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Ultrastructural Studies on the Myocardial Capillaries of the Experimentally Lathyritic Rat, Protective Effect of Certain Flavonoids

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Summary. The ultrastructure of rat myocardial capillaries was studied in the course of experimental lathyrism. Endothelial cells were hypertrophic, with a sinuous profile of the plasma membrane facing the lumen and with a consistent increase of pinocytotic vesicles; the nuclei were irregular in shape; ATPase activity was no more demonstrable. Therefore, various and well distinct structural endothelial mechanisms seem to be primarily involved, causing an alteration of the dynamics of transcellular exchanges and of the permeability of the vascular wall.

Simultaneous treatment with certain flavonoids, (O-(β-hydroxyethyl)-rutosides and Na(+)-epicatechin-2-sulfonate), resulted in a less pronounced alteration and a more rapid recovery. The possibility of the existence of a common site of action of lathyrogens and flavonoids is raised in the discussion.

Key words: Lathyrism — Capillaries — Rat — Ultrastructure — Flavonoids.

It is known that in experimental animals, lathyrogenic substances cause changes also at the level of the vascular system. Hence, in addition to osteolathyrism and neurolathyrism, processes which have been amply studied by now (Selye, 1957), it is possible to speak of experimental angiolathyrism. Hithertoe, investigation has been focused mainly on the action of these substances on collagen fibres, which action is considered to be primordial in causing the changes typical of experimental lathyrism. It is generally agreed that lathyrogens (at least some of them) inhibit lysyloxidase, an enzyme responsible for the formation of cross-linkages between elementary collagen chains (Siegel *et al.*, 1970; Nayaranan *et al.*, 1972).

Research into angiolathyrism has been largely biochemical and, given the above-mentioned primary effect of the lathyrogens, has been devoted to the study of the soluble collagen of the aorta (Hosoda and Iri, 1967; Cetta *et al.*, 1971, 1972). Histological investigations revealing the existence in the microvessels of alterations typical of lathyrism have also been conducted (in the retina: Heath and Rutter, 1966; Paterson and Heath, 1968; Williams, Paterson and Heath, 1968; in the myocardium: Gerzeli *et al.*, 1971).

In particular, the histochemical and histoenzymatic study of the vascular membrane conducted by Gerzeli and co-workers has, by describing the initial stages of cell injury, furnished considerable data on the functional state of this membrane. These data have led the authors to believe that harmful substances such as lathyrogens are capable of causing a disturbance of vessel wall dynamics, independent of their effect on collagen fibres.

Along with the destruction of pericytes (as demonstrable with the PAS reaction), distinct morphological alterations of the epithelium—which is found to be hypertrophied—and the histochemical response to certain enzymes have been revealed; the most significant changes have been observed in connection with ATPase (activated by Na^+ with neutral pH), an enzyme involved in the transepithelial passage of electrolytes.

These investigations could be of considerable value in that they offer an interesting and easily reproducible experimental model in the form of lesions of individual vessel walls. Using this model, it is possible to test the possible protective effect of various substances. For instance, special emphasis has already been placed on the flavonoids (Paterson and Heath, 1968; Gerzeli, 1971; Gerzeli *et al.*, 1971; Cetta *et al.*, 1971, 1972).

As the histochemical and histoenzymatic studies already mentioned have emphasized, the state of the vascular membrane and consequently the characteristics of the endothelium, we felt it worthwhile to undertake an ultrastructural morphological study of the capillaries of the rat myocardium for the purpose of identifying the ultrastructural sites of action of lathyrogens.

We also felt it worthwhile to study the modifications resulting from the concomitant administration of flavonoids—which have already been found to have a beneficial effect on the maintenance of vessel integrity and permeability (Böhm, 1968)—and their effect on collagen in lathyrism (Cetta *et al.*, 1971, 1972).

Material and Methods

24 adult male rats with an initial average weight of ca. 245 g were divided into 4 groups of 6 rats each:

A. Lesion. Administration of $\beta.\beta'$-iminodipropionitrile (IDPN), 25 mg/100 g body weight i.p. per day for 7 days. From the 4th to 5th day onward, all IDPN-treated animals exhibited a syndrome characterized by motor excitation with circling and choreiform movements (termed ECC syndrome by Selye, 1957) as well as severe ocular lesions with haziness of the cornea and conjunctivitis, accompanied by considerable loss of weight.

