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.

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Calmodulin in epithelial intestinal cells during rat development

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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,

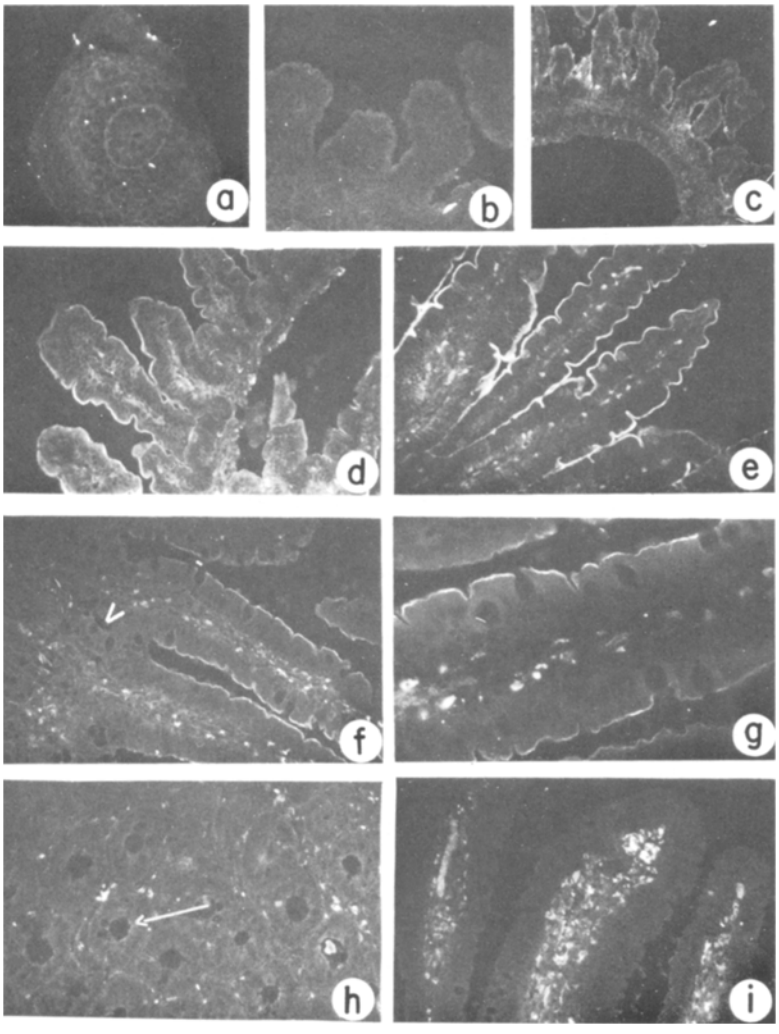

Figure 1. Immunofluorescence staining of calmodulin in rat jejunum at different representative stages. *a* 14–15-day-old fetus (× 95); *b* 18-day-old fetus (× 110); *c* 21-day-old fetus (× 44); *d* 3-day-old newborn (× 95); *e* 20-day-old newborn (× 95); *f* adult (× 95); *g* adult (× 190); Note the bright fluorescence in the brush border of the top villus epithelial cells and the faint almost undetectable fluorescence in the crypt zone (arrow). Note that the fluorescence is interrupted in some places by mucous granules ejecting from goblet cells. It should be noted that numerous mast cells in the lamina propria show autofluorescence of histamine which is easily distinguished from the greenish immunoreaction of calmodulin by its yellowish color[24]. *h* adult (× 151) transverse section at the crypt zone; *i* adult (× 95) control section after neutralization of calmodulin antibodies with native calmodulin.

