Electroimmunodiffusion – a Powerful Tool for Quantitative Determinations of Both Soluble and Membrane Bound Chloroplast ATPase, CF_1

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Z. Naturforsch. **38 c,** 799–805 (1983); received May 18, 1983

Electroimmunodiffusion, Chloroplast, CF_1-Content

1. Electroimmunodiffusion can be used for routine specific quantitative microdeterminations of photosynthetic ATP-synthase. The method has been worked out for samples of both the dissociated (CF_1) and the membrane bound enzyme (CF_0CF_1).

2. Only a monospecific antiserum against CF_1 is required for relative determinations of CF_1 in any fraction. If beyond this an absolute determination is intended of either the separated or the membrane bound ATPase, in addition a pure CF_1 is needed for calibration.

3. A varying CF_1/chlorophyll ratio between 1/700 and 1/1200 has been found for thylakoids of spinach plants grown under various light intensities.

4. Since the amount of CF_1 was the limiting factor of photophosphorylation capacity in the electron transport system used (PMS), from simultaneous measurement of CF_1 content and photophosphorylation capacity a turn over time of the ATPsynthase of 4 ms/ATP/CF_0CF_1 could be recalculated.

The main function of the protein complex called CF_0CF_1 in chloroplasts is the synthesis of ATP from ADP and phosphate, probably converting the energy of a transmembrane proton gradient. Although the photophosphorylation capacity of intact thylakoids reflects the CF_1 content of the membranes on a chlorophyll basis [1, 9], there is no linear correlation between photophosphorylation capacity and CF_1 content in partially CF_1 depleted thylakoids [1]. Thus, ATP formation cannot be used for quantitative determinations of membrane bound CF_1.

Various treatments of soluble and membrane bound CF_1 result in an unmasked ATPase; this enzymatic property is widely use for quantitative determinations of CF_1 [1–4]. The treatment with trypsin [5] is rather laborious and thus not suitable for high sample numbers, for example necessary in studies on CF_1 binding to thylakoids (Roos and Berzborn, in prep.). Moreover different CF_1 preparations exhibit different specific ATPase activities not due to a varying CF_1 content [1]. Thus a calibration via this activity of the enzyme cannot be performed.

Another way of unmasking the ATPase activity of CF_1 is heat treatment in the presence of thiols [6]. This method is only feasible for soluble CF_1 and at high protein concentrations (higher than 0.5 mg/ml). Therefore it is useless for our intended binding studies and absolute determinations of the membrane bound enzyme.

Because of the disadvantages of enzymatic methods we tried to use electroimmunodiffusion for the quantitative determinations of either soluble or membrane bound CF_1 [7–9]. The established method is much simpler than the one described earlier [10]; cp. discussion. In this paper the method will be used to demonstrate the variability in CF_1/chl content of thylakoids and to recalculate the turnover time of the enzyme.

Materials and Methods

Chemicals were of reagent grade (p.A.) and purchased from Merck, except PMS, BSA, Coomassie brilliant blue G 250, acrylamide, Na-desoxycholate and Triton X-100 from Serva; ADP from Boehringer; N,N-methylene-bis-acrylamide and am-

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Abbreviations: Chl, chlorophyll; EDTA, ethylendiaminetetraacetic acid; EID, electroimmunodiffusion; PMS, phenazine-methosulphate.

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moniumheptamolybdate from Fluka; agarose from Sigma.

Spinach was grown in a growth chamber at 19 °C with 13 h light and 11 h darkness under various light intensities.

Chloroplast thylakoid systems were prepared as described in [1]. Standard procedures were used for chlorophyll determination [11], isolation of CF_1 [12], cyclic photophosphorylation with PMS [13], ^{32}P-determination [14] and protein determination [15].

Preparation of CF_1-depleted thylakoids: Thylakoid membranes (0.1 mg Chl/ml) were gently stirred at room temperature in 0.75 mM EDTA for 10 min. The pH must be kept at 7.2 or slightly higher values. By addition of small amounts of NaCl (2–5 mM) a different degree of CF_1-depletion can be obtained [1]. After extraction the membranes were sedimented for 10 min at 27000 × *g* (4 °C) and resuspended in an appropriate buffer.

SDS gel electrophoresis using the gel system of Laemmli [16] with an acrylamide concentration of 12.5% in the separating gel was done as described by Lughtenberg *et al.* [17].

