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Ultrastructure and Ion Distribution of the Intestinal Cell during Experimental Vitamin-D Deficiency Rickets in Rats*

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Summary. Rats with an initial weight of 40–50 g developed significant rickets, when fed on a vitamin-D free, low-phosphorus diet continuously for 4 weeks. In contrast, a vitamin-D free, normal-phosphorus diet had no rachitic effect. The rachitic animals showed an elevation of alkaline phosphatase and a decrease of inorganic phosphorus in serum, a reduction of growth and body weight and marked rachitic alterations in bone. Surprisingly, the serum calcium showed a significant elevation, the cause of which will be discussed. Light and electron microscopy revealed only nonspecific signs of metabolic damage, such as swelling of mitochondria, endoplasmic reticulum and Golgi complex in the duodenal cell. A sensitive precipitation method, using potassium pyroantimonate for ultrastructural demonstration of calcium as well as sodium ions, however, showed a characteristic difference in distribution pattern of the precipitates normally found at the surface of the microvilli, in the endoplasmic reticulum, in Golgi apparatus, mitochondria and extracellular space. In rickets, there were additional deposits within the inner space of microvilli and a marked reduction of mitochondrial granules. After vitamin-D substitution these deposits within microvilli disappeared, and mitochondria were temporarily overloaded with ions. If the precipitates in microvilli and mitochondria mainly represent calcium and not sodium, which seems probable but cannot be proved at present, these findings support the concept that in rickets at least a part of the calcium is able to pass the microvillar membrane and that the following step of transcellular calcium transport is impaired. After vitamin-D substitution the carrier system for ion transport at the top of the cell seems to recover earlier than the ion-exclusion mechanism in the basal portion of the enterocyte.

Zusammenfassung. Bei den an Ratten mit einem Ausgangsgewicht von 40–50 g durchgeführten Versuchen führte nur eine Vitamin D-freie, phosphatarme, nicht jedoch eine allein Vitamin D-freie Diät nach 4 Wochen zu einer eindeutigen Rachitis. Die rachitischen Tiere zeigten eine starke Erhöhung der alkalischen Phosphatase und eine Erniedrigung des Phosphats im Serum, eine Reduktion des Wachstums und des Körpergewichtes sowie deutliche rachitische Knochenveränderungen. Das Serumcalcium wies überraschenderweise eine deutliche Erhöhung auf, deren mögliche Ursache diskutiert wird. Am rachitischen Duodenum fanden sich licht- und elektronenmikroskopisch die Zeichen einer Störung des Zellstoffwechsels mit Schwellung von Mitochondrien, endoplasmatischem Reticulum und Golgiapparat. Die Anwendung einer empfindlichen Präzipitationsmethode zum elektronenmikroskopischen Nachweis von Calcium- und allerdings auch Natriumionen ergab zusätzlich einen charakteristischen Verteilungsunterschied der Präzipitate, die normalerweise nur an der Oberfläche der Mikrovilli, im endoplasmatischen Reticulum, in den Mitochondrien, im Golgiapparat und im Extracellularraum nachweisbar waren. Bei Rachitis traten zusätzliche Niederschläge innerhalb der Mikrovilli bei starker Abnahme der Mitochondrien-Präzipitate auf, während nach Vitamin D-Substitution die Präzipitate in den Mikrovilli verschwanden und die Mitochondrien vorübergehend von Ionen überschwemmt wurden. Wenn die Präzipitate in Mikrovilli und

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Mitochondrien vorwiegend Calcium und nicht Natrium darstellen, was wahrscheinlich ist aber gegenwärtig nicht bewiesen werden kann, sprechen die Befunde für die Vorstellung, daß bei der Rachitis wenigstens ein Teil des Calciums die Mikrovillusmembran passieren kann und daß der darauffolgende Schritt des transcellulären Calciumtransportes gestört ist. Nach Vitamin D-Substitution scheint sich im Enterocyten der apikale Ionenzustrom schneller zu erholen als der basale Ionenausschleusungsmechanismus.

In recent years many important data concerning the mechanism of normal and rachitic calcium absorption in intestine were obtained. There are for instance the elucidation of vitamin-D metabolism, the demonstration of calcium transporting enzymes and the detection of calcium binding proteins (CaBP). At the cellular level, however, the exact localization of these factors, associated with the intracellular calcium homeostasis, is still lacking. Furthermore the role of the different cell organelles in physiology and pathophysiology of calcium absorption remains still obscure. The literature in this subject gives only few morphologic details about rachitic intestine (Nordio *et al.*, 1968; Sampson *et al.*, 1970; Matthews *et al.*, 1971; Sampson *et al.*, 1972). Therefore the purpose of this paper is to study the morphological changes of the duodenal enterocyte during rickets by light and electron microscopy. In addition it is attempted to get some information about the site of the defective mechanism of calcium transport within the cell by comparing the intracellular ion distribution in normal and rachitic state. For ion detection we used a method, which differs from that used by Sampson *et al.* (1970) in an in situ precipitation of calcium. Artificial ion translocation during tissue preparation is thereby avoided.

