10 McCall, C., Schmitt, J. Cousart, S., O'Flaherty, J., Bass, D., and Wykle, R., Biochem. biophys. Res. Commun. *126* (1985) 450.
11 Witter, L.A., Vater, C.A., and Lienhard, G.E., Nature, Lond. *315* (1985) 777.
12 Derr, J.T., and Smith, G.L., J. cell. Physiol. *102* (1980) 55.
13 Markwell, M.A., Hass, S.H., Bieber, L.L., and Tolbert, N.E., Analyt. Biochem. *87* (1978) 637.
14 Collins, M.K.L., and Rosengurt, E., J. cell. Physiol. *118* (1984) 133.
15 Rodriguez-Pena, A., and Rosengurt, E., Biochem. biophys. Res. Commun. *120* (1984) 1053.
16 Rosengurt, E., Rodriguez-Pena, A., Coombs, M., and Sinnett-Smith, J., Proc. natn. Acad. Sci. USA *81* (1984) 5748.
17 Vara, F., Schneider, J.A., and Rosengurt, E., Proc. natn. Acad. Sci. USA *82* (1985) 2384.
18 Castagna, M., Takai, Y., Kikkawa, U., Mori, T., and Nishizuka, Y., J. biol. Chem. *275* (1982) 7847.
19 Kelley, D.S., Evanson, T., and Potter, V.R., Proc. natn. Acad. Sci. USA *77* (1980) 5953.

0014-4754/86/091038-03$1.50 + 0.20/0

Ammonia photoproduction by *Cyanospira rippkae* cells 'entrapped' in dialysis tube

M. Vincenzini, R. De Philippis, A. Ena and G. Florenzano

Istituto di Microbiologia Agraria e Tecnica dell'Università degli Studi, Piazzale delle Cascine 27, I-50144 Florence (Italy), and Centro di Studio dei Microrganismi Autotrofi del C.N.R., Florence (Italy), 29 November 1985

Summary. The photoproduction of ammonia by cells of the heterocystous cyanobacterium *Cyanospira rippkae* in the presence of the glutamine synthetase inhibitor, L-methionine D,L-sulfoximine (MSX), was investigated. The time course of changes in protein, pigment and carbohydrate concentrations and the C_2H_2-reducing activity of nitrogenase in MSX treated and untreated filament suspensions was also determined. The results show that nearly 40 h after MSX addition the cells are able to recover from the nitrogen starvation induced by the inhibitor by themselves, without the removal of MSX or the addition of nitrogenous compounds. Biliproteins, mobilized as a consequence of MSX addition, seem to play a key role in the process of cell recovery. These findings were exploited in a semicontinuous ammonia producing process with cells 'immobilized' in a dialysis tube photobioreactor.
Key words. Heterocystous cyanobacteria; nitrogenase; nitrogen fixation; MSX-inhibition; ammonia photoproduction.

Photobiological ammonia production by nitrogen fixing cyanobacteria, treated with the glutamine synthetase inhibitor L-methionine D,L-sulfoximine (MSX), is under investigation in different laboratories employing free living[1-3] and immobilized cells[4,5]. From these studies it appears that the ammonia production rate is generally lower in immobilized cells, probably because of diffusional limitations. Nevertheless, cell immobilization stabilizes the NH_3-producing activity for a longer time and gives many operational advantages over free cells.

In our study we have utilized a special 'immobilization' technique consisting of an 'entrapment' of the microbial cells inside a dialysis tube. In this way the high substrate diffusion typical of free living cells, and the operational benefits of working with immobilized cells, were both obtained.

Certainly, this 'entrapment' technique does not achieve all the specific advantages of the classical immobilization methods[6] but it constitutes an effective system for evaluating the ability of microbial cells to produce low molecular weight extracellular metabolites.

Cyanospira rippkae strain Mag II 702, the type strain of the new genus *Cyanospira* described in this Research Centre[7], was the N_2-fixing organism selected for this study. The choice was suggested by the high nitrogenase activity of the organism and by the high pH required for its optimal growth, both properties being suitable for the ammonia production process.

