Derepression of Arylsulfatase Activity by Sulfate Starvation in *Chlorella fusca*

Inge Niedermeyer, Susanne Biedlingmaier, and Ahlert Schmidt

Botanisches Institut, Universität München, Menzinger Str. 67, D-8000 München 19, Bundesrepublik Deutschland

Z. Naturforsch. **42c,** 530–536 (1987); received October 27, 1986

Chlorella, Arylsulfatase, Sulfate Starvation, Derepression by Starvation

Arylsulfatase activity was detected in the green alga *Chlorella fusca* 211-8b during sulfur starvation. No activity was present during growth on sulfate, whereas activity was found with sulfur sources as growth-limiting factor including L-cysteine and L-methionine. Arylsulfatase activity was found after transfer to sulfate starvation conditions after a lag-period of about 3 h; addition of cycloheximide prevented arylsulfatase formation, suggesting the participation of protein synthesis. Addition of sulfate to cultures with active arylsulfatase led to a decay of this enzyme, whereas addition of sulfate and cycloheximide prevented this decay suggesting that protein synthesis is also needed for degradation of arylsulfatase activity. The K_m for *p*-nitrophenyl sulfate was determined to be 0.8 mM. The activity of the arylsulfatase is not affected by sulfate or phosphate, however it is inhibited strongly by sulfite, cyanide and boric acid and therefore is classified according to Dogson and Spencer (Meth. Biochem. Anal. **4**, 211–254 (1957) as an arylsulfatase of type I.

Introduction

Arylsulfatase activity has been detected in bacteria [1, 2], fungi [3, 4], cyanobacteria [5], the green algae *Chlamydomonas reinhardti* [6, 7]; and the higher plant *Zea mays* L. [8]. Arylsulfatases are normally induced by corresponding substrates as shown for the bacteria *Comamonas, Pseudomonas* [9, 10], and *Klebsiella* [11], the mold *Aspergillus oryzae* [3, 4] and the cyanobacterium *Plectonema* [5]. Evidence for a regulation of arylsulfatase activity by sulfur starvation was obtained from *Chlamydomonas* [7]. Since we could demonstrate recently that enhanced sulfate uptake and a sulfonic acid permease are regulated in *Chlorella fusca* 211-8b by the availability of sulfate and not by sulfur compounds in general [12, 13] it was of interest to study the regulation of arylsulfatase expression in this alga in comparison to sulfate uptake and sulfonic acid permease activity.

Materials and Methods

Organism

Chlorella fusca strain 211-8b was obtained from the Sammlung von Algenkulturen (Pflanzenphysiologisches Institut der Universität Göttingen) and cultured according to Schmidt [14] using a final sulfate concentration in the medium of 0.2 mM. Cultures were gased with normal air and illuminated with 10,000 lux.

Preparation of algal extract

Cells were harvested by centrifugation at $7{,}000 \times g$ for 10 min. The algae (1 g wet weight) were taken up in 5 ml of 0.1 M Tris-HCl buffer pH 8.0 containing 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. Cells were disrupted in a french press at 12,000 PSI ($\sim 84 \times 10^6$ Pa). The extract was clarified by centrifugation for 10 min at $12{,}000 \times g$ and used as crude extract.

Arylsulfatase measurement

This activity was determined with either *p*-nitrophenyl sulfate or *p*-nitrocatechol sulfate as a substrate. The following test conditions were used: 50 mM Tris-HCl pH 7.5; 2 mM substrate (normally *p*-nitrophenyl sulfate), enzyme as needed in a total volume of 0.5 ml. After incubation for 60 min at 37 °C the reaction was stopped with 0.5 ml of 0.5 M NaOH. The product was determined spectrophotometrically using an ε of $17.5 \times 10^6 \times \mathrm{cm}^2 \times \mathrm{mol}^{-1}$ for *p*-nitrophenol at 401 nm and an ε of $14.5 \times 10^6 \times \mathrm{cm}^2 \times \mathrm{mol}^{-1}$ for *p*-nitrocatechol at 514 nm with a Beckman spectral photometer DU 7. This test was linear for either *p*-nitrophenyl sulfate or *p*-nitrocatechol sulfate

Reprint requests to Prof. Dr. A. Schmidt.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0500–0530 $ 01.30/0

from 1 to 200 nmol product formed under the conditions used.