Materials and Methods

27 female Wistar albino rats (initial weight 40–50 g) were divided in four groups. The rats were kept in total darkness. Demineralized water was given ad libitum.

Group 1. 4 rats were given a vitamin-D free, calcium phosphorus normal diet (Altromin® Nr. C 1000 without vitamin D) for 4 weeks.

Group 2. 5 rats were given a vitamin-D free, low phosphate (0.2%), normal calcium (0.8%) diet (Altromin® rachitogenic diet Nr. C 1420) for 4 weeks.

Group 3. 9 rats were kept as described in group 2. After 4 weeks the rats were given 200 IU vitamin D_3 (Vigantol®) diluted in corn oil by oral tube and killed 15, 22, and 36 hours after vitamin-D substitution.

Group 4. 9 rats as controls were given a normal standard diet (Altromin®) for 4 weeks.

At the end of the period the rats were killed by ether anaesthesy. 24 hours prior and just before death blood was collected from the retroorbital plexus for chemical analysis. The duodenum of the groups 2, 3, and 4 was taken and immediately fixed for light and electron microscopic observation.

For light microscopy tissue was fixed in buffered formaline and embedded in paraffine. Sections were stained with H. E., PAS, Astrablue and the v. Kossa reaction. Kryostat sections were stained with Fettrot 7B for demonstration of fat and with an azo method for demonstration of alkaline phosphatase (Pearse, 1968).

For electron microscopy tissue was fixed by immersion fixation in an ice-cold Caulfield-solution (Veronal buffered OsO_4), dehydrated in ethanol and embedded in Epon 812 in the usual way.

For electron microscopic ion detection small tissue samples were fixed for 2 hours in a solution of ice-cold 2% potassium-pyroantimonate in 1% OsO_4 (Komnick and Komnick, 1963). The fixative was adjusted to pH 7.3 by 0.01 n acetic acid. After a short rinsing in 8% sucrose the tissue was dehydrated in ethanol and embedded in Epon.

0.5 μ sections were stained with 1% toluidine blue for light microscopic observation. Ultrathin sections were visualized unstained and after staining with uranylacetate or uranylacetate and lead citrate. Examination was carried out with a Zeiss EM 9 electron microscope.

In some animals of group 3 and 4 undecalcified sections of the left tibia were examined, to prove the existance of rickets in group 3.

In serum levels of calcium (Eppendorf flame photometer), alkaline phosphatase (Merckotest Nr. 3304), magnesium (Merckotest Nr. 3338) and inorganic phosphorus (Merckotest Nr. 3331) were determined by microliter methods.

Results

In our first experiment we wanted to explore, whether a diet, which is only free of vitamin-D (group 1), would be sufficient to produce rickets in rats or whether a vitamin-D free, low phosphorus diet would be necessary for this purpose. Group 1 showed no changes in all biochemical parameters (Table 1). The body weight was not significantly reduced. In contrast the rats, fed a vitamin-D free, low phosphorus diet (group 2), developed the signs of severe rickets: (1) growth and body weight were markedly reduced. (2) In serum a marked increase of alkaline phosphatase and a decrease of inorganic phosphorus was observed. (3) The serum magnesium was slightly increased, while the serum calcium showed a significantly elevated level in all animals of group 2 and 3. Vitamin-D substitution at the end of the period (group 3) had no significant effect on the serum levels of the rachitic animals within the studied time.

Undecalcified sections of the tibiae of group 2 showed the signs of severe rickets, too, with extreme broadening of the growth plate and the osteoid seams[1].

Table 1. Results of chemical serum analyses and body weights in the different experimental groups at the time of death (in brackets values 24 hours ante exitum). Mean values ± standard deviations

