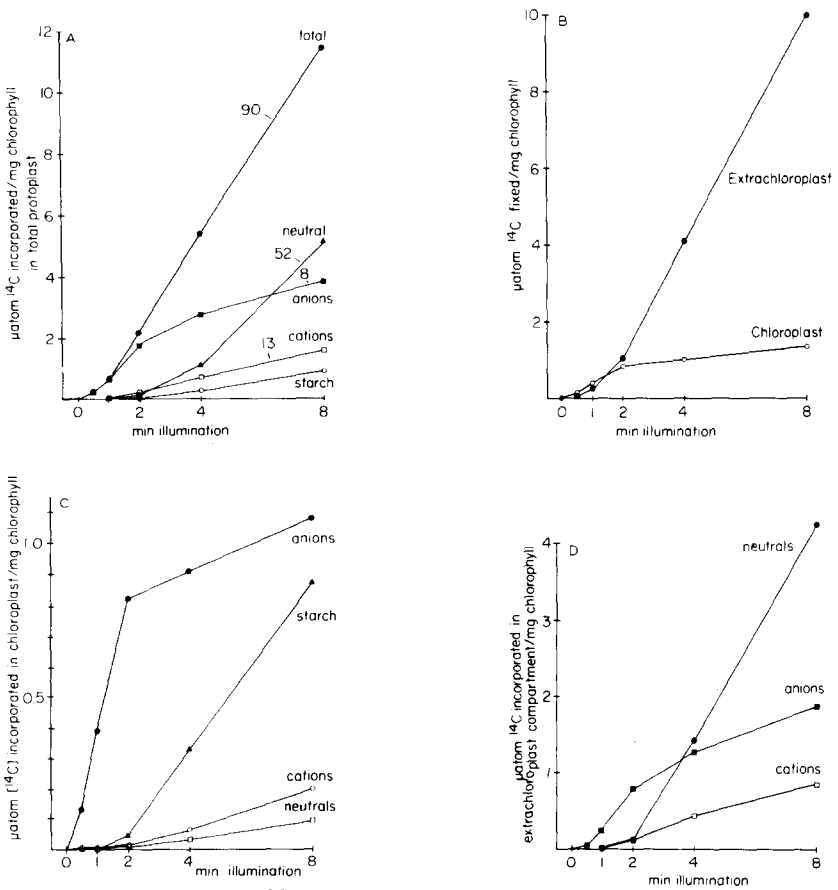

Fig. 1. The fixation of $NaH^{14}CO_3$ and the distribution of the fixed carbon in neutral, anionic and cationic compounds and starch in the chloroplast and extrachloroplast compartments of spinach palisade protoplasts. Protoplasts (50 µg/ml chlorophyll) in 0.42 M sorbitol/20 mM Hepes (pH 7.6)/1.25 mM Mes/7.5 mM $CaCl_2$/7.5 mM $NaH^{14}CO_3$ (spec. act. 3.4 Ci/mol) were incubated in the dark for 2 min and then illuminated and fractionated. The results are corrected for cross contamination. A, CO_2 fixation and distribution of radioactivity in whole protoplasts; B, distribution of radioactivity between chloroplast and extrachloroplast compartments; C, distribution of radioactivity in the chloroplast compartment; D, distribution of radioactivity in the extrachloroplast compartment.

contaminating free chloroplasts was selectively inhibited by 7.5 mM $CaCl_2$ [11]. Radioactivity was incorporated first in anionic compounds. There was a marked incorporation into cations from 1 to 2 min onward, and into neutral soluble compounds and, to a lesser extent, starch from 2 to 4 min onwards. Over 80% of the activity in neutral compounds was identified as sucrose. The high incorporation of radioactivity into carbohydrate endproducts (at least 56% of the total CO_2 fixed) and the high rates of carbon fixation suggest that protoplasts resemble whole leaves, qualititatively, in their photosynthetic metabolism.

The subcellular distribution of the radioactivity was studied. All measurements were corrected for metabolites in the medium deriving from contaminating free chloroplasts and for cross contamination between the fractions. At the beginning of illumination, the majority of the radioactivity was retained in the chloroplast (Fig. 1B). The amount of radioactivity in the chloroplast soon saturated, rising only slowly after 2 min. That in the extrachloroplast compartment rose steeply. The vast majority of the radioactivity in soluble compounds in the chloroplasts was in anionic compounds (Fig. 1C). By 8 min, a large fraction of the total radioactivity retained in the chloroplasts was in starch. In the extrachloroplast compartment (Fig. 1D) radioactivity was first incorporated into anions, but by 4 min accumulation in neutral soluble compounds was the major feature. Over 97% of the radioactivity in neutrals was recovered from the extrachloroplast compartment. This is in accordance with the view that sucrose synthesis occurs in the cytoplasm [15—17]. Of the radioactivity present in cations, most was in the extrachloroplast compartment but a proportion was found in the chloroplasts. Further experiments are required to establish precisely which amino acids are labeled in the chloroplasts and whether this is due to synthesis in the chloroplasts, as has been suggested for some amino acids [18], to import from the cytoplasm [19] or transaminase reactions. When wheat protoplasts are supplied with $^{14}CO_2$, glutamate, aspartate, serine and alanine are labeled in the chloroplasts (Giersch, C., Heber, U., Kaiser, G., Walker, D.A. and Robinson, S.P., unpublished observations).