Sulfate and sulfonic acid uptake measurements

Uptake measurements for sulfate and ethanesulfonic acid were performed according to Biedlingmaier *et al.* [13] using ^{35}S-labeled compounds with a specific activity of 65 kBq/nmol.

Protein determination

Protein was determined by the Coomassie blue method according to Bradford [15] with the dye reagent preparation from Biorad (München, W. Germany). Bovine serum albumin was used as a reference.

Chemicals

The arylsulfates and cycloheximide were purchased form Sigma (München, W. Germany), ^{35}S-labeled sulfate was obtained from Buchler (Braunschweig, W. Germany) and ^{35}S-labeled ethanesulfonic acid was prepared as described by Biedlingmaier and Schmidt [12]. All other reagents were obtained from Merck (Darmstadt, W. Germany).

Results

Arylsulfatase activity as a function of sulfur nutrition

Arylsulfatase activity was not found in *Chlorella fusca* growing on normal sulfate concentrations (0.2 mM). However, high arylsulfatase activity was detected if algae were grown on certain sulfate esters and sulfonic acids as summarized in Table I. Obviously, this activity was not a response to a specific class of sulfur compounds, since it was detected after growth on a variety of sulfur sources not being related to sulfate esters, including cysteine and methionine (Table I). However, arylsulfatase activity seems to be correlated to the availability of sulfate, since addition of sulfate to any of the sulfur compounds used for growth inhibited expression of arylsulfatase activity, as shown in Table I. Even

Table I. Arylsulfatase activity in *Chlorella fusca* during growth on different sulfur sources. Algae were grown for 5 days on the sulfur source given at the concentration as indicated. Cells were harvested, broken in a french press and the specific activity of the arylsulfatase was determined.

Sulfur source	Concentration [mM]	Arylsulfatase activity [nmol/mg prot. × h]
minus sulfate	none	0
Sulfate	0.001	6
Sulfate	0.002	22
Sulfate	0.004	58
Sulfate	0.02	26
Sulfate	0.2	0
L-Methionine*	0.2	26
D-Methionine	0.2	17
D,L-Methionine	0.2	18
L-Cysteine*	0.2	9
Sulfopon	0.1	35
Sulfopon*	0.3	33
Sodiumdodecyl sulfate*	0.2	14
p-Nitrophenyl sulfate*	0.2	26
Texapon	0.1	32
Texapon	0.3	45
Taurine*	0.3	4
Ethanesulfonic acid*	0.3	23
Ligninsulfonic acid*	0.3	24
β-Mercaptopyruvic acid*	0.3	24
α-Keto methylthio-butyric acid*	0.3	23

* No arylsulfatase activity was detected if grown with addition of 0.2 mM sulfate.

sulfate starvation in the absence of any "substrate signal" induced arylsulfatase activity in this alga.

Arylsulfatase activity in response to sulfate starvation

The data of Table I suggested that sulfate starvation could be the signal for arylsulfatase activity. Therefore *Chlorella fusca* was grown for 5 days on different sulfate concentrations in the medium and arylsulfatase activity was subsequently determined. It is evident from the data of Table I that arylsulfatase activity responds to sulfate limitation, however, best activity was detected within a concentration range of 4–20 μM initial sulfate in the growth medium. It should be emphasized that practically no arylsulfatase activity was detected in a control without any sulfate addition (zero sulfate with practically no growth) as well as in controls run with the normal 0.2 mM sulfate supply, suggesting that a specific sulfate concentration is needed for optimal arylsulfatase activity.