	Vitamin D-free normal phosphorus diet (group 1)	Vitamin D-free low phosphorus diet (group 2)	Vitamin D-free low phosphorus diet + 200 IU vitamin D_3 (group 3)	Controls standard diet (group 4)
Calcium (mg-%)	10.9 ± 0.5 (10.7 ± 0.4)	14.1 ± 1.0 (14.5 ± 1.3)	14.1 ± 1.2 (14.4 ± 1.1)	10.6 ± 0.7 (10.8 ± 0.6)
Magnesium (mg-%)	2.06 ± 0.1 (2.08 ± 0.08)	2.32 ± 0.12 (2.35 ± 0.09)	2,29 ± 0,10 (2.31 ± 0.12)	2,09 ± 0,09 (2.1 ± 0.08)
Inorganic phosphate (mg-%)	7.5 ± 1.2 (7.8 ± 1.0)	3.5 ± 1.5 (3.7 ± 1.3)	3.9 ± 1.4 (3.8 ± 1.2)	8.2 ± 1.0 (7.9 ± 1.1)
Alkaline phosphatase (mU/ml)	90 ± 30 (103 ± 24)	205 ± 71 (225 ± 83)	190 ± 52 (205 ± 41)	95 ± 31 (110 ± 25)
Body weight (g)	134 ± 9	76 ± 15	80 ± 13	149 ± 18

1 Preparation of undecalcified sections and histologic examination of bone was kindly carried out by Dr. G. Delling, Department of Pathology, University of Hamburg.

Table 2. Distribution of precipitates within the enterocyte

	rickets	rickets + vitamin D_3	
		15–24 hrs	36 hrs
Microvilli outside	n	n	n
Microvilli inside	++	+	n
Endoplasmic reticulum	n	n	n
Mitochondria	−−	++	+
Golgi complex	n	n	n
Lateral space	n	n	n

n = normal, + = slightly increased, ++ = markedly increased, − = slightly reduced, −− = markedly reduced.

Morphologic examination of duodenum was carried out in animals of group 2, 3, and 4.

Light Microscopy. Sections stained with PAS, H. E., Astrablue and the v. Kossa reaction showed no differences in any group. Length of villi and number of goblet cells were normal in all rachitic rats. The distribution of mitochondria within the enterocyte, as studied in 0.5 μ sections, was equal in all groups. In some rachitic intestinal cells especially in the upper part of the villi there was an increase of fat droplets within the cytoplasm. The light microscopic evaluation of alkaline phosphatase showed no difference in distribution pattern and activity.

Electron microscopically with conventional fixation the intestinal cells of rachitic rats showed only unspecific signs of metabolic damage like swelling and vesiculation of the endoplasmic reticulum and a dilatation of the Golgi complex. The mitochondria were swollen, too, with lysis or concentric curling of the cristae (Fig. 6). The microvilli showed no changes. The alterations increased from the base to the top of the villi. After vitamin-D substitution especially the mitochondrial damages tended to normalize.

The reaction with potassium-pyroantimonate for electron microscopic ion detection gave a characteristic pattern of precipitate distribution for each group. Position and extent of precipitation is summarized in Table 2 and Fig. 1.

In the *normal* enterocyte there were heavy precipitates in the lumen of the intestine (Fig. 7). They became even more dense at the microvillar surface within the fuzzy coat, where ion accumulation just before passage of the microvillar membrane occurs. In normal state the space within the microvilli was completely free of deposits (Fig. 2). But precipitates were found in the channels and along the membranes of the endoplasmic reticulum; the cisterns and vesicles of the Golgi apparatus contained many pyroantimonate granules (Fig. 3) and the lateral space and the basal interstitial room were filled with precipitates, too (Fig. 4). In mitochondria precipitation occurred mainly at the so-called matrix granules; each mitochondrium contained about 1–4 granules normally.

In *rickets* (group 2) there were characteristic changes in the precipitate distribution. In the space within the microvilli, which was normally free of precipitates, now deposition of pyroantimonate salts occurred (Fig. 5). The granules were situated mainly along the inner membrane of the microvilli. The mito-

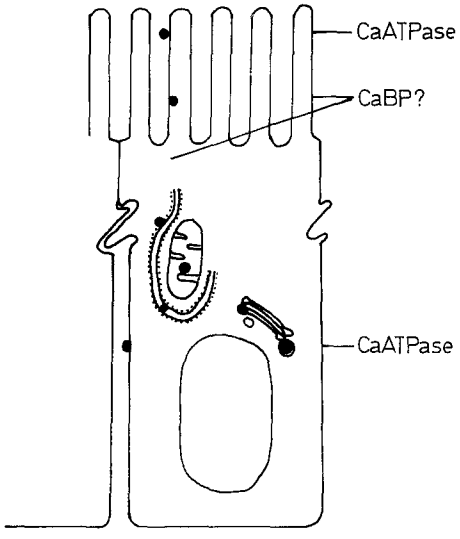

Fig. 1. Diagram of an enterocyte with some cell organelles, showing the typical sites of pyroantimonate precipitation (•). Calcium-dependent adenosine triphosphatase (CaATPase). Calcium binding protein (CaBP)