Materials and methods. Organism and culture conditions. Cyanospira rippkae strain Mag II 702 was grown in 2-l Erlenmeyer flasks, containing 800 ml of the synthetic mineral medium previously described[7], under continuous white fluorescent illumination (80 μ E/m^{2}s on the liquid surface) at 28 °C. Air was bubbled through the culture at a flow rate of 500 ml/min in order to avoid cell stratification on the liquid surface.

Cells from 2-day-old cultures (late exponential phase) were used for the experiments on NH_3-photoproduction.

Description of the dialysis tube photobioreactor. The reactor was derived from the cooling jacket of an LKB 2137 chromatography column (16 mm i.d.) by replacing the glass column with dialysis tubing (Visking size 2-18/32″) as shown in figure 1. A volume of 125 ml of cyanobacterial trichome suspension was poured into the dialysis tube and immediately air was bubbled through the culture. Outside the dialysis membrane 150 ml of growth medium were introduced. When necessary, the outer medium was replaced without washout of the cells.

The bioreactor containing the cell suspension was incubated under continuous white fluorescent light (70 μ E/m^{2}s) at 28 °C.

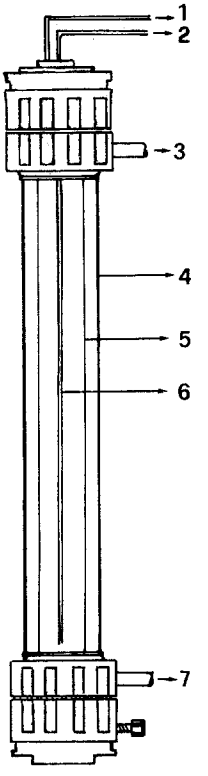

Figure 1. Dialysis tube photobioreactor. 1, air outlet; 2, air inlet; 3, inlet for outer medium; 4, cooling jacket of LKB chromatography column; 5, dialysis tube containing cell suspension; 6, connection for air bubbling; 7, outlet for outer medium.

Analytical methods. Protein content of the cells was determined according to the Lowry procedure as described by Herbert et al.[8]. Total cell carbohydrates were estimated by the phenol-sulfuric acid method of Dubois et al.[9] using glucose as a standard. Phycobiliproteins were extracted from the cells and determined in crude extracts according to the equations given by Bennett and Bogorad[10]. Chlorophyll *a* was determined spectrophotometrically in 90% acetone extracts using the extinction coefficient, 89, given by Parson and Strickland[11].

Ammonia dissolved in the medium was estimated by the colorimetric method of Solorzano[12].

The acetylene reduction test for determining the nitrogenase activity was performed in 35 ml flasks containing 10 ml of culture under an atmosphere of air: $C_2H_2 = 9:1$. Ethylene produced after 30 min of incubation in the light (100 μ E/m^{2}s) at 28 °C was measured using a gaschromatograph (Fractovap series 2150 Carlo Erba) fitted with a flame ionization detector and Porapak R column according to the method described by Turned and Gibson[13].

Results. Effect of MSX concentration on the NH_3-production rate. Different molar ratios MSX/chlorophyll *a* were assayed on free living cultures of *Cyanospira rippkae* in order to select the optimal MSX concentration for the NH_3-production experiments. As can be seen in figure 2, the optimal ratio MSX/chl.*a* was in the range 2.5–5. No trace of ammonia was found in the medium with a molar ratio of 1.3. It is interesting to note that 5 h after the addition of MSX the NH_3-production rate with a molar ratio of 2.4 was markedly lower than that obtained with ratios of 3.5 and 4.6, whereas after 25 h the difference was practically negligible. Probably at a low MSX concentration (i.e. with a molar ratio of 2.4) a longer time is required for the expression of the effects induced by the inhibitor so that the ammonia production rate found after 5 h, which would include the lag period, might be underestimated, whereas after 25 h the difference would be insignificant.

