Physiological Characterization of *Cyanophora paradoxa*, a Flagellate Containing Cyanelles in Endosymbiosis

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Z. Naturforsch. 33 c, 981 – 987 (1978); received October 9, 1978

*Cyanophora paradoxa*, Endosymbiosis, Cyanelles, Cell-Metabolism, Nitrogen Assimilation

Culture conditions are described under which *Cyanophora paradoxa* grows with a generation time of less than two days. The organism is an obligate photoautotrophic flagellate unable to degrade exogenous carbohydrates via respiration. It does not synthesize nitrogenase but can form a hydrogenase with similar properties as in blue-green algae. The photosynthetic $\text{O}_2$-evolution proceeds with essentially the same rate as in green or blue-green algae. Besides $\text{CO}_2$, $p$-benzochinon, nitrate and nitrite also stimulate the photosynthetic $\text{O}_2$-evolution. Nitrite reduction is strictly light-dependent where the stoichiometry between nitrite-disappearance and photosynthetic $\text{O}_2$-evolution is 1:1.5. It is concluded that the cyanelles supply the eucaryotic host both with carbon and nitrogen compounds.

*Cyanophora paradoxa* is a small, unicellular flagellate difficult to catagorize systematically. It was classified either with cryptomonads [1] or with primitive dinoflagellates [2], and there were also proposals to consider an own class of organisms comprising *Glaucocystis* and *Gloeochaete* besides *Cyanophora* [3, 4]. More interesting to note, *Cyanophora* contains 1–6 endosymbiotic, blue-greenish structures which are termed cyanelles, which reproduce in an autonomous way [5] and which obviously take over functions of the chloroplasts of higher plants [6]. The nature of these cyanelles is also unclear at present. Cytological investigations [7] and the pigment analysis [8] indicated that the cyanelles were of cyanophycean origin. However, Schnepf and Brown [9] considered the cyanelles as chloroplasts and the intact organisms as primitive algae of the rhodophycean type. A recent study [10] of the DNA content and of the genome size also indicated that the cyanelles are chloroplast type organelles rather than endosymbiotic blue-green algae.

The unresolved taxonomic affinity is not the only feature which deserves attention with *Cyanophora*. Such an organism where blue-green algae or chloroplast type organelles are housed in an eucaryotic cell may constitute an ancient relic in the evolution of chloroplasts and mitochondria. Moreover, there are now attempts to bring nitrogen fixing blue-green algae into protoplasts of higher plants [11]. These have as their final goal the establishing of symbiotic organisms which would be self-supplying in nitrogen compounds. With *Cyanophora*, nature may already have provided such a stable symbiosis which was, however, not yet investigated with respect to its nitrogen metabolism. This was probably due to the fact that *Cyanophora* and all other organisms containing cyanelles grew very slowly which impeded physiological experiments.

The present investigation was undertaken to improve the culture conditions for *Glaucocystis geitleri* and *Cyanophora paradoxa*. Whereas all attempts to obtain short growth rates failed with the former organism, conditions were found where *Cyanophora* consistently grew with a generation time of less than two days. Thus sufficient quantities of cells were available for a physiological characterization of *Cyanophora*. This communication reports experiments on photosynthesis, respiration, hydrogen metabolism and nitrogen assimilation with emphasis on the latter process.