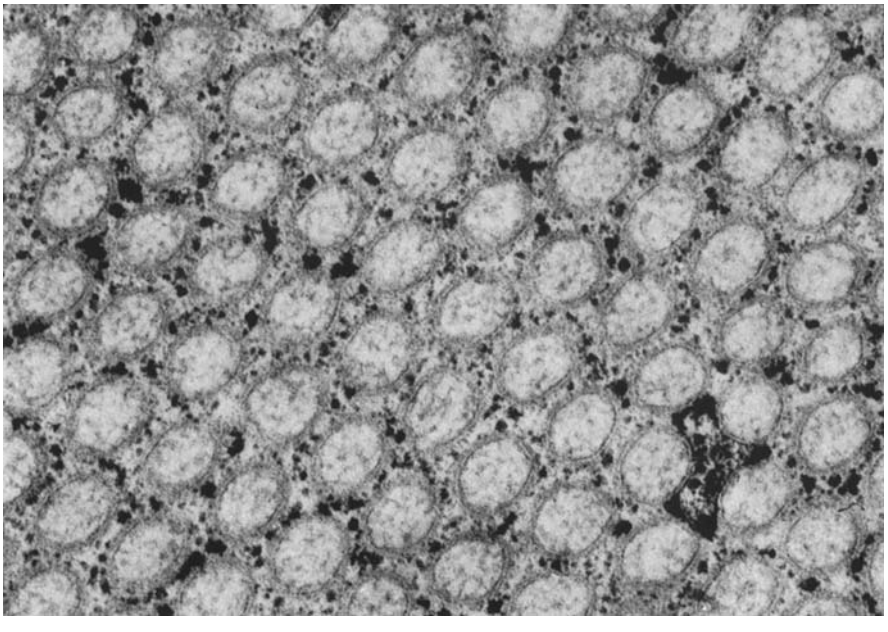

Fig. 2. Normal rat: cross section through microvilli. Precipitates at the outer surface of the microvillar membranes; no deposits within the microvilli. K-pyroantimonate reaction. × 75000

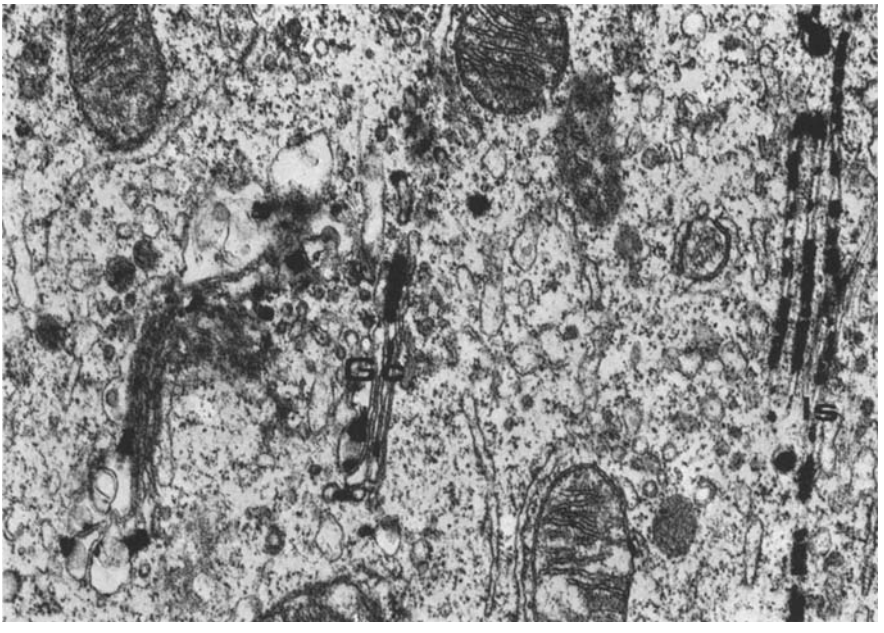

Fig. 3. Normal rat: central portion of an intestinal cell. Precipitates within the Golgi complex (*Gc*) and in the lateral space (*ls*) between two enterocytes. K-pyroantimonate reaction. × 30000