At values of the ratio higher than 5, the extracellular amount of NH_3 produced decreased regularly showing that high concentrations of the glutamine synthetase inhibitor had a negative effect on metabolic pathways other than ammonia assimilation. Nevertheless neither complete suppression of NH_3-producing activity nor cellular lysis were observed up to molar ratios greater than 10. In view of these results the subsequent experiments on NH_3-photoproduction were carried out with a molar ratio of 4.0.

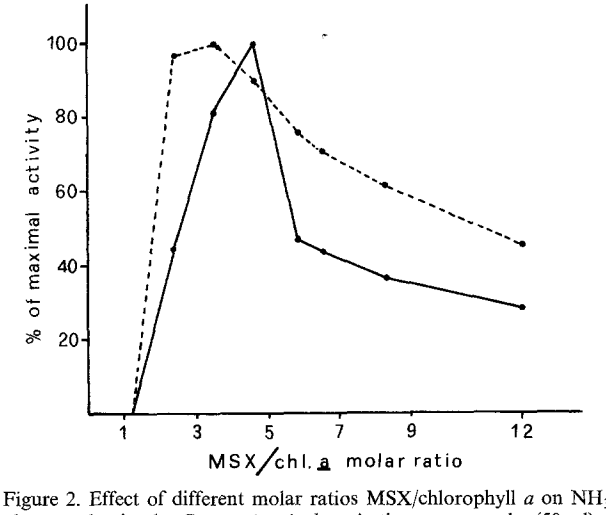

Figure 2. Effect of different molar ratios MSX/chlorophyll *a* on NH_3-photoproduction by *Cyanospira rippkae*. At time zero samples (50 ml) of cultures at different cell densities (5.6, 8.2 and 14 μg chlorophyll *a*/ml) were supplemented with MSX (in molar ratios with chl.*a* as indicated in the figure) and incubated under continuous incandescent light (100 μ E/m^{2}s) at 28 °C. The ammonia in the medium was determined after 5 (solid line) and 25 h (dashed line). 100% activity was in the range 3.4–18 μmoles NH_3/mg initial chl.*a* depending on the incubation time and chlorophyll *a* concentration.