Chloroplast metabolite levels during the induction of photosynthesis

Subcellular metabolite levels were measured in unilluminated protoplasts, and after 0.5, 2 and 4 min illumination. These corresponded to times at which the rate of carbon fixation was still low, when carbon fixation was nearly maximal but newly fixed carbon was not rapidly entering the major carbohydrate end-products of photosynthesis, and when both photosynthesis and end-product synthesis were maximal. The experiment shown was carried out with the same preparation of protoplasts as was used for the experiments shown in Fig. 1.

After the onset of illumination, marked perturbations of individual metabolites in the chloroplasts were observed (Fig. 2A—C). There was a rapid and maintained decrease in the hexose monophosphates. Levels of fructose 1,6-bisphosphate, triose phosphates and ATP increased sharply and then fell again. The level of 3-phosphoglycerate decreased sharply at first, and then went through a maximum. Ribulose 1,5-bisphosphate doubled during the first 2 min and the declined. It is stressed, however, that the total level of the combined

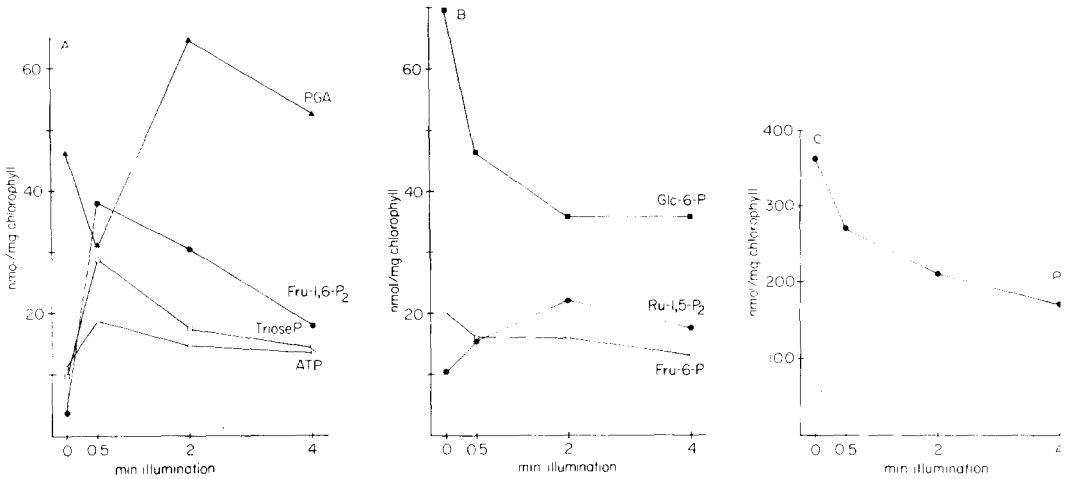

Fig. 2. Metabolite levels in the chloroplast compartment during the first 4 min of illumination of spinach palisade protoplasts. Protoplasts were incubated (for conditions see Fig. 1) for 2 min in the dark and then fractionated or illuminated and fractionated after 0.5, 2 or 4 min in the light. Metabolite levels were determined by enzymic substrate analysis and the results corrected for cross contamination and metabolites in the medium. PGA, phosphoglycerate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Triose-P, triose phosphate.

intermediates scarcely changes (Fig. 3) during the induction period. Attempts to measure the pentose and heptose phosphates indicated their presence in only very low quantities. In another experiment, fructose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate were determined separately in a concentrated

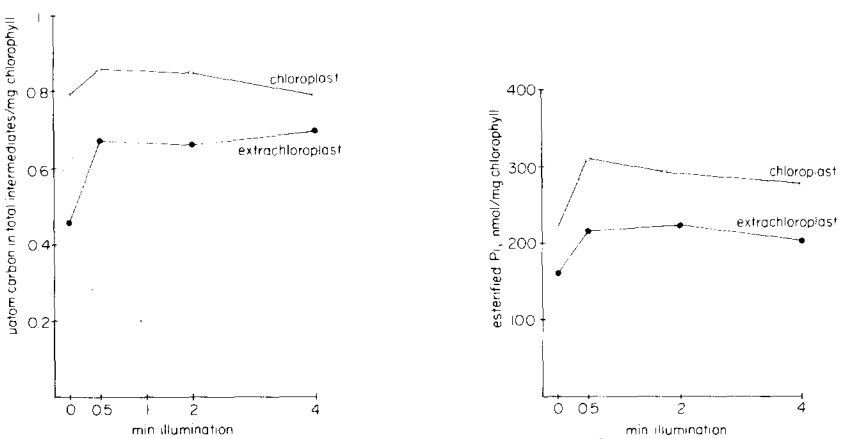

Fig. 3. The levels of carbon in total phosphorylated intermediates in the chloroplast and extrachloroplast compartment during the induction period. The data are taken from Fig. 2 and Fig. 5 for chloroplast and extrachloroplast compartment, respectively. For each intermediate, the nmol/mg chlorophyll is multiplied by the number of carbon atoms per molecule. The individual totals for each intermediate are then summed.