Development of arylsulfatase activity during sulfate starvation

The kinetics of arylsulfatase induction were analyzed using sulfate grown algae, which were transferred, after washing in sulfurfree medium, to sulfate starvation conditions with or without addition of cycloheximide (Fig. 1). Arylsulfatase activity developed after a lag-period of 2 to 3 h increasing steadily over a period of 24 h, whereas in the culture with cycloheximide practically no arylsulfatase activity was detected within 24 h. However, after prolonged time periods arylsulfatase activity was found, possibly due to cycloheximide detoxification in this alga. It should be noted that the arylsulfatase activity declined after prolonged sulfur starvation, thus no activity was found after 5 days of growth in a sulfurfree medium (see Table I).

Further evidence for the induction of arylsulfatase activity by sulfate starvation, rather than by general sulfur starvation was obtained by the comparison of sulfate uptake capacity, ethanesulfonate uptake and arylsulfatase activity in identical cultures. These measurements are summarized in Table II. It is evident, that arylsulfatase activity was only detected in cultures with ethanesulfonate uptake corresponding to enhanced sulfate uptake, with one exception: in cultures starting without any sulfate addition, no arylsulfatase activity was found, however sulfate and sulfonate uptake were clearly enhanced in comparison to the normal sulfate control.

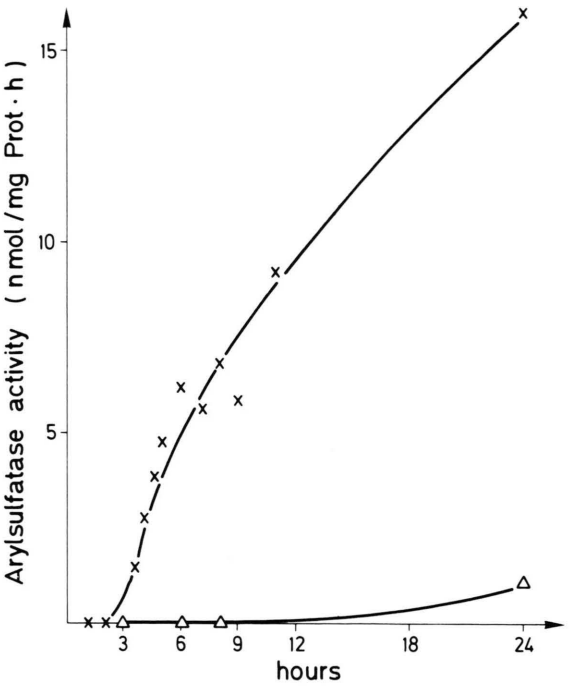

Fig. 1. Induction of arylsulfatase activity after transfer to sulfate-free medium. Sulfate-grown algae were harvested, washed once with sulfur-free medium and resuspended in sulfate-free medium. Aliquots were withdrawn at the time intervals given and analyzed for arylsulfatase activity.
×––× = Cultures without cycloheximide;
△––△ = cultures with 50 μM cycloheximide.

Decrease of arylsulfatase activity after sulfate addition

Addition of sulfate to cultures with derepressed arylsulfatase activity resulted in a decline of this activity; the presence of cycloheximide prevented this decline in the 8-h period analyzed (Fig. 2), suggesting that protein synthesis is involved in the decrease of arylsulfatase activity. The decline of arylsulfatase activity after sulfate addition was slow in comparison to that of sulfate or sulfonate uptake (see Fig. 3) suggesting that the signal for a repression of the arylsulfatase is different from that of sulfate or sulfonic acid permeases.