**Materials and Methods**

*Cyanophora paradoxa* was generously given to us in pure culture by Dr. Kies, Hamburg. *Glaucocystis geitleri* was No. 229 – 5 of the Sammlung von Algenkulturen of the Pflanzenphysiologisches Institut, University of Göttingen, Germany. *Cyanophora* was grown in sterile media with three different nitrogen sources. The nitrate-medium contained the following salts in $m$: $\text{KNO}_3$, $10^{-3}$; $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$.
4 \times 10^{-5}; \text{CaCl}_2 \times 2\text{H}_2\text{O}, 5 \times 10^{-5}; \text{CoCl}_2, 10^{-6}; 
\text{H}_3\text{BO}_3, 10^{-3}; \text{MnCl}_2 \times 4\text{H}_2\text{O}, 10^{-5}; \text{ZnSO}_4 \times 7\text{H}_2\text{O}, 10^{-6}; 
\text{CuSO}_4 \times 5\text{H}_2\text{O}, 10^{-7}; \text{NaMoO}_4 \times 2\text{H}_2\text{O}, 10^{-7}; 
\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}, 7.5 \times 10^{-5}; \text{FeSO}_4, 2.5 \times 10^{-5} \text{as the EDTA complex, vitamin B}_1, 4 \times 10^{-6}; 
\text{vitamin B}_12, 1.2 \times 10^{-8}; \text{a mixture of Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4 
adjusted to pH 8.0, 8 \times 10^{-4}. \text{In the ammonia-medium, nitrate was replaced by (NH}_4\text{)}_2\text{HPO}_4, 3.75 \times 10^{-4}, 
\text{and the nitrate-ammonium medium contained KNO}_3, 1 \times 10^{-3} \text{plus (NH}_4\text{)}_2\text{HPO}_4, 7.5 \times 10^{-5}. \text{The phosphate salts and the iron-EDTA complex were}
\text{autoclaved separately, and the vitamins were added after sterile filtration. With all media, the pH-value was adjusted to 8.0. The same media were also tried}
\text{for Glaucocystis with different pH-values varying from 6.5 to 8.0.}

\text{Cyanophora} \text{was grown at 24}^\circ \text{C or 18}^\circ \text{C and}
1100 \text{lux in 100 ml Erlenmeyer or 21 Fernbach}
flasks closed with cotton wool, and diluted with
fresh culture medium at the rate of approximately
1:5 every three to four days. The motile cells distributed almost homogeneously throughout the culture
medium, although some accumulation was often
observed on the surface of the solution. For the
experiments, the cultures were concentrated 10—20
fold of the original volume by centrifugating at
5000 \times \text{g and 4}^\circ \text{C for 10 min and suspending the}
cells in the medium desired in the assays.

The photosynthetic and respiratory activities were
assayed by conventional Warburg manometry. The
photosynthetic O_2-evolution rate was determined
under argon. Before the start of the experiments, the
cells were routinely illuminated in the vessels
for 30—60 min. This completely terminated the O_2-
evolution activity probably caused by the endo
genous CO_2 which could not be removed by gassing.
After tipping the Hill-reagents from the side arms of
the vessels, the assays were conducted at 25 \degree\text{C}
for 1—2 h and 35000 \text{lux. Such high light intensi}
ties did not affect the photosynthetic activities of
the organism in these short-termed experiments. In order
to determine the respiratory O_2-uptake capa
bilities, the center well of the Warburg vessels con
tained 0.2 ml of 10% KOH to bind the CO_2 evolved.

The measurement of the C_6H_5-reduction (nitrogen
fixation) and H_2-uptake activities were described in
detail in the preceeding publications [12, 13].

In order to determine the nitrate and nitrite assimila
tion rates, the organisms were washed twice in
a medium free of nitrate before the assays were
conducted for 1 h and 25 \degree\text{C. They were terminated by}
boiling the organisms for 15 min to completely
release the nitrate and nitrite not assimilated. After
centrifugation, the amount of nitrite could directly
be measured with Gries-Ilovay’s reagent (α-naphthyl-
amine and sulfanilic acid) in the supernatant.
Nitrate was also determined as nitrite. For this
purpose, it had to be converted enzymatically with
nitrate reductase from \text{Escherichia coli} using a
slightly modified version of the procedure described
by Ohmori and Hattori [14]. The reaction mixture
to convert nitrate to nitrite contained in a final
volume of 3.8 ml in \mu mol: Tris-HCl-buffer pH 7.6, 
200; Na_2S_2O_4, 30; methyl viologen, 3; NaHCO_3,
30; NaNO_3 to be converted 0—5; and 2 ml of the
nitrate reductase preparation (see below). The re
action mixture was incubated for 30 min at 30 \degree\text{C}
in test tubes, and during this period care was taken
that the solutions remained deep blue due to reduced
methyl viologen. To end the reaction, the dye was
oxidized by air, and the test tubes were boiled.
After centrifugation, the Gries-Ilovay’s reagent was
added to the supernatant, a resulting precipitate was
removed by centrifugation and the extinction of the
diazo couple was determined at 546 nm 15 min
after the addition of the reagent. As a reference, a
standard of nitrate, treated in exactly the same way
as the samples, was required, since nitrate was only
partially recovered as nitrite in these assays with
\text{Escherichia coli} nitrate reductase.