Fig. 4. Levels of esterified phosphate in chloroplast and extrachloroplast compartments of spinach palisade protoplasts during induction of photosynthesis. The totals are calculated from Figs. 3A and 3B (phosphate esters) and Fig. 6A (adenine nucleotides), and from Fig. 7 (phosphate esters) and Fig. 6B (adenine nucleotides). for the chloroplast and extrachloroplast compartment, respectively.

TABLE I

METABOLITE LEVELS IN THE CHLOROPLAST COMPARTMENT IN INDEPENDENT EXPERIMENTS

These results are from five different protoplast preparations, obtained over a period of 3 months. Protoplasts were incubated and fractionated exactly as in Fig. 1. The levels of metabolites were determined by enzymatic substrate analysis, and were corrected for cross contamination.

Time in light (min)	Expt. No.	Amount (nmol/mg chlorophyll) in chloroplast compartment of								CO_2 fixation (μmol/mg chlorophyll per h)
		Rib-1,5-*P*$_2$	Glc-6-*P*	Fru-6-*P*	*Fructose*-1,6-*P*$_2$	Triose-*P*	3-Phospho-glycerate	ATP	P_i	
0	1	11.1	53.1	18.9	10.1	7.3	66.4	—	—	
	2	11.0	69.2	20.2	3.9	9.8	46.2	11.2	362	
	3	—	59.7	27.2	6.1	17.2	33.7	—	—	
	4	13.2	—	—	12.2	21.7	70.1	5.5	336	
	5	23.1	48.1	—	3.1	16.6	40.1	5.2	262	
	μ ± S.E.	14.6 ± 2.9	57.5 ± 1.6	22.1 ± 1.6	7.1 ± 1.7	14.5 ± 2.6	51.3 ± 7.1	7.4 ± 2.0	320 ± 59	
0.5	1	12.9	43.4	12.1	42.1	28.1	52.4	—	—	
	2	15.3	46.2	16.0	37.9	38.6	38.0	19.0	271	
	3	—	31.8	10.3	23.8	26.7	17.0	—	—	
	4	20.3	—	—	43.1	43.9	40.1	11.3	280	
	5	53.1	42.0	—	39.2	23.6	20.0	12.4	112	
	μ ± S.E.	25.4 ± 9.3	40.8 ± 3.1	12.8 ± 1.7	37.2 ± 3.5	32.2 ± 3.8	33.5 ± 6.6	14.2 ± 2.4	221 ± 32	
2	1	24.2	23.7	12.5	32.9	24.6	103	—	—	
	2	21.9	35.7	15.8	30.1	27.5	64.5	14.4	211	
	3	—	24.3	24.0	24.7	26.1	58.7	—	—	
	4	36.2	—	—	34.8	21.8	81.7	8.4	207	
	5	47.1	35.1	—	39.3	42.0	90.2	5.8	56	
	μ ± S.E.	32.3 ± 5.8	29.7 ± 3.3	17.4 ± 3.4	32.3 ± 2.4	28.4 ± 3.5	79.8 ± 8.3	9.5 ± 2.5	158 ± 53	
4	1	12.5	23.2	10.5	27.9	23.4	76.3	—	—	135
	2	17.5	35.6	12.8	22.9	24.3	52.9	13.5	171	89
	3	—	11.8	6.8	12.2	20.0	27.8	—	—	70
	4	27.5	—	—	37.0	32.4	59.2	8.5	114	73
	5	41.5	35.3	—	31.8	37.2	59.9	8.6	132	85
	μ ± S.E.	24.7 ± 6.3	26.5 ± 5.6	10.0 ± 1.7	26.4 ± 4.2	27.4 ± 3.1	55.2 ± 7.8	10.2 ± 1.6	139 ± 28	

chloroplast sample. After 0, 0.5, 2 and 4 min illumination the sedoheptulose 1,7-bisphosphate was approximately 6, 10, 24 and 48% of the fructose 1,6-bisphosphate levels, respectively. Independent evidence that the unmeasured intermediates were present in low amount is provided by comparison of Fig. 1C and Fig. 3. Incorporation of ^{14}C radioactivity into the chloroplast anions saturated at a level corresponding closely to that found by summing the amounts of the metabolites found by the reported enzymic analysis.