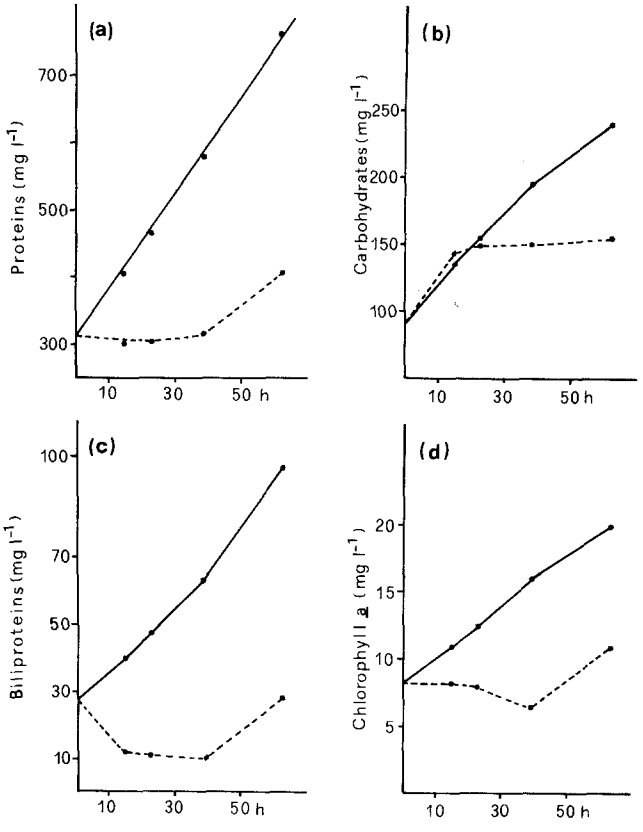

Figure 3. Effect of MSX on the synthesis of different cell components in *Cyanospira rippkae*. A 2-day-old culture was divided into two 250 ml aliquots, one of which was supplemented at time zero with MSX at its optimal molar ratio with chlorophyll *a* (4:1) Both samples were successively incubated under continuous incandescent light (100 μ E/m^{2}s) at 28 °C. The analyses of the control cell culture (solid line) and the MSX treated filament suspension (dashed line) were performed at the times indicated (a-protein; b-carbohydrates; c-phycobiliproteins; d-chlorophyll *a*).

Effect of MSX on some cell components. The effects of the glutamine synthetase inhibitor on cell constituents were determined by following the time course of changes in protein, carbohydrate, biliprotein and chlorophyll *a* concentrations in MSX treated and untreated cell suspensions (fig. 3a–d).

The results clearly demonstrate that the addition of MSX to filament suspensions prevented cell growth (protein synthesis) for at least 40 h (fig. 3a). The carbohydrate synthesis in MSX treated cells was higher in the first 15 h than in untreated cells (fig. 3b) so that the MSX treated trichomes in this period may be regarded as normal cells placed under nitrogen starvation. It is in fact known that in actively CO_2-fixing photosynthetic cells nitrogen starvation leads to an increase in the cellular C/N ratio[14].

The inhibitor also caused a remarkable depigmentation of the trichomes mainly due to a decrease (more than 50%) in biliprotein concentration as shown in figure 3c–d. This biliprotein degradation was already noticed in other MSX treated N_2-fixing cyanobacteria[3,15], and can be ascribed to a specific proteinase activity induced by intracellular glutamine limitation and hence by nitrogen starvation[15,16].

Filaments treated with MSX plus chloramphenicol (CAM), an inhibitor of protein synthesis, maintained the initial biliprotein concentration (table) indicating that the biliprotein degradation in MSX treated *Cyanospira rippkae* cells is dependent on de novo protein synthesis.

Experientia 42 (1986), Birkhäuser Verlag, CH-4010 Basel/Switzerland

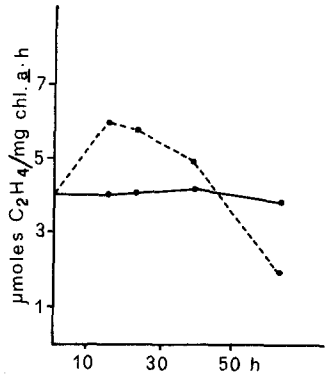

Figure 4. Effect of MSX on the C_2H_2-reducing activity in *Cyanospira rippkae*. Conditions were the same as for figure 3. C_2H_2-reducing activity of MSX treated (dashed line) and untreated cultures (solid line) was measured at the times indicated.

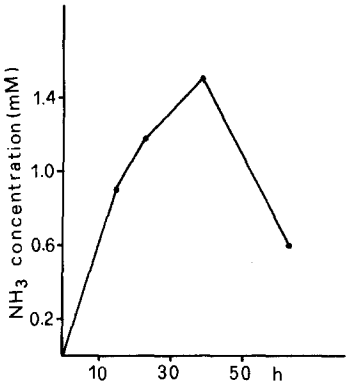

Figure 5. Time course of change in ammonia concentration in the culture medium of MSX-treated *Cyanospira rippkae* cells. Conditions were the same as for figure 3. Ammonia dissolved in the medium was determined at the times indicated.