Nitrate reductase was either purchased from
Sigma, St. Louis, No. N 3255 or (preferentially)
prepared by suspending 2 g of \text{Escherichia coli} cells
(obtained from Merck, Darmstadt, No. M 1132) in
10 ml of 0.2 M Tris-HCl buffer pH 7.6 containing
2 mg of dithiothreitol. The cells were broken by
sonication and centrifuged for 10 min at 10000 \times \text{g}.
The supernatant was used as the nitrate reductase
preparation.

Chlorophyll was determined in cells broken by
sonication using the extinction coefficient of 82 \text{mm}^{-1}
\times \text{cm}^{-1} at 663 [15]. Protein was measured accord
ing to Lowry et al. [16]. Cell numbers were counted in
a Thoma chamber after having immobilised the
organisms by adding a drop of formaldehyde to the
suspension. Chlorophyll and carotinoids were se
parated by thin layer chromatography and phyco
biliproteins by gel-filtration on Sephadex G-200.
The spectra were record with a Perkin-Elmer 200
photometer and compared to standards.
Results

In the literature [6], Cyanophora was reported to grow very slowly. Using the three media described under Materials and Methods, the organism consistently showed fast growth (Fig. 1). The exponential phase lasted from the second to the sixth day after the inoculum, and the generation time was 1.8—2.4 days in the nitrate-ammonia medium and 1.6 to 2.0 days when nitrate was the only source for the cultures. In the ammonia-medium, the generation time was somewhat longer with 2.0—3.0 days. Cyanophora grew strictly photoautotrophically. The addition of organic carbon compounds (glucose, fructose, lactose, galactose, maltose, sucrose, succinate, pyruvate, acetate) to the media did not enhance the growth rate; on the contrary, concentrations above 0.1 osmolar of all these compounds caused the organisms to bleach out and finally to die. Vitamin B\textsubscript{12} was routinely added to the media but the absolute dependence of the growth on these substances was not seriously checked. The temperature could vary between 18 ° and 24 °C and low light intensities (under 1500 lux) were required. Cyanophora was easy to handle and thus is an organism suitable for physiological experiments. By comparison, the generation time is 20 h for the nitrogen fixing species Anabaena cylindrica and Nostoc muscorum.

Contrary to the situation with Cyanophora, the generation time of Glaucocystis geitleri was 15—20 days under best obtainable conditions with all nutrient solutions tried. Moreover, this organism tended to adhere tightly to the glass of the vessels which could only partially be prevented by covering the bottom of the flasks with a sheath of agar. In addition, the cells did not homogeneously suspend in solutions. Therefore the following experiments were exclusively done with Cyanophora.

The pigment analysis confirmed the results of Chapman [8]. Cyanophora contained chlorophyll a, c- and allo-phycocyanin, $\beta$-carotene and zeaxanthin. Chlorophyll b, lutein, echinenone as well as myxoxanthophyll were not detected. In NO\textsubscript{3}~--grown cultures harvested from the exponential growth phase, 1 mg chlorophyll corresponded to 3.25 × 10\textsuperscript{9} cells and to 95 mg soluble protein, whereas the ratio between chlorophyll, cell number and protein content was 1:2.43 × 10\textsuperscript{9}:62 in NH\textsubscript{4}+-grown cells.