Illumination led to a decline in chloroplastic P_i (Fig. 2C), although it sometimes increased again later (Table I). Total esterified phosphate (calculated from Figs. 2 and 5A) showed a reciprocal trend, rising during the onset of illumination (Fig. 4). It was not possible to demonstrate a quantitative stoichiometry between the fall in inorganic phosphate level and the rise in organic phosphate, such as has been demonstrated in isolated chloroplasts [3]. We are inclined to attribute this discrepancy to technical problems. In spinach protoplasts, over 95% of the inorganic phosphate is extrachloroplastic, presumably mainly in the vacuole. Small errors in the estimation of cross contamination will produce large errors in the absolute amount ascribed to the chloroplasts [11]. Although the present data point to a decrease in chloroplast unesterified inorganic phosphate upon illumination, more work on the precise location of phosphate in the cell, with plant material having lower overall phosphate levels, is required to allow precise analysis of this change.

These results shown in Fig. 2 were very reproducible. The same trends were found in five experiments carried out over a period of three months (Table I). While the absolute levels of metabolites vary over a range of 2, the relative amounts remain fairly constant. In view of the inherent variation in substrate levels found in plant tissues [20] the agreement between the absolute levels in different experiments is also reasonable. The rate of photosynthesis varied between protoplast preparations, but it was not correlated to the overall substrate levels found in the protoplasts; obviously factors other than general substrate levels determine the final rate of photosynthesis in spinach protoplasts. The ATP measurements were extended in a separate experiment in which ATP, ADP and AMP levels were determined (Fig. 5A). As ATP increased after 0.5 min light, ADP and AMP declined. Between 0.5 and 4 min ATP declined, although to a level still above that found in the dark, and both ADP and AMP levels increased accordingly.

On the mechanism of the light activation of the Calvin cycle

The data shown here strongly support the notion that the lag of CO_2 fixation by protoplasts after the onset of illumination is caused by the gradual activation of regulatory enzymes. The observed perturbations during the induction period allow these enzymes to be identified. During a period when flux through photosynthesis is increasing, the enzymes which are being most rapidly activated at a particular stage can be identified by a fall in their substrate levels. Conversely, enzymes which are being activated more gradually, and hence become rate-limiting, should be recognized by a rise in the amount of the corresponding substrate [21]. A concomitant fall in the product can be taken as indicating that subsequent reactions have surplus capacity to use the supplied intermediates. A complication is raised by the possibility that substrates may

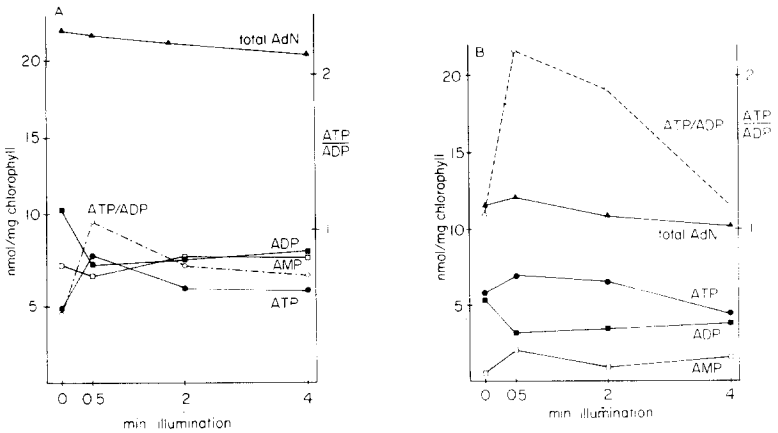

Fig. 5. The levels of adenine nucleotides in the chloroplast (A) and extrachloroplast compartment (B). For details see Fig. 2. The results are corrected for cross contamination and metabolites in the medium.

be bound on enzymes; in this case the total amount of a metabolite would not reflect accurately the free level of the substrate. Particular caution is necessary in the case of ribulose 1,5-bisphosphate, of which a large proportion may be bound to ribulose-1,5-bisphosphate carboxylase [22]. However, the relative changes of fructose 1,6-bisphosphate, triose phosphate, 3-phosphoglycerate, ATP and ADP, and of glucose 6-phosphate and fructose 6-phosphate are fully self-consistent. This supports the contention that the total amounts of these substrates provide a satisfactory picture of the amounts available to interact with their enzymes.

The following conclusions can be drawn from the reported data. During the onset of photosynthesis, the rate of carbon fixation is limited by the activity of the enzymes rather than by the general level of substrates available to them. The enzymes activate rapidly, O_2 evolution and carbon fixation starting immediately and reaching maximal rates by 2 min. As a large accumulation of carbon

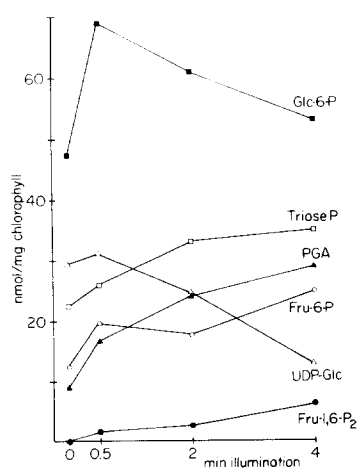

Fig. 6. Metabolite levels in the extrachloroplast compartment during the first 4 min illumination of spinach palisade protoplasts. For details see Fig. 2.