Ouchterlony double diffusion tests [18] were performed at room temperature in 0.8% agarose in barbital buffer, pH 8.6, $\mu = 0.02$. After about 24 h the diffusion was stopped by washing the gels twice for about 24 h in 2% NaCl and another 24 h in distilled water. The gels were dried and the precipitation lines stained in 0.1% amidoblack/45% methanol/10% acetic acid for 10 min. Destaining was done in 45% methanol/10% acetic acid three times for 10 min.

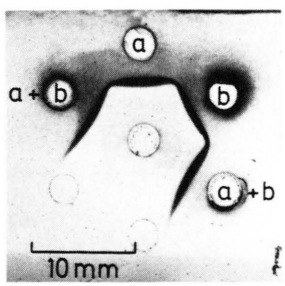

Fig. 1. Test for immunochemical relation of soluble CF_1 and solubilized CF_0CF_1 in Ouchterlony double diffusion. (Central well: anti – CF_1, 152-t; antigens: a) purified CF_1, 6 µl of 0.8 mg/ml; b) thylakoid membranes, solubilized by 3% Triton X-100, 6 µl of 1.3 mg Chl/ml; a + b: 3 µl of a) mixed with 3 µl of b).

Electroimmunodiffusion according to Laurell [19] was modified: Agarose concentration and barbital buffer, both in the gel and for the electrode, are the same as for the Ouchterlony double diffusion. The antiserum was mixed with the gel after boiling and cooling down the gel to 58 °C. The amount of the antiserum to be used is dependent on its precipitation titer and the expected antigen concentration in the samples. Microscopic slides in LKB-Gelman-Camag frames were used. 10 ml of antibody containing agarose gel per 3 slides yield a thickness of the separation gel of 1.35 mm.

Electrophoresis was performed for 18 h at 15 °C at a field strength of 3.4 to 5.3 V/cm with sample volumes of 6 µl.

Results

1) Conditions for electroimmunodiffusion of soluble CF_1

Quantitative determinations of CF_1 by EID requires a monospecific antiserum against CF_1. The monospecificity of the antiserum used (152-t) is demonstrated in Fig. 1 by an Ouchterlony double diffusion test: There is only one precipitation line with a crude extract of CF_1. To exclude a fortuitous superposition of more lines by the reaction of the antiserum with more than one antigen in the crude extract we also tried to displace and resolve such lines by adding CF_1 to the crude extract without success. Moreover we always obtained only one "rocket" in EID with various crude and purified CF_1 fractions (*e.g.* Fig. 2).

Rocket formation with soluble CF_1 is obtained without any difficulty. Fig. 2A shows the precipitation pattern of 3 samples containing different amounts of CF_1. Using a field strength of 3.5 to 5.3 V/cm (120 V with the LKB frame) electrophoresis should be performed for at least 18 h at 15 °C (Fig. 3). Prolonged electrophoresis did not affect the final rocket length (Fig. 3). As usual in EID a higher field strength (300 V, the suitable condition for migration in an immunoelectrophoretic analysis within 30 to 40 min) produced longer and narrower rockets, but often resulted in a double contoured rocket [20] also with our antigen. Due to lateral diffusion of both the CF_1 and the antibodies, a lower field strength leads to wider and shorter rockets. Under the described conditions the

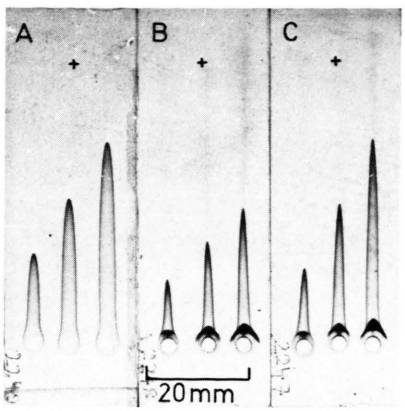

Fig. 2. Precipitation pattern ("rockets") in electroimmunodiffusion into an antibody containing gel with different amounts of CF_1. Direction of migration: upwards to the anode. Antigens A: purified soluble CF_1, 6 µl each with 1 µg, 2 µg and 3 µg, resp. B: thylakoids from spinach, grown at 5000 lux (6 µl each with 1.5 µg, 3 µg and 6 µg Chl, resp.). C: thylakoids from spinach grown at 14 000 lux (amounts as in B). All samples contained 3% triton X-100. gel: 0.8% agarose in barbiturate buffer, $\mu = 0.02$; pH = 8.7 25 µl antiserum 152-t against CF_1/10 ml gel. Electrophoresis: 16 h at 80 V (*i.e.* 3.5 V/cm) at 15 °C (an unspecific green precipitation triangel was in this case not removed during washing).