The photosynthetic O\textsubscript{2}-production at the expense of NaHCO\textsubscript{3} was approximately 120 $\mu$mol/h × mg chlorophyll and hence is in the same range of the rates observed with blue-green or green algae. Of the other electron acceptors tested (Table I), p-benzochinon, nitrite and to some extent also nitrate could stimulate the photosynthetic O\textsubscript{2}-production,
Table I. The photosynthetic O₂-evolution by intact *Cyanophora paradoxa* in the presence of various electron acceptors. The Warburg experiments were conducted for 30—60 min at 25 °C and 35 000 lux under argon. The vessels contained in a final volume of 3 ml: chlorophyll, 0.03—0.09 mg; electron acceptors, 10 µmol, unless indicated otherwise. DCMU was 1 × 10⁻⁵ M in No. 9 and 10.

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>µmol O₂ evolved/h × mg Chl</th>
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<tbody>
<tr>
<td>1. —</td>
<td>—</td>
</tr>
<tr>
<td>2. NaHCO₃</td>
<td>+122</td>
</tr>
<tr>
<td>3. K₃Fe(CN)₆</td>
<td>+ 3</td>
</tr>
<tr>
<td>4. p-benzochinon</td>
<td>+ 28</td>
</tr>
<tr>
<td>5. K₃Fe(CN)₆+p-benzochinon (0.5 µmol)</td>
<td>+ 29</td>
</tr>
<tr>
<td>6. p-benzochinon (0.5 µmol)</td>
<td>+ 29</td>
</tr>
<tr>
<td>7. NaNO₂</td>
<td>+ 13</td>
</tr>
<tr>
<td>8. NaNO₂ (5 µmol)</td>
<td>+ 55</td>
</tr>
<tr>
<td>9. p-benzochinon + DCMU</td>
<td>— 7</td>
</tr>
<tr>
<td>10. NaNO₂ + DCMU</td>
<td>± 0</td>
</tr>
</tbody>
</table>

whereas K₃Fe(CN)₆ did not do so. The latter compound was also ineffective in the presence of catalytic amounts of p-benzochinon, contrary to the situation in *Anacystis nidulans* [17]. The O₂-production in *Cyanophora* was affected by the herbicide DCMU similar as in other photosynthetic organisms having two light reactions.

The endogenous respiratory rate accounted to 15 µmol O₂ consumed/h × mg chlorophyll in the dark. This activity was not stimulated by glucose, fructose, maltose, galactose, sucrose and succinate, which might reflect the obligate photoautotrophic nature of this organism. Some enhancement of the rates was observed by pyruvate (18 µmol O₂ consumed/h × mg chlorophyll) and acetate (22 µmol) which was, however, marginal. The endogenous respiration was not stimulated by the uncouplers dinitrophenol and FCCP.

Table II indicates that *Cyanophora* did not reduce acetylene when the cultures were kept either aerobically or anaerobically in the absence of any combined nitrogen. Since the organism did not grow under these conditions, it is obviously unable to synthesize nitrogenase. It can, however, form hydrogenase. This enzyme was not present in air-grown cells but was induced by anaerobic conditions and became fully active when the cultures were continuously flushed with molecular hydrogen (Table II).

Table II. H₂-consumption and N₂-fixation (C₂H₂-reduction) by *Cyanophora paradoxa*. The organism was grown for 3—6 days in the nitrate medium a) anaerobically under flushing with a mixture of 20% H₂/ 75% N₂/ 5% CO₂, b) anaerobically under flushing with 95% N₂/ 5% CO₂, c) aerobically without flushing, in 1 liter flasks at 22 °C and 1100 lux. Rates are given per h × mg Chl.

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>µmol C₂H₄ formed</th>
<th>µmol H₂ consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td>anaerobically H₂/N₂/CO₂</td>
<td>0</td>
<td>52</td>
</tr>
<tr>
<td>anaerobically N₂/CO₂</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>aerobically —</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 2. Dependence of the H₂-consumption on the presence of varying concentrations of oxygen. a) *Cyanophora* grown under H₂/N₂/CO₂ (see Table II) ■ — ■ assayed in the dark; X — X assayed in the light. b) *Cyanophora* grown under N₂/CO₂. ■ — ■ assayed in the dark; X — X assayed in the light.
Similar as recently observed for several blue-green algae [18], hydrogen uptake in *Cyanophora* was dependent on the presence of oxygen in the vessels. Fig. 2 shows that the optimal concentration of this gas was approximately 10 mmolar which corresponds to 20% in the gas phase. High levels of oxygen in the assays did not significantly decrease the H₂-consumption activity, indicating that hydrogenase in intact *Cyanophora* is virtually insensitive to exposure to oxygen.