is not found in any individual substrate pools, the enzymes must increase their activity roughly in synchrony. However, limited perturbations point to small differences in the rate at which the different enzymes are activated. Fructose 1,6-bisphosphatase activates relatively slowly, as evidenced by the rise in fructose 1,6-bisphosphate in the first half minute and its slow subsequent fall. The subsequent enzymes initially have surplus activity, and hexose monophosphate levels fall. It is likely that ribulose-1,5-bisphosphate carboxylase approaches its steady activity more slowly than ribulose-5-phosphate kinase in view of the rise and subsequent small decrease in the ribulose 1,5-bisphosphate levels. It is unlikely that phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase are rate-limiting at any stage. The initial build-up of ATP, and probably also of NADPH, occurs because the light reactions reach their full potential for activity before the Calvin cycle. The fact that such rises are accompanied by an absolute fall in the 3-phosphoglycerate points to the regeneration, rather than the use, of 3-phosphoglycerate being rate-limiting. This is borne out by the reciprocal movement of ATP and of the 3-phosphoglycerate/triose phosphate ratio at all stages, as is expected when these compounds are being kept near equilibrium.

The model can be related to the known properties of the enzymes. Fructose-1,6-bisphosphatase can be activated by the pH and magnesium changes produced in the stroma by illumination, both in vitro [6,7] and in intact chloroplast [23]. It can also be activated by thioredoxin [5]. In wheat chloroplasts, it was the enzyme found to show the most marked light-activation [8]. Ribulose-1,5-bisphosphate carboxylase slowly activates when incubated in alkaline conditions with Mg^{2+} and CO_2 in vitro [24]. Moreover, the extracted activity increases markedly when chloroplasts are incubated in the light [25, 26]. In protoplasts, only minor changes in the extracted activity of the ribulose-1,5-bisphosphate carboxylase were reported to be caused by illumination [27]. We sometimes found larger changes with protoplasts (Wirtz, W., unpublished observations). Assay and incubation conditions may be crucial in determining whether a change in activity in vivo can be detected after disrupting protoplasts or chloroplasts.

Extrachloroplast metabolite levels during the induction of photosynthesis

The measurement of metabolites in the extrachloroplast compartment was technically more difficult than in the chloroplast compartment. The cross contamination from the chloroplast was larger than that in the reverse direction. The results also had to be corrected for metabolites in the medium [11]. Such corrections are largest for triose phosphate and 3-phosphoglycerate. Nevertheless, in three separate experiments a qualitatively consistent picture was obtained. The results presented (Fig. 6) are from the same experiment as those given for the chloroplast compartment in Fig. 2.

No dramatic change was found. Triose phosphates and 3-phosphoglycerate showed a general trend upwards. Hexose monophosphates rose slightly before declining to near the dark level. UDPglucose levels declined. The level of fructose 1,6-bisphosphate in the extrachloroplast compartment was much lower than that found in the chloroplast, so accurate measurement was not possible [11]. Since most of the extrachloroplastic P_i is probably vacuolar [11], changes

in cytoplasmic free P_i were probably masked (data not shown). The total measured esterified phosphate in the extrachloroplast compartment increased upon illumination (Fig. 4B), so free P_i may decrease. The total carbon in the measured intermediates rose somewhat, expecially in the first half minute. The adenine nucleotides were measured in a separate experiment (Fig. 5B). After 0.5 min illumination, the level of ATP had risen, and that of ADP declined. Subsequently the ATP level falls again. The AMP level was low throughout.

The onset of starch and sucrose synthesis after illumination

Since intermediates do not accumulate during the onset of carbon fixation (Fig. 3) there must be an almost immediate onset of end-product synthesis. The carbon fixed after 0.5 min, 1 and 2 min was 197, 670 and 2071 natoms/mg chlorophyll, respectively (Fig. 1A), yet after 0.5 and 2 min the total intermediate pools in the protoplasts had only increased by 268 and 267 natom carbon/mg chlorophyll, respectively (Fig. 3). Although maximal incorporation of $^{14}CO_2$ into starch and sucrose showed a lag of 2—4 min, most of this lag reflects the time needed for the supplied radioactivity to move through the intermediate pools to the immediate precursors for these end-products. A lag of 2 min found in the labeling of sucrose and starch when $H^{14}CO_3^-$ was supplied in steady-state photosynthesis was shown to reflect the time needed for the specific activity of the immediate precursors to rise to that of the supplied $H^{14}CO_3^-$ (Wirtz, W., Stitt, M. and Heldt, H.W., unpublished observations). Thus rapid sucrose and starch synthesis is initiated within the first 1—2 min of photosynthesis.