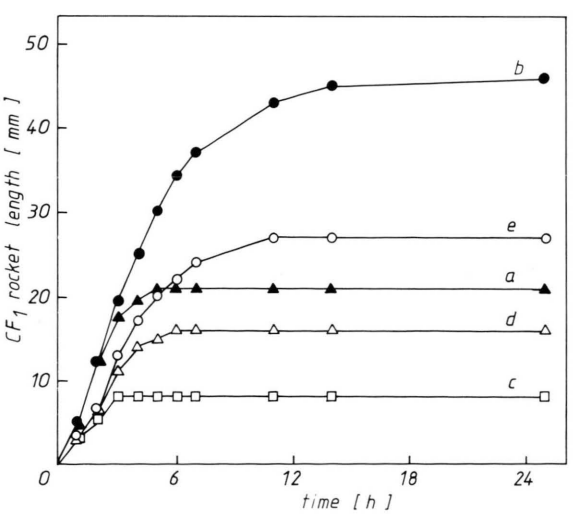

Fig. 3. Time course of rocket formation obtained with different amounts of CF_1, as measured by the rocket length. EID as described under methods, 3.5 V/cm. a) 2.5 µg CF_1; b) 5 µg CF_1; c), d), e) triton solubilized thylakoids with 2 µg, 3.5 µg and 6.9 µg Chl, resp. antiserum: 25 µl 152-t/10 ml gel.

dependency of the rocket length on CF_1 concentration is linear (Fig. 4). It is important that equal sample volumes are applied for the analysis.

In EID there is a reciprocal dependency between rocket length and serum dilution. With low amount of antibody in the gel a reliable measurement (rocket more than 1 mm) could be achieved with down to 0.02 µg CF_1, *i.e.* 6 µl of a solution with 4 µg/ml.

Same amounts of protein of different CF_1 preparations with about the same purity (estimated by SDS gel electrophoresis) produced an identical rocket length, although the ATPase activities were not identical (not shown). Therefore soluble CF_1 can be determined in absolute terms, *e.g.* in any crude extract or column fraction, after calibration of the rocket by means of pure CF_1.

2) Conditions for electroimmunodiffusion of membrane bound CF_1

For quantitative determinations of membrane bound CF_1 by this method, it is necessary either to extract CF_1 completely from the thylakoid membrane [10] or to dissolve the membrane. The simpliest way for membrane solubilization is to use detergents. Appropriate conditions were found to be: Incubation of suspended thylakoid membranes up to 1 mg Chl/ml with 3% Trition X-100 for 5 min at room temperature. By a short centrifugation starch was removed; a colourless pellet proves a complete solubilization of the thylakoids. The supernatant can be applied directly to the gels. Fig. 5 shows that again the length of the CF_1 rocket is a linear function of the amount of solubilized thylakoids applied, as measured by their chlorophyll content. It was found, that identical conditions can be used for electrophoresis of both soluble and membrane bound (solubilized) CF_1.

Addition of Triton X-100 to the gel was not necessary, although possible; it did not inhibit the antibody reaction up to a concentration of 1%; the rocket area was the same, but the rocket length was approximately 15% shorter.

To render possible an absolute determination of the membrane bound enzyme by calibration with purified soluble CF_1, the rocket lengths produced by soluble and "membrane bound" CF_1 – after solubilisation – have to be directly comparable. To varify this a comparative immunochemical and bio-