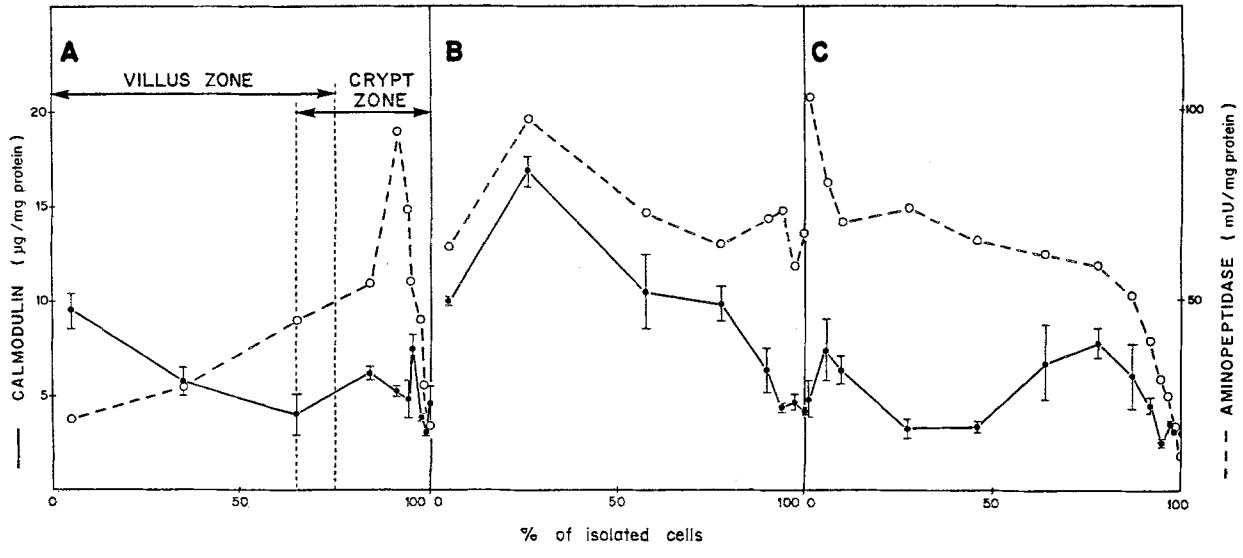

Figure 2. Representative gradient of calmodulin concentration in rat jejunal epithelial cells isolated along the villus-crypt axis at different development stages. *A* suckling; *B* weaning; *C* adult. The suckling stage corresponds to a 6-day-old state but similar profiles have been obtained for other stages of neonatal life (3, 6, 9, 12, 16 and 18 days after birth). The weaning stage corresponds to the 21st day of postnatal life. Each curve is representative of 2–3 experiments performed in duplicate.

During the 18 first days of postnatal life, aminopeptidase activity is preferentially localized at the crypt base (A), whereas in the adult, like all digestive brush border enzymes[7], it is maximal at the top of the villi and decreases progressively towards the crypt base (C). The shift is observed around weaning (B) when aminopeptidase is present almost uniformly along the whole villus-crypt axis[7].

especially at weaning. Much information is available about the pattern of appearance of digestive enzymes[6] or the modifications of their distribution along the villus-crypt axis during the neonatal period[7,8]. However, little is known about changes in the cytoskeletal proteins present in the intestinal brush borders. The microvillus core filament bundle is a highly ordered structure containing actin associated with a series of well characterized proteins[9-14]. Among them, calmodulin[9,10], an ubiquitous calcium-binding protein, functions as a calcium buffer[15], activates the actomyosin-based contractility system[16] and interacts with a number of actin binding proteins[15]. Therefore we studied the developmental pattern of calmodulin in the rat intestine, in order to attempt a correlation with the morphological and functional events accompanying intestinal ontogenic and spatial maturation.

Materials and methods. Wistar rats were used at different fetal and neonatal stages. The existence of a vaginal plug was designated as day 0.

For immunocytochemistry, the jejunums were removed, cut in 5 mm thick slices, fixed immediately with 2% paraformaldehyde in 0.1 M piperazin-1,4-bis-2 ethanesulfonate (PIPES) at pH 7.0, containing 5% sucrose, and frozen in melting freon. 4-5-µm-thick cryostat sections were processed for the immunofluorescence staining of calmodulin. After a 20-min pretreatment with 5% normal goat serum in Tris-buffered saline (TBS), the sections were incubated overnight with rabbit anticalmodulin antibodies diluted in TBS containing 0.2% bovine serum albumin. After washing with TBS, the sections were incubated for 1 h with fluorescein-labeled goat-anti-rabbit serum, extensively washed with TBS and mounted in glycerol as described by Schmit et al.[17].