Determination of K_m for p-nitrophenyl sulfate

The hydrolysis of *p*-nitrophenyl sulfate was linear with time over a 90 min period and also in the range

Table II. Correlation between sulfate uptake, ethanesulfonate uptake and arylsulfatase activity in *Chlorella fusca*. Algae were grown for 5 days on the sulfur source and concentration indicated; arylsulfatase activity, sulfate and sulfonate uptake were determined from identical cultures. Uptake rates for ^{35}S-labeled ethanesulfonate and sulfate were assayed in a Warburg apparatus using the following conditions: 27 °C, 4500 Lux, 30 min and a final concentration of 0.3 mM for labeled sulfate or sulfonate. A specific activity of 65 kBq/nmol was used in both cases. Data are normalized as nmol × 10^8 cell^{-1} × h^{-1}.

Sulfur source	Sulfate uptake [nmol/h × 10^8 cells]	Ethanesulfonate uptake	Arylsulfatase activity [nmol/mg prot. × h]
Sulfate (200) µM	18	0	0
Sulfate (20) µM	307	59	16
-Sulfate (~0) µM	193	132	0
Sulfopon (300) µM	159	21	30
Sodiumdodecyl sulfate (300) µM	102	76	55

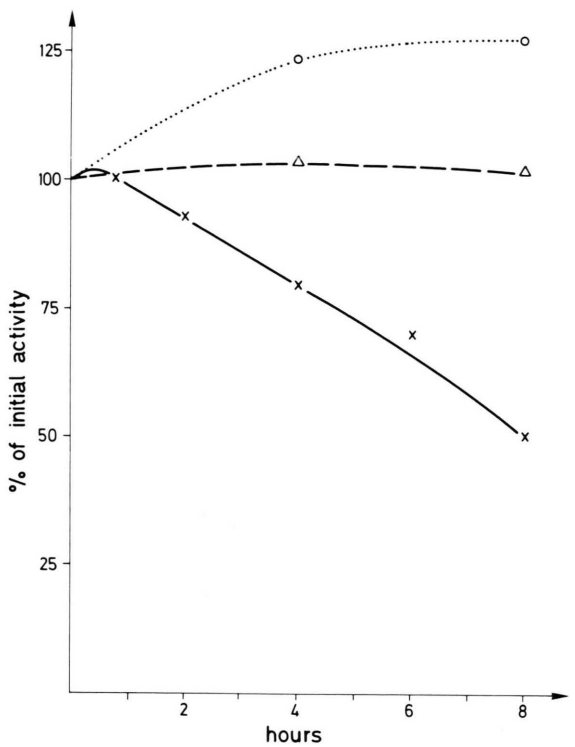

Fig. 2. Repression of arylsulfatase activity after addition of sulfate. Algae were grown for 4 days on 0.2 mM L-methionine as sulfur source; at zero time sulfate was added to a final concentration of 0.2 mM and the specific activity of the arylsulfatase was determined during an 8 h period. 100% are 32 nmol *p*-nitrophenyl sulfate hydrolyzed per mg protein and hour.
○--○ = Control without sulfate addition;
×--× = cultures with sulfate addition;
△--△ = cultures with sulfate and 50 µM cycloheximide addition.

of 20 to 600 µg protein using an extract of algae grown in 0.02 mM sulfate (Data not shown). The velocity of the reaction at different concentrations of *p*-nitrophenyl sulfate was measured. The reaction follows normal Michaelis-Menten kinetics, and from such data a K_m of 0.8 mM was calculated for *p*-nitrophenyl sulfate.

Influence of salts, thiols and detergents on arylsulfatase activity in vitro

The influence of compounds used to characterize arylsulfatase activities from bacteria was tested for *Chlorella* using extract obtained from algae grown

Table III. Influence of ions, inhibitors and effectors on the arylsulfatase activity *in vitro*. Crude *Chlorella* extract obtained from algal cultures grown for 5 days at 0.02 mM initial sulfate concentration was used.