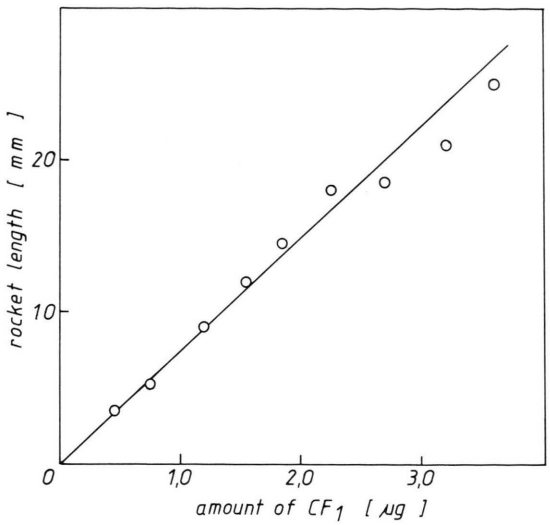

Fig. 4. Correlation of rocket length with amount of soluble, purified CF_1. (Antiserum: 40 µl 150-4/10 ml gel; electrophoresis: 5.3 V/cm).

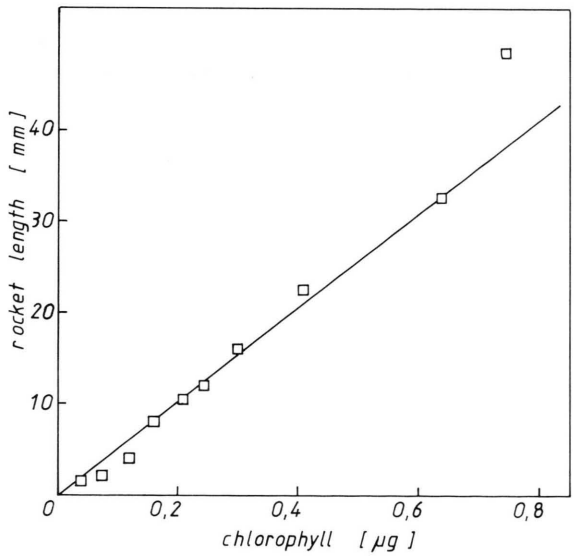

Fig. 5. Correlation of rocket length with amount of triton solubilized thylakoids, also demonstration of sensitivity of EID. (Conditions as in Fig. 4; antiserum: 10 µl 150-4/10 ml gel).

chemical analysis of the ATPases in purified form and after membrane solubilization was carried out, respectively.

For the ATPsynthase of *Rodospirillum rubrum* it has been shown that the entire enzyme complex (F_0F_1) is preserved after membrane solubilization with Triton X-100 [21, 22]. Analysis by SDS gel electrophoresis of an immunoprecipitate obtained with Triton solubilized thylakoids and a monospecific antiserum to CF_1 showed that this is true for the chloroplast enzyme (CF_0CF_1), too (Fig. 6). This means that the precipitation lines obtained with CF_1 or solubilized thylakoids in EID (and Ouchterlony) are different in their composition, *i.e.* containing antibody complexes with CF_1 or CF_0CF_1, respectively. In spite of this the two different antigens show immunological identity if tested with antisera against CF_1 (Fig. 1). Moreover Table I indicates that equal molar amounts of soluble and solubilized membrane bound CF_1 produce an identical rocket length under the described conditions: After partial resolution of CF_1 from the thylakoids by EDTA the sum of the rocket lengths obtained with the membrane and corresponding supernatant fractions is constant and identical to the value of untreated thylakoids (100%). Consequently an absolute and specific determination of membrane bound CF_1 is possible after solubilisation with Triton X-100 with this immunochemical method by calibration with purified CF_1.

The rocket length of either membrane bound or separated CF_1 are independent upon the presence in the sample of different substances in the following concentration ranges (other concentrations not tested): 5–100 mM NaCl; 0–7.5 mM EDTA; 0–50 mM $(NH_4)_2SO_4$; 0–100 mM sucrose; 5–50 mM Tricine-NaOH, pH 8.0; 1–3% Triton X-100 for CF_0CF_1 and 0–3% Triton X-100 for separated CF_1, respectively. Addition of increasing amounts of Na-desoxycholate resulted in reduced CF_1 rocket lengths, so that equal concentrations of this detergent have to be present, if different samples are

Table I. Comparison of CF_1 rocket lengths obtained with preparations of thylakoid membrane and corresponding supernatants. (Untreated and EDTA treated thylakoid membranes were solubilized in 3% Triton X-100. The supernatants were concentrated in a vacuum evaporator at 25 °C about ten times and Triton X-100 was added to a final conc. of 3%. Electrophoresis: 3.5 V/cm; antiserum against CF_1, 152-t, 25 µl/10 ml gel.)