Since *Cyanophora* grew on nitrate as the sole source of nitrogen, it is able to form the assimilatory nitrate and nitrite reductases. Table III shows that nitrate disappeared in the assays conducted in the dark. The rate of nitrate reduction was enhanced more than twofold when the samples were illuminated, and this light-dependent activity was accompanied by a production of oxygen. Contrary to nitrate, no nitrite was consumed in the dark, whereas the assimilation rate was high in illuminated samples (Table III). This already indicated that nitrite reduction was linked to the photosynthetic electron transport system of the cyanelles. The situation appeared to be similar as in chloroplasts of higher plants where nitrite is reduced at the acceptor side of photosystem I in a ferredoxin dependent reaction [19]. This would mean that nitrite reduction in *Cyanophora* should be accompanied by a stoichiometric release of oxygen according to the following equations:

\[
\begin{align*}
(I) \quad \text{NO}_2^- + 6 e^- + 8 H^+ & \rightarrow \text{NH}_4^+ + 2 H_2O \\
(II) \quad 3 H_2O & \rightarrow h^+ v \rightarrow 3/2 O_2 + 6 H^+ + 6 e^- \\
\text{(sum)} \quad \text{NO}_2^- + 2 H^+ + H_2O & \rightarrow 3/2 O_2 + \text{NH}_4^+. 
\end{align*}
\]

Table III. The reductions of nitrate and nitrite by *Cyanophora paradoxa*. The assays were conducted at 25 °C, 1 h and 35 000 lux or in the dark. The vessels contained in a final volume of 3 ml: nitrate or nitrite, respectively, 5 μmol, *Cyanophora* with 0.05 mg Chl. Negative data in the table are due to endogenous respiration.

<table>
<thead>
<tr>
<th>Nitrate reduction</th>
<th>Light</th>
<th>Dark</th>
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</thead>
<tbody>
<tr>
<td>a) Nitrate consumption</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td>b) O₂-evolution</td>
<td>6</td>
<td>-5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrite reduction</th>
<th>Light</th>
<th>Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Nitrite consumption</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>b) O₂-evolution</td>
<td>56</td>
<td>-5</td>
</tr>
</tbody>
</table>

Table IV. Stoichiometry between nitrite disappearance and photosynthetic O₂-evolution. Data are given in μmol. Experimental conditions as in Table III.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Light</th>
<th>Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. NO₃⁻ added at the start of the experiment</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>2. NO₃⁻ recovered at the end of the experiment</td>
<td>0.8</td>
<td>2.9</td>
</tr>
<tr>
<td>3. NO₃⁻ consumed</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>4. O₂ evolved photosynthetically</td>
<td>1.8</td>
<td>2.9</td>
</tr>
<tr>
<td>5. ratio O₂ formation: NO₃⁻ consumption</td>
<td>1.5:1</td>
<td>1.4:1</td>
</tr>
</tbody>
</table>

Table IV shows that the theoretical ratio of 1.5:1 between photosynthetic O₂-production and nitrite disappearance closely matched the results of the experiments with three different nitrite concentrations. Thus the conclusion is valid that nitrite reduction is exclusively confined to the cyanelles of *Cyanophora*.

Dinitrophenol is known to cause green algae to excrete nitrite to the medium [20]. This effect cannot yet unequivocally be interpreted, but it appears to be restricted to eucaryotic cells. Table V shows that such an excretion of nitrite was observed with the green alga *Ankistrodesmus braunii* as a control, but neither in the blue-green algae *Anabaena cylindrica* and *Anacystis nidulans* nor in *Cyanophora paradoxa*, regardless of whether high or low cell numbers were tried in the assays. From this we tentatively conclude that nitrate assimilation in *Cyanophora* follows the same mechanism as in blue-green algae, although more organisms should be investigated before such a generalization is safe.