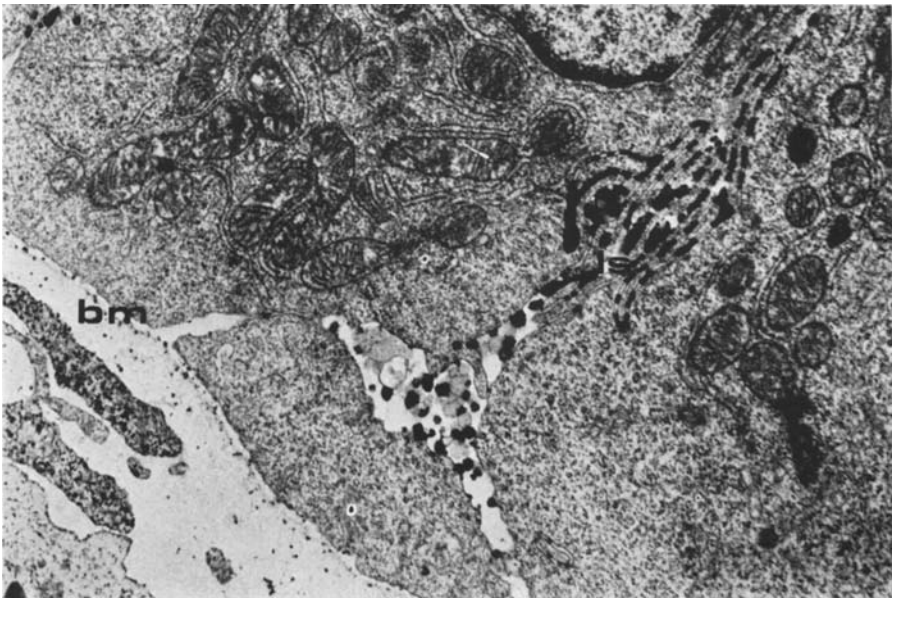

Fig. 4. Normal rat: basal portion of two intestinal cells. Only few mitochondrial (*m*) granules. Distinct precipitates at the basement membrane (*bm*) and in the lateral space (*ls*), which is in continuous connection with the basal interstitial fluid. K-pyroantimonate reaction. × 6000

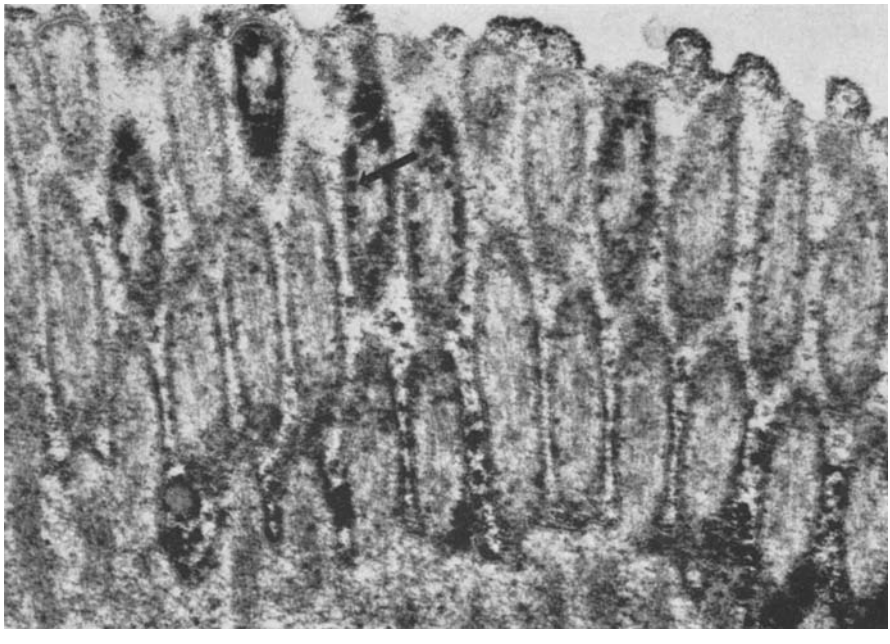

Fig. 5. Rachitic rat: intestinal microvilli. Dense deposits (arrow) within microvilli, mainly located along the inner microvillar membrane. K-pyroantimonate reaction. × 42000