Treatment of thylakoid membrane	CF_1-rockets (mm/10 µg Chl) membrane	supernatant	sum
control	50.9	0	50.9
EDTA, 3 mM NaCl added	19.4	31.8	51.2
EDTA, no NaCl added	8.5	43.2	51.7

compared in the presence of this detergent. This probably is also required in the presence of other negatively charged detergents.

Statistical analysis of the newly applied method including all steps of the procedure like chlorophyll and protein determination, dilution of the samples, preparing the agarose gels and application of the samples gave a standard deviation of 2.7–4.2% for rocket lengths above 10 mm (6 µl sample, hole 3 mm diameter). Below this value the deviation increased up to 7.5%. For various CF_1 preparations of about the same purity (estimated by SDS gel electrophoresis) the standard deviation of the obtained rocket lengths by equal amounts of protein was in the range of 3–5%; this is important for calibration with purified CF_1.

3) Quantitative determination of CF_1 in thylakoids

The absolute figures for the CF_1 content of thylakoid membranes determined on a chlorophyll basis by different methods vary in the range of 1:60 to 1:980 CF_1/Chl [3, 9, 10, 23–26]. After the publication of Strotmann *et al.* [4] a value of 1 CF_1/800 Chl is widely used, however, and recently confirmed by Frasch *et al.* [10]. Now we want to stress that the CF_1/Chl ratio is not a constant number. We found that this ratio varies in plants grown under different light intensities (Fig. 2 B and C, and Table II). Thylakoids of spinach plants grown under high light exhibit about the CF_1 content as was determined earlier [4, 10], but decreasing the light intensity resulted in a lower CF_1 content.

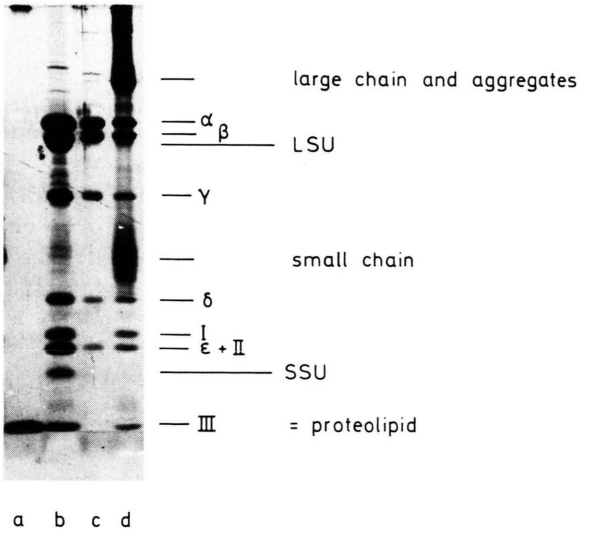

Fig. 6. Analysis by SDS gel electrophoresis of an immunoprecipitate obtained with an antiserum against CF_1 and thylakoid membranes solubilized by Triton X-100. (0.5 ml thylakoids, 1 mg Chl/ml, were incubated for 30 min with 0.3 ml antiserum, 152-t.) The obtained precipitate was washed twice with 5 mM tricine-NaOH pH 7.8 and 0.2% Triton X-100 [28], solubilized in 3% SDS and applied for analysis. Slot a: proteolipid of CF_0*; slot b: CF_0CF_1 complex*; slot c: purified CF_1; slot d: immunoprecipitate; *proteolipid and CF_0CF_1 complex were isolated by L. Klein-Hitpaß from our laboratory. The samples were not boiled for solubilization with SDS on purpurse; after boiling the large chain of the γ-globulin would be superimposed on the β subunit of CF_1.

Table II. Influence of the light intensity during growth on the CF_1 content and photophosphorylation capacity of isolated thylakoids. (The ratio of CF_1/Chl was determined by EID; photophosphorylation capacity was determined in cyclic PMS mediated electron transport [13] under saturating red light at 20 °C.)