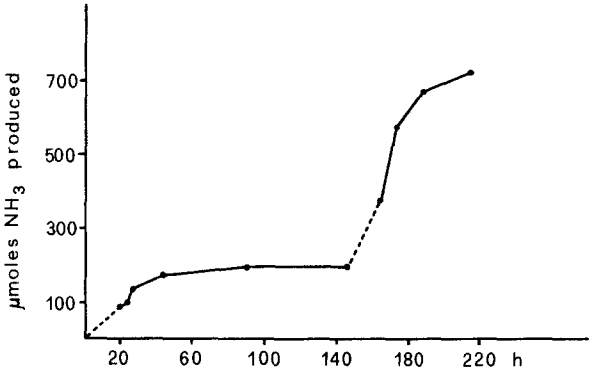

Figure 6. Semicontinuous ammonia production by MSX-treated *Cyanospira rippkae* cells 'entrapped' in the dialysis tube bioreactor. MSX, at its optimal molar ratio with chlorophyll *a*, was added at time zero and at 146 h. The starting chlorophyll *a* concentration in the cell suspension (125 ml) inside the dialysis tube was 4 μg/ml while at time 146 h, after cell recovery, it was 6.5 μg/ml showing that some cell growth occurred. At the times indicated the growth medium (150 ml) outside the dialysis membrane was replaced with fresh medium without the inhibitor and ammonia concentration was determined. Dashed portions of the curve refer to the periods in which the optimal amount of MSX was in the bioreactor (the replacement of the medium outside the dialysis tube partially removed the inhibitor).

In figure 3 it may be also seen that a considerable increase in protein and pigment concentration occurred 63 h after the addition of MSX to the filament suspension, showing that the cyanobacterial cells were able to grow again without the removal of the inhibitor from the medium.

Frequent additions (every 20 h) of MSX to *C. rippkae* cell suspensions led to complete pigment degradation and, in accordance with the results of Ramos et al.[3], to cell lysis after 60–70 h of exposure to the inhibitor.

Effect of MSX on nitrogenase activity. In figure 4 the time courses of changes in the rate of reduction of C_2H_2 by MSX treated and untreated *C. rippkae* cells are reported. The rate of acetylene reduction by cells treated with the inhibitor changed with time while that observed in untreated filaments was quite constant for a period of at least 55 h. After the addition of the inhibitor the nitrogenase activity increased and, up to the 40th h, it remained higher than in untreated cells. During this period cell growth was prevented, as shown in figure 3 (a and d), by the MSX dependent inactivation of glutamine synthetase. Since the amount of chlorophyll *a* remained substantially unchanged (fig. 3d), the increased C_2H_2-reduction rate in MSX treated cells reflects an effective increase in the nitrogenase activity. As no significant changes in C_2H_2-reduction rate were observed during 15-h incubation in the presence of MSX plus CAM (table), the observed increase in the nitrogenase activity of MSX treated filaments seems to be due to de novo protein synthesis. After 40 h of treatment the C_2H_2-reduction rate markedly declined.

As a consequence of MSX addition, ammonia produced by nitrogenase was excreted into the medium but, as shown in figure 5, only for about 40 h. The cessation in ammonia accumulation was not due to a loss of nitrogenase activity: 60 h after the addition of the inhibitor, the enzyme retained, as shown in figure 4, about 30% of its maximal activity.

After 63 h, when an increase in protein and pigment concentrations was observed (fig. 3), the amount of ammonia dissolved in the medium was lower than that found after 39 h. This result indicates that some recovery of glutamine synthetase activity in MSX treated cells occurs, without removal of the inhibitor, between 30 and 40 h after the start of the incubation. The uptake of the ammonia dissolved in the medium was concomitant with the observed decrease in the C_2H_2-reduction rate previously mentioned (fig. 4). After the exhaustion of the dissolved ammonia, the nitrogenase fully recovered its activity (data not shown).

Semicontinuous ammonia photoproduction. The finding that, nearly 40 h after MSX addition, *C. rippkae* filaments were able to recover by themselves from the deleterious effects induced by the glutamine synthetase inhibitor, was utilized in order to extend the process of ammonia photoproduction. This was achieved by allowing the cells to overcome the MSX induced effects before the next addition of MSX.