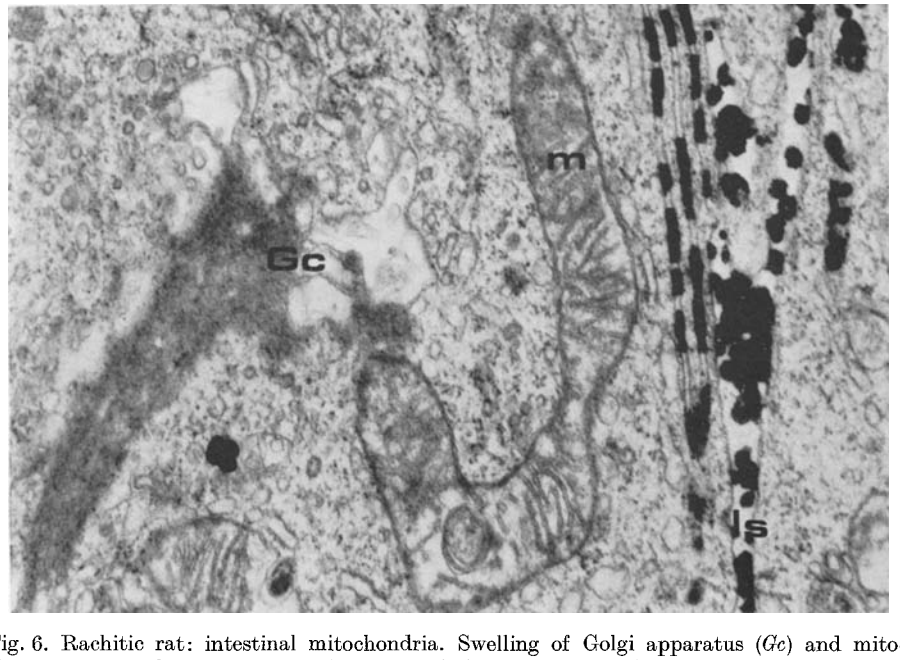

Fig. 6. Rachitic rat: intestinal mitochondria. Swelling of Golgi apparatus (*Gc*) and mitochondria (*m*) with irregularity and curling of the cristae. Mitochondrial granules are completely absent. Distinct deposits in the lateral space (*ls*). K- pyroantimonate reaction. × 26000

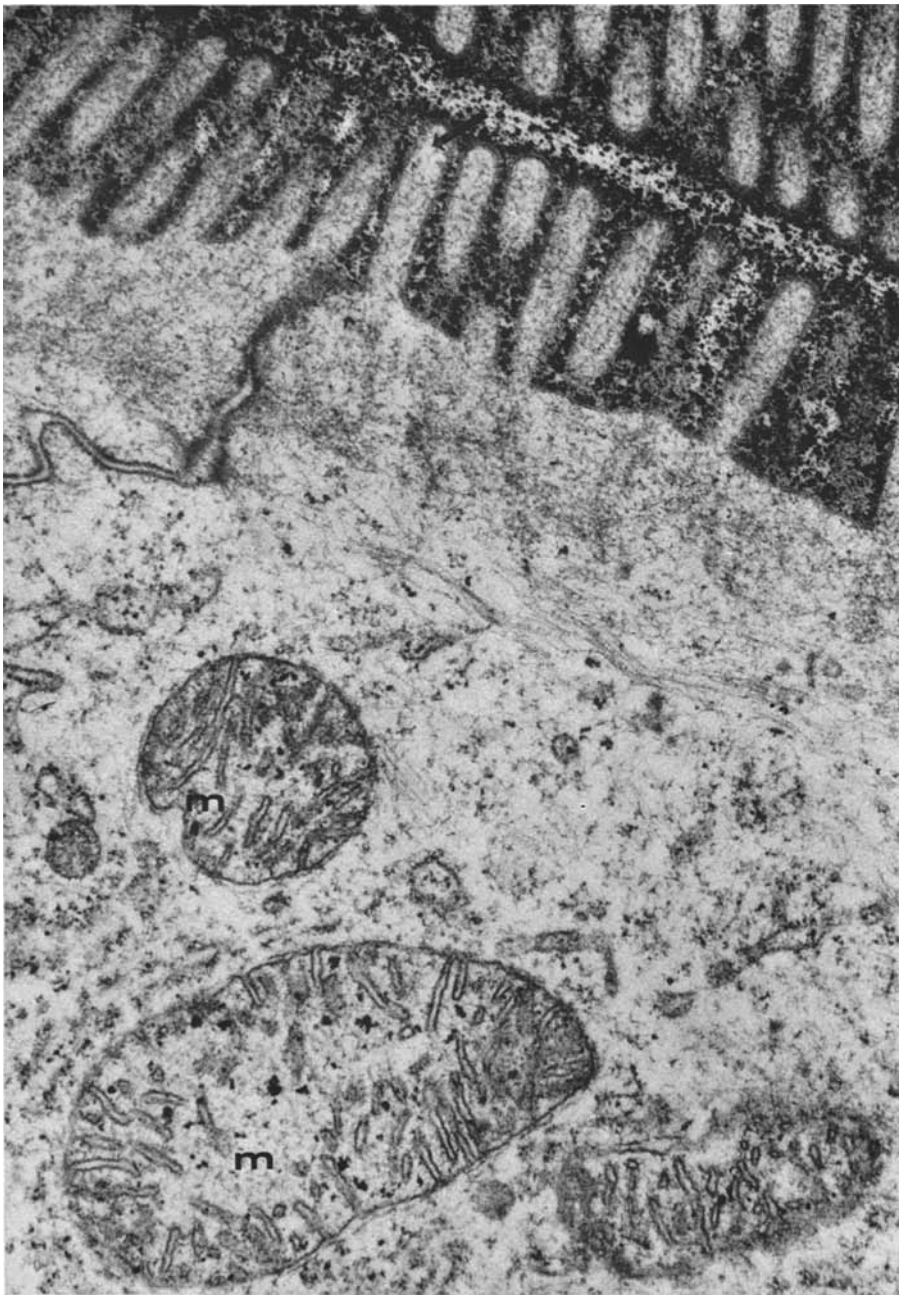

Fig. 7. Rachitic rat, substituted with 200 IU vitamin D_3 22 hours ante exitum: apical part of intestinal cell. Inner microvillar space (arrows) is now free of precipitates, as seen in controls. Mitochondria (*m*) show abundant granules. K-pyroantimonate reaction. × 40700