**Discussion**

The present investigation shows that *Cyanophora* is an organism easy to grow and to handle. It is an obligate photoautotrophic flagellate which is unable to consume sugars by respiration. Concentrations

<table>
<thead>
<tr>
<th>Alga</th>
<th>Concentration of dinitrophenol</th>
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<tbody>
<tr>
<td></td>
<td>1 × 10⁻⁴ M</td>
</tr>
<tr>
<td>1. <em>Ankistrodesmus braunii</em></td>
<td>0.2</td>
</tr>
<tr>
<td>2. <em>Anacystis nidulans</em></td>
<td>0.5</td>
</tr>
<tr>
<td>3. <em>Anabaena cylindrica</em></td>
<td>0.5</td>
</tr>
<tr>
<td>4. <em>Cyanophora paradoxa</em></td>
<td>0.5</td>
</tr>
</tbody>
</table>
of these substances above 0.1 osmolar cause irreversible damage to the organism, probably due to osmotic effects. It is possible that the permeases for the transport of sugars into the cells are lacking in *Cyanophora* or in the cyanelles similar to the situation in most blue-green algae [21].

The obligate photoautotrophic nature is not the only feature which *Cyanophora* shares with these organisms. There are close similarities in the pigment garniture, although echinonene and myxoxanthophyll, which are typical for blue-green algae [22], are lacking in *Cyanophora*. Molecular hydrogen is consumed in an oxygen-dependent, Knallgas-type reaction, and hydrogenase is virtually insensitive to exposure to oxygen, as in blue-green algae [18], but contrary to the situation in eucaryotic algae [23]. Also nitrate assimilation shares common characteristics. The experiments reported in this communication showed that nitrate release to the medium caused by dinitrophenol is demonstrable in the green algae *Ankistrodesmus*, but not in *Anacystis* and *Anaabaena* and also not in *Cyanophora*. In the blue-green alga *Nostoc* [24], nitrate as well as nitrite reductions are particle-bound and ferredoxin-dependent, whereas the formation of nitrite from nitrate catalyzed by nitrate reductase of higher plants requires NADH and is probably located in the cytoplasm [25]. In *Cyanophora* assayed in the light, both nitrate and nitrite reductions were accompanied by a release of oxygen produced in photosynthesis which should be expected if both reaction are ferredoxin dependent as in *Nostoc*. It should be stressed here, however, that these observations about the physiology are still too isolated to allow conclusions on the taxonomy of *Cyanophora*. More physiological as well as genetic and cytological investigations are required to establish the relationship between *Cyanophora* and blue-green algae or rhodophyta.

Schenk and Hofer [26] showed that *Cyanophora* has a regular Calvin cycle to fix carbon dioxide. It is obvious from the obligate photoautotrophy of this organism that the cyanelles must provide the eucaryotic cell with an organic carbon compound. The host must also be dependent on a supply of nitrogen from the organelles. This latter statement is based on the observation presented here that nitrite reduction is strictly light-dependent and coupled to the photosynthetic O₂-formation and therefore must occur in the cyanelles. It is unlikely that the disappearance of nitrite in the light is an artifact or a side-reaction to get rid of poisonous nitrite from *Cyanophora*, since the compound was shown to be an intermediate of the assimilatory nitrate reduction with all organisms so far investigated.

Further experiments have to show which carbon and nitrogen compounds are transferred from the cyanelles to the eucaryotic cell. Such a study requires the isolation of metabolic active cyanelles which is attempted. It is possible that *Cyanophora* will turn out to be one of the most useful systems for the study of the interrelationship between an organism of procaryotic nature and an eucaryotic host.

The authors are indebted to Dr. W. Koch, Göttingen, for helpful comments to grow the organisms and to Miss I. Pischel for skilful technical assistance. This work was kindly supported by grants from the Deutsche Forschungsgemeinschaft.