Compound added	Concentration [mM]	Activity as % of control
Sodium sulfate	1	86
Sodium sulfite	1	7
Ammonium sulfate	100	159
Ammonium sulfate	1000	145
KH_2PO_4/K_2HPO_4	10	66
H_3BO_3	2	14
KCN	2	29
EDTA	2	86
β-mercaptoethanol	10	63
Dithioerythritol (DTE)	10	33
Triton X-100	0.5%	8
Sodium dodecylsulfate	0.5%	90
Sodium dodecylsulfate	1%	53

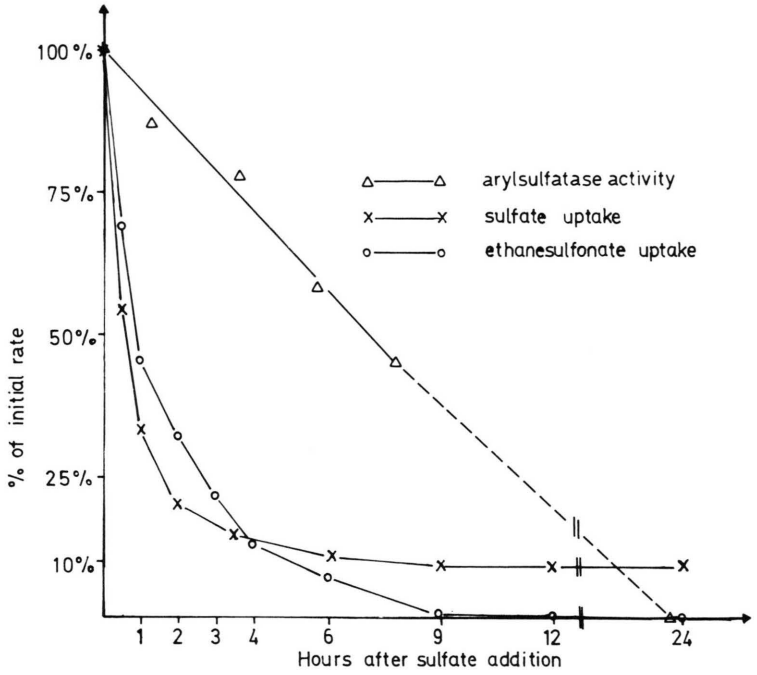

Fig. 3. Repression of arylsulfatase, sulfate permease, and sulfonic acid permease activity after addition of sulfate. Algae were grown on 0.3 mM ethanesulfonate for 5 days and sulfate to a final concentration of 0.2 mM was added at sero time. Sulfate and sulfonate uptake and arylsulfatase activity were determined according to Table II. 100% are: a) for arylsulfatase activity = 23 nmol/mg protein × h; b) for sulfate uptake = 2796 nmol × 10^9 cell^{-1} × h^{-1}; ethanesulfonate uptake = 380 nmol × 10^9 cell^{-1} × h^{-1}.

for 5 days with a starting concentration of 0.02 mM sulfate. Little interference was found for sodium sulfate (1 mM), EDTA (2 mM), or sodium dodecyl sulfate (0.5%), whereas sodium phosphate buffer (10 mM), β-mercaptoethanol (10 mM) and sodium dodecyl sulfate at 1% inhibited moderately. Severe inhibition was found for borate (2 mM), DTE (10 mM), sodium sulfite (1 mM) and Triton X-100 (0.5%). This characterizes the arylsulfatase of *Chlorella fusca* according to Dogson and Spencer [16] as type I.

Discussion

The results presented here for the green alga *Chlorella fusca* demonstrate clearly a derepression (induction) of an arylsulfatase in response to sulfate limitation: Neither cysteine nor methionine repress this activity, and arylsulfatase activity is present during growth on these two sulfur sources; addition of sulfate to arylsulfatase-active cultures causes the disappearance of this activity within one day. The onset of arylsulfatase activity can be determined after a lag-period of about 3 h. This period is in the same order as the development of increased sulfate uptake capacity and of sulfonic acid permease, which are derepressed simultaneously with arylsulfatase activity and which have been shown to be induced by sulfate limitation [12, 13]. A derepression of arylsulfatase by sulfate starvation is not a general property of all green algae, since we could not detect arylsulfatase activity in the two strains *Chlorella vulgaris* 211-1e and *Chlorella* k under sulfate starvation conditions described here.