The experiment was performed in the dialysis photobioreactor which allowed an easy and quick replacement of the medium without cell washout, and avoiding any mechanical cell separation. The replacement of the medium was necessary to avoid the ammonia uptake observed in batch experiments during the period of cell recovery. Obviously, with this procedure, the process of ammonia production had a semicontinuous time course. Figure 6 shows the results of such an experiment. The cyanobacterial cells, treated with the optimal amount of MSX at time zero, excreted ammonia for about 90 h, reaching the maximal production rate of 20 μmoles NH_3/mg chl.*a* h. The duration of ammonia excretion was longer than in the previous batch experiments probably owing to the frequent replacement of the medium outside the dialysis tube with fresh medium without the inhibitor (replacement times are reported in figure 6). In this connection it is to be noted that MSX was partially removed from the bioreactor just 20 h after it was added, without waiting for the cessation of ammonia accumulation in the medium. This removal, however, did not accelerate the cessation of ammonia production or the cell recovery.

At 146 h, when the cell constituents were fully recovered, the inhibitor was added again and the process of ammonia photoproduction started again. The maximal production rate reached in this phase was higher than 30 μmoles NH_3/mg chl.*a* h.

In another set of experiments we also tried to reduce the time required for cell recovery by adding glutamine to the MSX treated cell suspension at different times. When the amino acid (0.2 mM final concentration) was introduced into the bioreactor at time zero no ammonia production was observed, whereas when the addition was performed 17 or 40 h after the MXS treatment, the amount of ammonia excreted and the time for cell recovery were the same as with filaments treated with MSX only.

Discussion. The results of our investigation show that during the NH_3-producing phase an extensive degradation of biliproteins occurs in MSX treated *Cyanospira rippkae* cells. In the presence of chloramphenicol, however, the biliprotein content remains substantially unchanged (table), indicating the need of a de novo synthesis of a specific protease for the mobilization of these nitrogenous reserves. Moreover the disappearance of the biliproteins in MSX treated filaments was not accompanied by a significant reduction in protein concentration. Hence we may infer that the MSX dependent inactivation of glutamine synthetase does not prevent an active synthesis of protein. It is likely that new glutamine synthetase is also synthesized from degraded biliproteins because frequent additions of MSX to cell suspensions prevented cell recovery and led to cell lysis. This supposition may account for the ability of *C. rippkae* cells to recover by themselves from the deficiencies induced by the inhibitor. The initial level of phycobiliproteins could be the parameter that determines not only the possibility of a cell recovery but also the length of the period required to achieve a complete reestablishment of cell components and activities related to nitrogen fixation and assimilation.

In the experiment on semicontinuous ammonia production, cell recovery was favored by the partial removal of MSX about 20 h after its addition. The replacement of the medium outside the dialysis tube also removed the ammonia excreted avoiding the inhibition of nitrogenase function and the repression of its synthesis during the recovery period. The absence of ammonia in this phase had a stimulating effect on nitrogenase activity since in the second NH_3-producing period (fig. 6) the ammonia production rate, on a chlorophyll *a* basis, was greater than after the first MSX addition (20 and 30 μmoles NH_3/chl.*a* h, respectively). The results obtained by adding glutamine to MSX-treated filament suspensions with the purpose of accelerating the recovery of cell constituents and activities are not in agreement with those obtained by Ramos et al.[3] with *Anabaena* sp. strain ATCC 33047, but may be explained on the basis of the results reported by Chapman and Meeks[17] for *Anabaena variabilis* strain ATCC 29413. According to the latter authors glutamine inhibits MSX transport and is unable to support growth of the microorganism.

Effect of MSX and MSX plus CAM on the levels of some cell components and on the nitrogenase activity in *Cyanospira rippkae*

	Time zero	After 15 h		
		Control	MSX	MSX + CAM
Dry weight (mg/l)	480	648	530	552
Protein (mg/l)	310	402	305	300
Carbohydrates (mg/l)	90	135	145	160
Chlorophyll *a* (mg/l)	8.2	11	8.2	8.0
Biliproteins (mg/l)	27	40	12	26
C_2H_2-reducing activity (μmoles C_2H_4/mg chl. *a* h)	4.2	4	6	3.7
NH_3-production rate (μmoles NH_3/mg initial chl. *a* h)	–	–	7.3	2.0

Conditions were the same as for figure 3. Chloramphenicol (CAM) concentration was 5 μg/ml.