chondria in contrast were nearly free of deposits (Fig. 6); only very few granules could be detected. The other sites of deposition showed no significant changes.

After *vitamin-D substitution* of the rachitic animals again a significant alteration of the deposit pattern was observed. Within 36 hours the precipitates at the inner space of the microvilli disappeared nearly completely; but the mitochondrial deposit contents markedly increased to a much higher degree than normal (Fig. 7). Sometimes up to 20 granules were found per mitochondrium. After 36 hours already a tendency to normalization could be detected. Again the other sites of deposition showed no significant alterations.

Discussion

The first aim of the study was to find a reliable model for production of rickets in rats. If a vitamin-D free diet would be alone sufficient, as pointed out by Matthews *et al.* (1971), the experimental conditions would very much imitate the causes of human rickets. However, by other authors it was found that only a vitamin-D free, low phosphorus diet is truly rachitogenic in rats (De Luca, 1967). This may be due to the use of different rat strains by the single authors. In our experiments we could not observe any signs of rickets at all studied parameters, when a vitamin-D deficient diet was given for 4 weeks. This does, of course, not completely exclude the possibility that a longer period of diet administration in connection with more sensitive parameters may reveal discrete signs of rickets; but for our purpose this model was not reliable enough. Severe rickets, which was confirmed by histological examination of undecalcified tibia sections, was only found, when a vitamin-D deficient, low phosphorus diet was fed. Therefore we used, in contrast to Sampson *et al.* (1970), this diet for our morphologic investigations. Surprisingly this experimental procedure resulted in a marked elevation of serum calcium levels in all rachitic rats. A high calcium intake could not account for it, because of its low content of calcium (0.83%) and phosphorus (0.23%). An explanation may be that lack of phosphate results in an increased osteocytic osteolysis, as observed by Debove *et al.* (1972) in low phosphate, normal vitamin-D treated rats. The fact that this process was found to be PTH-independent may explain the elevation of calcium to values higher than normal.

Light microscopy and the conventional methods of electron microscopy gave disappointing results, which only pointed to a metabolic disorder of the intestinal cells in a rather unspecific way. Similar alterations with swelling of mitochondria and endoplasmic reticulum were observed by Nordio *et al.* (1968) in duodenal biopsy material of rachitic children. The fat droplets in intestinal cells, which are increased in number in rickets, may in part be absorbed nutritional fat; on the other hand vitamin-D deficiency is known to inhibit two steps of citric acid cycle (Neuman and Neuman, 1958; Rao and Patwardhan, 1962; Nordio *et al.*, 1968). Thus the increase of fat droplets may partly be understood as a result of disturbed energy metabolism of the intestinal cell during rickets.

To get information about the absorptive activity of the enterocytes, we studied the distribution of mitochondria within the cell. A polar congregation of mitochondria in the basal part of the cell was found to be a sign of absorptive activity in regard to certain constituents of food (Gonzalez-Licea, 1970, 1971,

1972). We could not find any difference in the normal and rachitic state. Also histochemical comparison of alkaline phosphatase, one of the enzymes, which seems to be closely related to calcium transport, did not reveal any changes in rickets. This enzyme was biochemically shown to be reduced in rachitic intestinal brush border preparations (Norman *et al.*, 1970; Haussler *et al.*, 1970). Perhaps the semiquantitative light microscopic method was not sensitive enough to detect small changes.

In summary all results obtained with conventional morphologic methods were only unspecific signs of impaired cell metabolism. In order to get more specific information about the rachitic defect of ion transport in intestine we tried to localize the substance itself, the transport of which is mostly impaired: the calcium.