Arylsulfatase activity was repressed by sulfate in all cases studied so far showing a close connection between sulfate ester degradation and normal sulfate metabolism. Arylsulfatase repression by sulfate could be overcome in certain bacterial species by the addition of tyramine [17, 18], suggesting the involvement of tyramine metabolism in arylsulfatase derepression. However, arylsulfatase activity could be induced by sulfur starvation in the absence of any sulfate ester (substrate) in the green alga *Chlamydomonas reinhardti* [6, 7]; thus, obviously different regulatory signals are involved in arylsulfatase repression/derepression.

The repression of arylsulfatase activity in *Chlorella fusca* by sulfate, and the derepression during growth on reduced sulfur compounds (cysteine and methionine) clearly points to a regulation by sulfate in a similar pattern as shown for the arylsulfatase of

Chlamydomonas [7]. Tyramine did not relieve sulfate repression in *Chlorella fusca* 211-8b, although a higher activity was found under growth conditions that allowed expression of arylsulfatase activity (unpublished). Sulfate uptake and sulfonic acid permease are reduced in *Chlorella* with a half-life of less than 1 h [12, 13], whereas the arylsulfatase activity is relatively stable being reduced to about 50% of initial activity within about 8 h; a similar pattern was found for *Chlamydomonas*. Obviously, different mechanisms for repression are used for sulfate uptake and sulfonic acid permease compared to arylsulfatase activity.

This arylsulfatase of *Chlorella fusca* was inhibited by sulfite, cyanide and borate, however sulfate and EDTA were without severe effects allowing to characterize this arylsulfatase according to Dogson and Spencer [16] as an arylsulfatase of type I. The K_m for *p*-nitrophenyl sulfate was determined to be 0.8 mM being in the same range as for the enzyme from *Chlamydomonas* with a K_m of 0.7 mM [6]. The pH-optimum for *p*-nitrophenyl sulfate hydrolysis in extracts obtained from sulfur-starved cells was around 7.5; thus, the arylsulfatases from *Chlamydomonas* and *Chlorella* appear to have similar properties.

Arylsulfatase activity could be measured using intact *Chlorella* cells, suggesting that this enzyme is localized on the periphery of the cells; similar observations were made for *Chlamydomonas reinhardti* [6] and the cyanobacterium *Plectonema* [5]. Probably sulfate esters are hydrolyzed in the periplasmic space and the sulfate liberated is transported by the sulfate permease which has a high activity if arylsulfatase activity is derepressed. In support of this suggestion is the observation that *p*-nitrophenol was detected in the growth medium when *p*-nitrophenyl sulfate was used as a sulfur source.

The presence of regulated arylsulfatase activity in algae leads to speculations about the modification of biopolymers by sulfation similar to phosphorylation. Tyrosine sulfation is widespread in nature [19–21] and tyrosine sulfotransferases have been characterized [22]. A second site for sulfation are carbohydrates of membranes and glycoproteins. Glycoprotein sulfation was observed in *Dictyostelium discoideum* [23], *Halobacterium halobium* [24] and in the green alga *Chlamydomonas reinhardti* [25]. Sulfated carbohydrates are found in algae [26–30] and sulfatases might be used for metabolization of these compounds; furthermore, sulfated flavonoids are present in higher plants [31–33]. The presence of sulfate esters in plants and algae could possibly be used as a regulatory signal for specific developmental stages as suggested for a control of differentiation in *Volvox carteri* [34].