Properties of isolated thylakoids	Light intensity during growth of spinach plants	
	5000 lux	14 000 lux
ratio of CF_1/Chl (mol/mol)	1:1260	1:740
photophosphorylation capacity (µmol ATP/mg Chl × h)	702	1240

Simultaneous measurement of the photophosphorylation capacity of the isolated thylakoids in cyclic electron transport mediated by PMS showed a direct correlation of CF_1 content and phosphorylation capacity [9], confirming that the number of CF_1 molecules is the limiting factor for phosphorylation in this electron transport system (the latter conclusion can be drawn from the fact, that oversaturating light increased PMS cyclic photophosphorylation of EDTA treated thylakoid systems, but not in the control; Fig. 3 in ref. 1). Due to this a turnover time of 4 ms per ATP and CF_1 molecule can be calculated from our results, a value already obtained by Witt *et al.* [27] by a completely different approach.

Discussion

For the elucidation of many problems of photosynthetic ATPsynthase a simple method for quantitative determination of membrane bound and soluble CF_1 is needed [8]. As explained in the introduction the enzymatic methods have disadvantages in this respect. Therefore we have adapted and established EID as a powerful tool for routine deter-

minations of CF_1. The newly applied method has the following advantages and characteristics:

1. High sample numbers (*i.e.* 100) can be analyzed by one person in one day.
2. The high sensitivity allows analysis of samples with CF_1 concentrations down to 4 µg/ml.
3. A small sample volume (6 µl) is appropriate; *i.e.* the method needs very little of a sometimes limited material.
4. The specificity is given with the monospecificity of the antiserum.
5. The determination is independent of the activity and purity of the enzyme. Many substances usually present in biochemical samples do not effect the final rocket length.
6. The results are reproducible with a standard deviation of about 4% for rocket lengths above 10 mm.
7. A direct comparison of the rocket lengths obtained with separated and membrane bound CF_1 is possible, allowing an absolute determination of the membrane bound enzyme by calibration with purified CF_1, in nearly any fraction of chloroplast preparations.
8. An unspecific precipitation of green material (chlorophyll-protein complexes?) can be removed from the gels by adding Triton X-100 (1%) to the washing NaCl.

For the application of the method the characteristics of the antiserum used have to be known. The antiserum has not only to be monospecific to CF_1 but must also have the same precipitation titer for CF_1 and CF_0CF_1. This is not necessarily always the case; antibodies directed against the CF_0 binding region of CF_1 could be present. Antibodies with this specificity would not be able to react with the entire undissociated complex. However, all the antisera produced and tested in our laboratory did not contain such antibodies (no spurs; cp. Fig. 1).

For the determination of the membrane bound CF_1 either extraction or membrane solubilization is necessary. Prior extraction carried out by Frasch *et al.* [10] has the disadvantage that either there is no complete CF_1 extraction ($CHCl_3$-method) or that a series of 5 extraction steps had to be carried out (EDTA-method). The supernatant of each washing step had to be analyzed, thus increasing the sample number by a factor of 5. Moreover repeated washing steps result in loss of material especially when using small sample volumes; this, however, is necessary in the intended binding studies (Roos and Berzborn, in prep.).

Considering the agreement in the CF_1/Chl determinations by Strotmann *et al.* [4] and Frasch *et al.* [10] one is induced to assume that the CF_1 content of thylakoids is a nearly constant value. In contrast to this our determinations show that the CF_1/Chl ratio of thylakoids can vary up to more than 100% because this value is dependent on the light intensity during growth of the spinach plants. A decrease of the CF_1/Chl ratio, correlated with a lower photophosphorylation capacity, was observed with spinach plants grown under low light in the growth chamber [9] or bought at winter time on the local market.

A physiological interpretation of this phenomenon would be that under low light the chlorophyll content of the thylakoid system is elevated, since light absorption is limiting photosynthesis under low light and not the enzymes, *e.g.* the ATPsynthase. A more detailed discussion in connection with the extent of grana formation is given in Berzborn *et al.* [9]. From our results we cannot decide whether the altered CF_1/Chl ratio is due a change in CF_1 and/or the chlorophyll content of the chloroplasts. This leads to the question of how and how fast the overall-composition of the thylakoid membrane is changed by light induced or light triggered processes, and how the composition of the thylakoid membrane system determines their structure, *i.e.* morphology.

Acknowledgements

This work was supported by grant Be 664 to R. J. B. by the DFG. Skillful technical assistance of Mrs. M. Tobien-Caesar and R. Heilek-Oworah is much appreciated.

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