During this period the chloroplast 3-phosphoglycerate/P_i ratio increases (Fig. 2). This would activate the chloroplast ADPglucose pyrophosphorylase [12,28,29]. However, it is not clear from the data that this change is maintained and it cannot be ruled out that the other factors are also involved in the regulation of starch synthesis in these conditions.

From the data it is clear that sucrose synthesis is not initiated by a large accumulation of cytoplasmic intermediates. Nor, in these conditions, was a large increase in ATP found in the light, although increases have been found elsewhere [30]. The steady level of hexose monophosphates and the fall in UDPglucose suggest that UDPglucose pyrophosphorylase is not the control enzyme, but that either sucrose phosphate synthetase or sucrose phosphate phosphatase are activated in the light as rapid sucrose synthesis begins. The latter two enzymes are unique to sucrose synthesis. UDPglucose is not unique to sucrose synthesis, being also a precursor for cell wall material and galactolipids. Thus UDPglucose pyrophosphorylase alone could not specifically control sucrose synthesis, although its activity is a prerequisite. An active cytoplasmic fructose 1,6-bisphosphatase is also needed. Given that glycolysis operates in the dark, this enzyme must be activated upon illumination. The data do not allow any clear conclusion to be drawn about how this is done, although a small rise in substrates appears to take place.

Metabolite gradients between the chloroplast and extrachloroplast compartments

In most cases the metabolite levels in the two compartments are of a similar

TABLE II

POOL SIZES, TURNOVER RATES AND CONCENTRATIONS OF METABOLITES IN THE CHLOROPLAST AND EXTRACHLOROPLAST COMPARTMENTS OF SPINACH PALISADE PROTOPLASTS AFTER 4 MIN ILLUMINATION

The metabolite levels are taken from Table I and Fig. 6. The molecular turnover is calculated in the chloroplasts for a photosynthetic rate of 80 μmol CO_2 fixed/mg chlorophyll per h, and in the extrachloroplast compartment for sucrose synthesis at 52 μatom C/mg chlorophyll per h (22 nmol CO_2/mg chlorophyll per s and 1.2 nmol sucrose/mg chlorophyll per s, respectively). The calculations assume a unidirectional flow of carbon. The chloroplast volume is taken as 47 μl (Ref. 14, Table VI) and the cytosolic volume is assumed to be equal to the chloroplast volume.

Compartment	Compound	Molecules turned over per molecule of CO_2 fixed (chloroplast) or molecule sucrose synthesized (extrachloroplast)	Amount (nmol/mg chlorophyll)	Consumption (nmol per second) when CO_2 fixation is 80 μmol/mg Chl per h, sucrose synth. = 4.3	Half-time of pool turnover (s) to regenerate Rib-1,5-P_2 or sucrose	Concentration (mM)
Chloroplast	3-Phosphoglycerate	2	55.2	44	0.63	1.17
	Triose-*P*	2	27.4	44	0.31	0.58
	Fru-1,6-P_2	0.33	26.4	7.3	1.8	0.56
	Hexose-*P*	0.33	36.5	7.3	2.5	0.78
	Rib-1,5-P_2	1	24.7	22	0.56	0.52
	ATP	3	10.2	66	0.08	0.22
Extrachloroplast	3-Phosphoglycerate	4	29.2	4.8	3.0	0.62
	Triose-*P*	4	35.1	4.8	3.6	0.74
	Fru-1,6-P_2	2	6.0	2.4	1.2	0.02
	Hexose-*P*	2	78.3	2.4	16.2	1.66
	ATP	1	6.1	1.2	5.0	0.13

order of magnitude (Figs. 1 and 6, and Table II), although there are some marked assymmetries. Almost all the fructose 1,6-bisphosphate is chloroplastic. The majority of the hexose monophosphates are extrachloroplastic. Also, during induction, the levels of these compounds change independently in the chloroplast and extrachloroplast compartments. Such distributions are consistent with the known impermeability of the chloroplast membrane to these compounds [9,31,32].

In both light and dark the ATP/ADP and triose phosphate/3-phosphoglycerate ratios are higher in the extrachloroplast than in the chloroplast compartment (Table II). Similar differences in the distribution of these compounds were found by non-aqueous fractionation of leaves [30,33], and also in that the NADPH/NADP and NADH/NAD ratios are higher outside the chloroplast. In isolated chloroplasts in the light, triose phosphate is preferentially exported and 3-phosphoglycerate, which is more negatively charged, is preferentially retained due to a pH gradient generated over the envelope in the light [9]. This provides a plausible mechanism for the generation of the observed 3-phosphoglycerate/triose phosphate and ATP/ADP gradient in the light in vivo. In the dark in isolated chloroplasts the envelope pH gradient disappears and the stroma acidifies, due to a Donnan potential between the proteinaceous stroma and the aqueous medium. Consequently, the 3-phosphoglycerate/triose phosphate ratios reverse in the dark [9]. Such a reverse is not seen in vivo. This discrepancy may be attributed to the contribution of cytosolic enzymes to the substrate concentrations observed in vivo, or to the pH gradient over the envelope in vivo not always exactly corresponding to those found in isolated chloroplasts. For example, in vivo the chloroplast will be surrounded by cytosolic protein, hence the contribution from a Donnan potential would not be expected. Further experiments are required to clarify these matters.