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

[1] K. S. Dogson, G. F. White, and J. W. Fitzgerald, Sulfatases of Microbial Orign, **Vol. I,** pp. 1–199, CRC Press, Inc. Boca Raton, Florida 1982.
[2] K. S. Dogson, G. F. White, and J. W. Fitzgerald, Sulfatases of Microbial Orign, **Vol. II,** pp. 1–199, CRC Press, Inc. Boca Raton, Florida 1982.
[3] K. Ohtsuki, Y. Sakurai, and H. Shiota, J. Ferm. Technol. **55,** 476–484 (1977).
[4] G. J. Burns and C. H. Wynn, Biochem. J. **166,** 415–420 (1977).
[5] S. Müller and A. Schmidt, Z. Naturforsch. **41c,** 820–824 (1986).
[6] T. Lien and O. Schreiner, Biochim. Biophys. Acta **384,** 168–179 (1975).
[7] O. Schreiner, T. Lien, and G. Knutsen, Biochim. Biophys. Acta **384,** 180–193 (1975).
[8] A. A. Farooqui and A. M. M. Samiullah, Planta **133,** 157–160 (1977).
[9] J. W. Fitzgerald and M. E. Cline, FEMS Microbiol. Letters **2,** 217–220 (1977).
[10] J. W. Fitzgerald and M. E. Cline, FEMS Microbiol. Letters **2,** 221–224 (1977).
[11] T. Adachi, H. Okamura, Y. Murooka, and T. Harada, J. Bacteriol. **120,** 880–885 (1974).
[12] S. Biedlingmaier and A. Schmidt, Biochim. Biophys. Acta **861,** 95–104 (1986).
[13] S. Biedlingmaier, H.-P. Köst, and A. Schmidt, Planta **141,** 518–523 (1986).
[14] A. Schmidt, Arch. Microbiol. **84,** 77–86 (1972).
[15] M. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[16] K. S. Dogson and B. Spencer, Meth. Biochem. Anal. **4,** 211–254 (1957).
[17] T. Adachi, Y. Murooka, and T. Harada, J. Bacteriol. **116,** 19–24 (1973).
[18] T. Harada and Y. Murooka, Mem. Inst. Sci. Ind. Res., Osaka Univ. **37,** 45–58 (1980).
[19] W. B. Hutter, Nature **299,** 273–276 (1982).
[20] M.-C. Liu and F. Lipmann, Proc. Natl. Acad. Sci. USA **81,** 3695–3698 (1984).
[21] P. Rosa, G. Fumagalli, A. Zanini, and W. B. Huttner, J. Cell Biol. **100,** 928–937 (1985).
[22] F. Vargas, O. Frerot, M. D. T. Tuong, and J. C. Schwartz, Biochem. **24,** 5938–5943 (1985).
[23] H.-P. Hohmann, G. Gerisch, R. W. H. Lee, and W. B. Huttner, J. Biol. Chem. **260,** 13869–13878 (1985).

[24] F. Wieland, W. Dompert, G. Bernhardt, and M. Sumper, FEBS Lett. **120,** 110–114 (1980).
[25] K. Roberts, M. R. Gay, and G. J. Hills, Physiol. Plant. **49,** 421–424 (1980).
[26] T. Akahane and S. Izumi, Agr. Biol. Chem. **40,** 285–289 (1976).
[27] L. V. Evans, M. Simpson, and M. E. Callow, Planta **110,** 237–252 (1973).
[28] S. A. Huntsman and J. H. Sloneker, J. Phycol. **7,** 261–264 (1971).
[29] M. M.-D. Hussein, A. Abdel-Aziz, and H. M. Salem, Phytochemistry **19,** 2131–2132 (1980).
[30] J. Ramus and S. T. Groves, J. Cell Biol. **54,** 399–407 (1972).
[31] J. H. Harborne, Phytochemistry **14,** 1147–1155 (1975).
[32] J. H. Harborne and C. A. Williams, Phytochem. System. Ecol. **4,** 37–41 (1976).
[33] C. A. Williams and J. F. Harborne, Phytochemistry **14,** 1141–1149 (1974).
[34] M. Sumper and S. Wenzel, FEBS Lett. **114,** 307–312 (1980).