For this purpose Sampson *et al.* (1970) tried to stabilize the diffusible calcium in tissue by addition of calcium chloride to the fixation fluid. After this treatment they showed electron dense deposits, which were shown by microincineration to be of mineral nature and which changed their position during rickets. This method, however, did not exclude translocation of calcium during fixation or accumulation of calcium from the fixative to calcium binding sites in tissue. This latter event proved to occur as recently shown by the studies of Oschman and Wall (1972). After treatment with calcium rich fixative, in situ calcium and secondarily accumulated calcium was indistinguishable with this method. Therefore we used another method. We added potassium pyroantimonate to the fixative, which precipitates calcium in situ to an highly insoluble electron dense salt. This method, first used for electron microscopic sodium detection (Komnick and Komnick, 1963), was later shown to be highly sensitive for calcium ion detection in tissue, as well (Legato and Langer, 1969; Thureson-Klein and Klein, 1971; Yarom and Meiri, 1971). It must therefore be pointed out that the method is not specific for calcium. But despite of this lack of specifity we were able to obtain a significant difference in the distribution pattern of precipitates in normal, rachitic and vitamin-D substituted rachitic animals.

In the intestine of controls intraluminal precipitates are most dense at the surface of the microvilli, which may indicate ion accumulation just before absorption, perhaps within the mucopolysaccharide layer of the fuzzy coat. The lack of precipitates within the microvilli suggests a quick ion transport to the deeper portions of the cell after passage of the microvillar membrane under normal conditions. The significance of the precipitates at the endoplasmic reticulum is not yet clearly understood. Perhaps they are related to the intracellular pathways of the transported calcium or to that fraction of calcium, which acts as an intracellular messenger for specific cell functions (Rasmussen, 1970). At the present time also the heavy deposits within the Golgi apparatus cannot exactly be interpreted. Further investigations, which are in progress, should answer the question, whether it is really calcium or sodium, which can be found in such great amounts within the Golgi apparatus. Perhaps the Golgi apparatus is involved in the ion exclusion mechanism of the cell. This interpretation may be supported by observations of Bernard and Pease (1969) and Anderson (1969), who found mineralized spherules in chondrocytes and osteoblasts, possibly

secreted by the Golgi apparatus. The mitochondrial deposits are very important. They occurred mainly in connection with the matrix granules, which are purified and biochemically analyzed (Matthews *et al.*, 1971). Mitochondria are able to accumulate calcium (Greenawalt *et al.*, 1964; Cassidy *et al.*, 1969) and are known to deposit it in the inactive form of tertiary calcium phosphate and apatite within the matrix granules. The mitochondria are the site of the slowly exchangeable intracellular calcium pool (Borle, 1971) and act as a buffer system against toxically high intracellular calcium levels. Therefore size and number of mitochondrial deposits can be interpreted as an indicator of intracellular calcium level. Finally, the precipitates in the lateral and basal intercellular spaces may demonstrate the high extracellular levels of calcium as well as sodium. In rickets changes in the distribution pattern of deposits mainly consisted of precipitate accumulation within the microvilli and a subnormal level of precipitates within the mitochondria. After vitamin-D substitution microvillar deposits disappeared and the deeper parts of the cell were overloaded with ions, as indicated by the abundance of mitochondrial deposits. There is no proof at the present time that the precipitates in microvilli and mitochondria mainly represent calcium, because the pyroantimonate method demonstrates sodium as well. The above mentioned biochemical findings on mitochondria, the demonstration of calcium binding sites within microvilli of insect intestine (Oschman and Wall, 1972) and the predominance of a calcium absorption defect in rickets make it more probable, however, that the observed variation in electrolyte distribution is correlated with the calcium ion. If this assumption is correct our findings can be interpreted in the following way: there is an ion transport defect in rickets between the inner microvillar membrane and the deeper cell portion, where the mitochondria are localized; at least a part of the calcium is able to pass the microvillar membrane and accumulates in the close vicinity, because the next transport step seems to be impaired; after vitamin-D substitution the transport mechanism in the apical cell portion recovers, the intramicrovillar precipitates disappear and the lower cell part, containing the mitochondria, becomes overloaded with calcium. This interpretation would confirm the concept of Sampson *et al.* (1970). It would provide further evidence that the site of a vitamin-D dependent calcium transport, which is thought to be closely related to the calcium binding protein, is located in the apical portion of the enterocyte, but not in the microvillar membrane. In contrast, immunofluorescent studies of Taylor and Wasserman (1970a) localized CaBP in goblet cells and at the microvillar surface. However, the optical resolution of the immunofluorescent method is limited.

The temporary ion overloading of the cell after vitamin-D supplementation suggests that the apical transport mechanism recovers earlier than the basal ion exclusion mechanism, which may be linked with Ca-ATPase, mainly localized at the basal cell membrane (Parkinson and Radde, 1971). In fact, Taylor and Wasserman (1970b) could demonstrate a time lag between the appearance of intestinal CaBP and Ca-ATPase (Melancon and De Luca, 1970) after vitamin-D substitution of rachitic animals. But up to now a proved correlation between these findings cannot be given.

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