For the determination of calmodulin content, the epithelial cells were sequentially released, from the villus tip to the crypt base, as described previously[7]. The progressive cell isolation procedure was assessed by determining the enzymatic profile of a representative brush border enzyme which has been shown to exhibit different crypt-villus distribution as a function of intestinal development[7]: aminopeptidase assayed by the method of Maroux et al.[18]. At the fetal stages, owing to the small size of the intestines, the sequential isolation of the cells was not possible and calmodulin content was determined on the whole jejunum. Each fragment or cell pellet was then sonicated in 20 mM Tris/Hcl buffer pH 7.4 containing 1 mM ethylene glycol-bis (β-aminoethylether) N, N, N′,N′-tetraacetic acid (EGTA), 1 mM mercaptoethanol and 1 mM phenyl methylsulphonylfluoride. After heating at 80 °C for 5 min in the presence of 1 mM EGTA in order to inhibit Ca^{++}-activated proteases, and centrifugation, calmodulin contained in the supernatant was assayed by its ability to activate a standard quantity of calmodulin-dependent cyclic AMP phosphodiesterase according to the method of Rochette-Egly and Egly[19].

Protein was determined by the method of Lowry et al.[20] and standard calmodulin was purified from rat liver by chromatography on immobilized fluphenazine as previously described[21].

Results and discussion. At the 14th day of fetal life, no specific fluorescence could be detected in the pluristratified epithelium which is devoid of differentiated brush borders (fig. 1a). However, at day 18 of gestation (fig. 1b), a weak fluorescence appeared in the apical part of the epithelial cells paralleling the formation of still sparse and irregular microvilli. At day 21, just before birth (fig. 1c), the fluorescence increased markedly in parallel to the maturation of the apical brush borders. Then despite the fact that villus elongation proceeds from birth to the adult stage, no variation in fluorescence intensity was obvious at the various post natal (fig. 1d and 1e) and adult stages (fig. 1f–1g). From day 18 of fetal life onwards (fig. 1b–1f), the apical staining intensity appeared identical in the epithelial cells from the tips to the bases of the jejunal villi. It should be stressed that crypt formation occurs during the neonatal period[22] and that calmodulin fluorescence is difficult to characterize in this zone. However at the adult stage, when crypts are clearly defined, calmodulin fluorescence is undetectable in the crypt zone (fig. 1f). This observation is particularly evident in transverse sections (fig. 1h). and corroborates the presence of sparse and irregular microvilli[23].

However, quantitative evaluation of calmodulin led to results contrasting with immunofluorescence labeling; a) in the whole jejunum of either 15–18- or 21-day fetal rat intestine, calmodulin was present at the mean level of 3.7 ± 0.3 µg/mg protein; b) in sequentially isolated epthelial cells of suckling (fig. 2a) and adult (fig. 2c) jejunums, calmodulin levels were found to range around similar and uniform values (4.9 ± 1.8 µg/mg protein). Moreover, at weaning (20–21th day of postnatal life) there was an unexpected finding; a significant and transient 2–3-fold increase in calmodulin levels of villus cells but not of crypt cells could be observed (fig. 2b). The fact that this increase in calmodulin content in the villus epithelial cells was transient rather than definitive, strengthens the hypothesis that the passage to the adult functional state is paralleled by important maturation processes[1,3] and marked changes in membrane composition at the time of weaning[25,26]. The acquiring of mature digestive functions, mainly in the villus epithelial cells, is illustrated by the modification of the crypt-villus distribution of a brush border marker enzyme, aminopeptidase (fig. 2).

The present work clearly shows that calmodulin is localized in the brush border of intestinal epithelial cells, confirming some earlier reports[9,10] and supporting the assumption that calmodulin is one of the major components of the microfilamentous core. Of particular interest in the present study is the observation that calmodulin is immunocytochemically almost undetectable either in the early fetal epithelial cells (day 14) or in the adult crypt zone, whereas by the biochemical assay it was found in amounts equal to that in the mature top villus epithelial cells. This discrepancy suggests that in the immature epithelial cells, calmodulin would be evenly distributed in the cytoplasm, and thus be undectectable by immunocytochemistry. Then one can speculate that during the maturation of the fetal cells, as during the migration of crypt cells towards the villus-tip, calmodulin would segregate in the microvilli, whereas its mean concentration remains roughly constant in the cells. A similar phenomenon has already been reported for other brush border cytoskeleton proteins (villin and 140 Kd)[27,28]. This suggests that in contrast to membrane hydrolases[29] the differentiation of brush borders is paralleled by a modification in the localization of calmodulin as of other cytoskeleton components rather than in their expression.