Metabolite turnover rates in the chloroplast and extrachloroplast compartments

The amounts of metabolites in the chloroplast and extrachloroplast compartments are of similar magnitudes, but the turnover rates of the metabolites vary greatly between the compartments in the light. In Table II the concentrations, and the half-time of pool turnover, are calculated from the average substrate levels in five experiments (Table I) for rates of photosynthesis and sucrose synthesis of 80 and 50 μatom C/mg chlorophyll per h, respectively. The chloroplastic pools all turn over 5—10-times faster than the corresponding cytosolic pools. These rapid turnover times emphasize the need for a rapid killing method and for checks that metabolite levels do not change during the fractionation procedures [11]. Even so, caution is needed in interpreting the absolute values for compounds which have turnover rates as high as that of ATP in the light.

The rapid turnover times emphasize that the contents of a pool are negligible compared with the flux through it; very little time is needed to change drastically the size of the intermediate pools. This can be illustrated by considering the source of the fructose 1,6-bisphosphate during the first half minute of illumination. Even although the carbon fixation over this period averages only about 30% of the maximal rate (Fig. 1A), it would need only about 7 s for the

operation of the Calvin cycle to produce the observed increase in fructose 1,6-bisphosphate. Thus, there is no need for import of carbon from the cytosol during the onset of photosynthesis. Conversely, the chloroplasts export carbon and the cytosolic intermediates increase over the first half minute (Fig. 3).

Changes in metabolite levels in whole leaf tissue

Although the results obtained with protoplasts point to enzyme activation rather than substrate accumulation being the cause of the initial lag in photosynthesis in vivo, protoplasts kept in the dark for only a short time might not faithfully represent the metabolism of a leaf after a night period. Therefore, metabolite levels were also measured in intact leaf tissue taken from spinach plants after 14 h absolute darkness, and then after 15 min subsequent light. For comparison, measurement were also made after 10 h light, and after 15 min subsequent dark. The light regime and temperature were identical to those under which the plants had been previously grown.

The results (Table III) clearly demonstrate that there was no large build-up of intermediates when leaves were first illuminated. Indeed, the metabolite levels decreased more during the initial period of darkness than they increased during the initial minutes of illumination. Moreover, metabolites did not decline during the night; in this experiment a substantial increase was found during the hours of darkness. Nor did the plant leaf run out of respiratory reserves. After 14 h darkness, soluble sugars were still present at 36% of the levels at the start of the night, sufficient for another 2—4 h at a total rate of

TABLE III

METABOLITE LEVELS IN WHOLE LEAF TISSUE ON PLANTS IN A 24 h LIGHT-DARK REGIME

Intact spinach plants were grown in a 10 h light/14 h dark regime in hydroponic culture. Three samples, each of four leaf discs (fresh weight 90 mg), were collected from matched leaves after: 10 h light; 10 h light and 0.25 h dark; 14 h dark; and 14 h dark plus 0.25 h light. The discs kept under the prevailing light regime, were quenched in liquid N_2, extracted at $-5^{\circ}C$ in 20% (v/v) $HClO_4$, neutralized and assayed for substrates (see Methods). Chlorophyll was determined in parallel samples extracted in darkness in 80% acetone. The data for intact protoplasts are taken from experiments reported in Table II. All results are given as the mean ± S.E. of three replicates. The starch was measured in a separate experiment as in Ref. 35). n.d., not determined.

Compound	Amount (nmol/mg chlorophyll) in					
	whole leaf tissue after				isolated protoplasts	
	10 h light	10 h light and 0.25 h dark	14 h dark	14 h dark and 0.25 h light	dark	4 min light
Glc-6-*P*	176 ± 17.0	40 ± 5.5	132 ± 11.1	148 ± 8.5	142 ± 28	84 ± 17
Fru-6-*P*	61 ± 10.3	17 ± 4.3	44 ± 3.8	41 ± 4.9	48 ± 14	33 ± 6
Fru-1,6-P_2	41 ± 1.8	5 ± 0.5	10 ± 1.6	26 ± 0.8	8 ± 3	32 ± 5
Triose-*P*	40 ± 5.2	10 ± 0.6	19 ± 3.8	35 ± 3.7	33 ± 2	58 ± 1
3-Phosphoglycerate	251 ± 17.9	108 ± 7.4	263 ± 13.3	369 ± 12.6	87 ± 9	94 ± 5
Rib-1,5-P_2	62 ± 4.5	7 ± 0.3	<0.5	44 ± 5.1	12 ± 1	19 ± 4
UDPglucose	18 ± 0.7	10 ± 3.7	19 ± 2.2	18 ± 2.2	30 ± 3	13 ± 3
Free hexose	11 284	10 920	4149	5669	528	n.d.
Hexose in starch	29 900	n.d.	970	n.d.	n.d.	n.d.

carbohydrate oxidation of 1—2 μmol hexose/mg chlorophyll per h. In addition, some starch remained. From the data in Table III, assuming a cytosol volume equal to the chloroplast volume of 47 μl/mg chlorophyll [11] and a distribution of the 3-phosphoglycerate similar to that found in spinach protoplasts in the dark (Table II), the 3-phosphoglycerate concentration in the cytosol can be estimated as about 1 mM. With isolated chloroplasts, 0.1 mM 3-phosphoglycerate is sufficient to relieve the lag period [1].