The data presented show that the special dialysis photobioreactor employed in this study is a promising tool for research and applications as it combines the advantages of immobilized cell reactors and of conventional reactors with free cells.

Acknowledgments. The authors are grateful to Dr C. Sili who grew the cultures of *Cyanospira rippkae* and to Mr G. Tassinato for his valuable technical assistance.

1 Stewart, W. D. P., and Rowell, P., Biochem. biophys. Res. Commun. *65* (1975) 846.
2 Guerrero, M. G., Ramos, J. L., and Losada, M., Experientia *38* (1982) 53.
3 Ramos, J. L., Guerrero, M. G., and Losada, M., Appl. envir. Microbiol. *48* (1984) 114.
4 Musgrave, S. C., Kerby, N. W., Codd, G. A., and Stewart, W. D. P., Biotechnol. Lett. *4* (1982) 647.
5 Musgrave, S. C., Kerby, N. W., Codd, G. A., and Stewart, W. D. P., Biotechnology *17* (1983) 133.
6 Hallenbeck, P. C., Enzyme Microbiol. Technol. *5* (1983) 171.
7 Florenzano, G., Sili, C., Pelosi, E., and Vincenzini, M., Archs Microbiol. *140* (1985) 301.
8 Herbert, D., Phipps, P. J., and Strange, R. E., in: Methods in Microbiology, Vol. 5B, pp. 209–344. Eds J. R. Norris and D. W. Ribbons. Academic Press, London/New York 1971.
9 Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F., Analyt. Chem. *28* (1956) 350.
10 Bennett, A., and Bogorad, L., J. Cell Biol. *58* (1973) 419.
11 Parson, T. R., and Strickland, J. D. H., J. mar. Res. *21* (1963) 155.
12 Solorzano, L., Limnol. Oceanogr. *14* (1969) 799.
13 Turner, G. L., and Gibson, A. H., in: Methods for Evaluating Biological Nitrogen Fixation, pp. 111–138. Ed. F. J. Bergersen. J. Wiley and Sons, New York 1980.
14 Allen, M. M., and Smith, A. I., Archs. Microbiol. *69* (1969) 114.
15 Wood, N. B., and Haselkorn, R., J. Bacteriol. *141* (1980) 1375.
16 Foulds, I. J., and Carr, N. G., FEMS Microbiol. Lett. *2* (1977) 117.
17 Chapman, J. S., and Meeks, J. C., J. Bacteriol. *156* (1983) 122.

0014-4754/86/091040-04$1.50 + 0.20/0

Calmodulin in epithelial intestinal cells during rat development

C. Rochette-Egly, J. Cl. Garaud, M. Kedinger and K. Haffen

Unité INSERM 61, 3, avenue Molière, F-67200 Strasbourg (France), 18 December 1985

Summary. Calmodulin was immunocytochemically localized in the brush borders of rat intestinal epithelial cells from the tip to the base of the villi, from day 18 of fetal life up to the adult stage. The early (14th day) fetal cells, like the adult crypt cells, were not immunoreactive, although their calmodulin content was equal to that of the mature cells from the tips of the villi.
Key words. Calmodulin; immunofluorescence; epithelial cells; brush borders; rat development.

The structural and functional changes that occur during development of the mammalian small intestine have been investigated by several authors (for review see refs 1–3). In the rat, short and sparse microvilli start to appear around the 17th day of fetal life[4], and at the time of birth, brush borders are well differentiated[5]. The digestive and absorptive functions necessary to cope with suckling are already acquired, but further physiological functions continue to mature during the neonatal period,