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Laser microbeam-induced fixation for electronmicroscopy: Visualization of transient developmental features in nematode embryos

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Summary. In order to study development of embryos of *Caenorhabditis elegans* at an ultrastructural level, a new method of fixation has been developed. With a laser microbeam coupled to a microscope the impermeable eggshell is punctured to allow penetration of the fixative. At specific stages of embryogenesis further development can be arrested at will under visual control. As fixation occurs instantaneously, transient events (e.g. different phases of mitosis and cytokinesis) can be visualized.
Key words. Electronmicroscopy; laser; embryogenesis; mitosis; germline.

Cellular development of the nematode *Caenorhabditis elegans* has been extensively studied under the light microscope. The complete cell lineages from fertilization to adulthood have been described including cell fates[1-4]. In addition, electronmicroscopical analysis of various stages of development and of various parts of the hatched organism has been performed [5-10].

Because the embryo of *C. elegans* develops so reproducibly we are able to predict reliably the fate of each cell at any stage of development[4]. Thus, it is desirable to arrest individual embryos at specific stages for ultrastructural analysis of selected cells.

During early embryogenesis many typical events occur which are transient (e.g. pronuclear migration[11], pseudocleavage[11], asymmetric growth of membranes[10], formation of cleavage spindles[12] and segregation of germline-specific granules[13,14]). Because of their short duration and the impermeability of the eggshell it is difficult to fix an embryo exactly at the desired stage.

To facilitate this, it is necessary to observe the developing embryo under the microscope and then, at the chosen time, induce an instantaneous fixation of the egg for preservation of the structures of interest.

Here we describe a new method for fixation of the embryo at given developmental stages using a laser microbeam coupled to a microscope.

The developing embryos are dissected out of the mother in a drop of filter-sterilized water. Early embryos, well before the stage at which they will be fixed, are selected under a dissecting microscope and transferred with a drawn-out pasteur pipette to

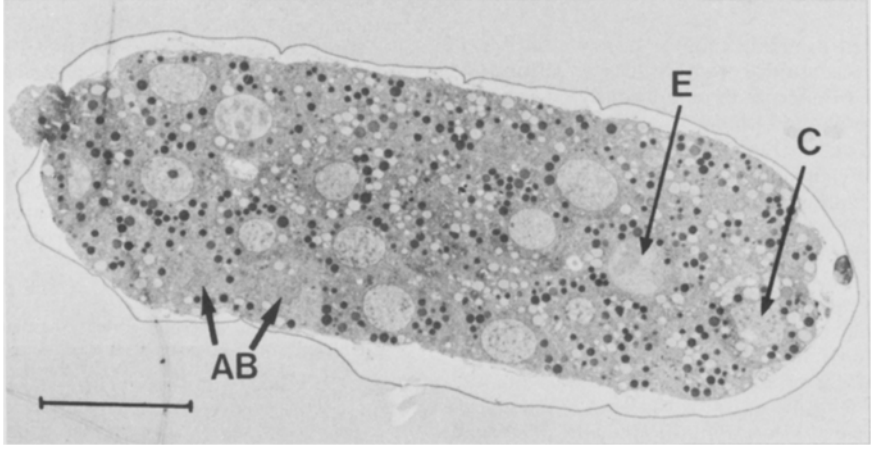

Figure 1. Longitudinal section through a 101-cell embryo after laser-induced fixation. At the point of laser penetration some cytoplasm has leaked out. Arrows point to nuclei which have been taken as landmarks to identify cells. AB = pharyngeal precursor in division, C = body muscle precursor in early mitosis, E = gut precursor at the onset of mitosis. Orientation: anterior left, dorsal top. Bar = 10 µm.