There is an additional argument to support the view that the results obtained with isolated protoplasts reliably show what happens in vivo. The total metabolite levels of three different protoplast preparations were fully representative of the range of values found in whole leaves (Table III). Also, the general response of metabolites to illumination is similar in leaves and protoplasts.

It is of interest that metabolite levels in leaves vary somewhat during a 24 h illumination cycle. More experiments are needed to establish the exact relation of protoplast metabolism to this apparent range of metabolic states in the whole leaf, and to understand the significance of this variation. Particularly striking are the gradual rise in metabolite levels over the long period of illumination, and the sharp decrease of most metabolites found upon initial darkening and their subsequent rise again through the night. A general fall in metabolites after darkening also occurs in the extrachloroplast fraction of barley protoplasts (Stitt, M., unpublished results). A sharp fall of ribulose 1,5-bisphosphate upon darkening has been seen in *Chlorella* [34], in isolated spinach chloroplasts [35], and in barley protoplasts (Stitt, M., unpublished results). In these experiments some ribulose 1,5-bisphosphate always remained in the dark, whereas in leaves it totally disappears in the course of the night. Presumably the gradual total disappearance can be ascribed to small differences in any low residual activity of ribulose-5-phosphate kinase and ribulose-1,5-bisphosphate carboxylase left in the dark.

On the mechanism of induction

In the dark, leaf cells have a metabolism based on a pattern of carbohydrate metabolism typical of that found in non-photosynthetic plant tissue [36]. Upon illumination, they switch within 1—2 min to rapid photosynthesis and cytosolic gluconeogenesis for sucrose synthesis. It is becoming clear that these often mutually opposed metabolic states are not segregated by compartmentation. Gluconeogenesis for sucrose synthesis occurs in the cytosol [15—17]. It is also clear that a significant proportion of the total carbohydrate oxidation in the dark occurs in the stroma [37—39]. Unless enzymes are efficiently regulated, futile cycles will occur between, for example phosphofructokinase and fructose-1,6-bisphosphatase. It can be estimated from published data [39] that fructose-1,6-bisphosphatase activity in isolated pea chloroplasts in the dark is only about 1% of that required in the light for photosynthesis. The present results show that the enzymes are so efficiently regulated that they can rapidly switch carbon flow from carbohydrate oxidation, to photosynthesis and gluconeogenesis, without large changes in the energetic state of the cell and with minimal changes in substrate levels.

Thus, the basic causes of the induction lag can differ between chloroplasts and protoplasts. In situ, even after a period of darkness, chloroplasts contain

sufficient intermediates to allow rapid photosynthesis as soon as the enzymes are active. On the other hand, in isolated chloroplasts in the dark, stromal levels of the Calvin cycle intermediates are very low [3] and must be built up during the induction period. Apparently almost all the triose phosphate and 3-phosphoglycerate have been lost from the isolated chloroplasts, in exchange for P_i. After the onset of illumination, the building of the stromal levels is further delayed by exchange with P_i in a surrounding medium the volume of which is 1000-times greater than the stromal volume. Even when chloroplasts are prepared from protoplasts in essentially P_i-free medium, it is likely that substantial P_i is generated by phosphatase hydrolysis of released intermediates and by breakage of the vacuole. Protoplasts from hydroponically grown spinach contain over 5 μmol P_i/mg chlorophyll [11]; when disrupted at a concentration of 100 μg chlorophyll/ml this would give a final concentration of 0.5 mM.

It is not yet clear that intermediate accumulation never occurs in protoplasts and leaves during induction. It is possible that plants grown in less favourable conditions might deplete their leaf substrates to a point where the autocatalytic capacity of the Calvin cycle becomes a prerequisite for the onset of photosynthesis. Plants which retain less of their photosynthate as starch might have small chloroplast metabolite pools in the dark. Certainly, the length of the induction period varies between species, and with the growing conditions [1]. A study of the relation between substrate levels and the characteristics of the induction period in whole leaves should throw further light on this question.

Acknowlegements

The authors are grateful to Prof. M. Klingenberg (Munich) for providing us with the laboraty facilities of carrying out this work. We are indebted to Prof. D. Walker (Sheffield) and Prof. U. Heber (Würzburg) for reading of the manuscript and stimulating discussions. This research has been supported by the Deutsche Forschungsgemeinschaft.

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