B. Protection. Administration of O-(β-hydroxyethyl)-rutosides (HR), 50 mg/100 g body weight s.c. per day or Na(+)-epicatechin-2-sulfonate (ES), 5 mg/100 g body weight s.c. per day[1].

C. Lesion and Protection. Protective treatment was begun on the 1st experimental day and continued till sacrifice. Lesion-producing treatment was administered, in adjunction to the former, from the 8th to the 15th experimental day.

D. Controls. The animals received neither of the substances under examination.

Half of the animals of each group were sacrificed by decapitation on the 3rd day after discontinuation of treatment with IDPN; the remaining half were sacrificed (by decapitation) on the 17th day after discontinuation of IDPN treatment.

Dissection of the animals revealed no visible alterations of the heart or largest vessels. Immediately after sacrifice, tissue samples from the left ventricle were fixed in 2.5% glutaraldehyde (buffered with 0.2 M cacodylate buffer to pH 7.4) for 3 hrs at 4°C. For the localization of adenosine triphosphatase activity (pH 7.4), several samples were incubated for 1 hr at 37°C according to the technique of Wachstein and Meisel (1957) as modified by White and Krivit (1965) in the following mixture:

20 ml ATP (125 mg/100 ml)
20 ml 0.2 M trismaleate buffer (pH 7.2)
3 ml 2% Ph $(NO_3)_2$
5 ml 0.1 M $MgSO_4$
2 ml bidistilled H_2O.

1 We are indebted to Zyma SA, Nyon, Switzerland, who kindly supplied us with O-(β-hydroxyethyl)-rutosides and Na(+)-epicatechin-2-sulfonate.

All samples were successively post-fixed for 2 hrs in 1% OsO_4 solution, dehydrated and embedded in Epon.

From the embedded tissue samples, ultra-thin sections (ca. 600 Å) were obtained. They were stained in uranyl acetate and lead citrate according to the technique of Venable and Coggeshall (1965). Microscopic observation was made with the Philips EM 300 electron microscope and Agfa Gevaert 23056 flat film was used in the photographic stage.

Results

The myocardial capillaries observed, exhibited patterns which were rather easy to classify. Upon microscopic examination, special attention was given to the overall appearance of the endothelial cells, the position of the nucleus, the number and orientation of pinocytotic vesicles and the state of the plasma and basal membrane. In addition, the sicles and the state of the plasma and basal membrane. In addition, the concentration and distribution of ATPase reaction products were studied. Hence it was possible to establish for the control rats, a sufficiently reliable frame of reference for the subsequent evaluation of the various experimental situations.

In the preparations from the control rats (transverse capillary sections), the endothelial cells were rather thin. However, their thickness varied with the area observed and was, of course, greater in the vicinity of the nucleus. In the cytoplasm of the endothelial cells, there were numerous evenly distributed vesicles rather small in size (400–600 Å) facing the lumen and the basal lamina along the inner and outer plasma membrane. These are ultrastructural patterns attributable to pinocytosis (Majno, 1965). The basal membrane was found to be thin and adherent to the endothelium. It generally showed a continuity all round the vessel. The inner plasma membrane exhibited a certain degree of unevenness due to minute extensions into the lumina of the capillaries (Fig. 1).

Special emphasis is to be placed on the structure and position of the endothelial cell nucleus, whose greater axis was found to be parallel to the cell wall. The lumina of the capillaries were generally spacious, since the nuclei bulged inwards very little.

Around the capillaries, pericytes were recognized; some of them were complete with nuclei, while others showed but a few lamellae of cytoplasm. In the surrounding spaces were distributed bundles of collagen fibres; in addition, single fibres could be seen adjacent to the basal membrane.

The preparations examined for ATPase activity showed numerous precipitates along the endothelium, which were distributed mainly along the outer plasma membrane (Figs. 9, 10). However, the localizability of this reaction product, which was rather coarse, was quite limited, as Hoff, 1968, 1969, also observed.

In the preparations from the IDPN-treated rats, deviations from the normal appearance were observed. These changes were of considerable magnitude, especially in the preparations obtained from the animals sacrificed on the 3rd day after discontinuation of treatment (Figs. 2, 3).

On the whole, the endothelium appeared uneven and considerably thickened. There was, firstly, a great increase in pinocytotic processes: numerous small vesicles (400–600 Å), intermingled with larger ones, occupy a large portion of the total section area.

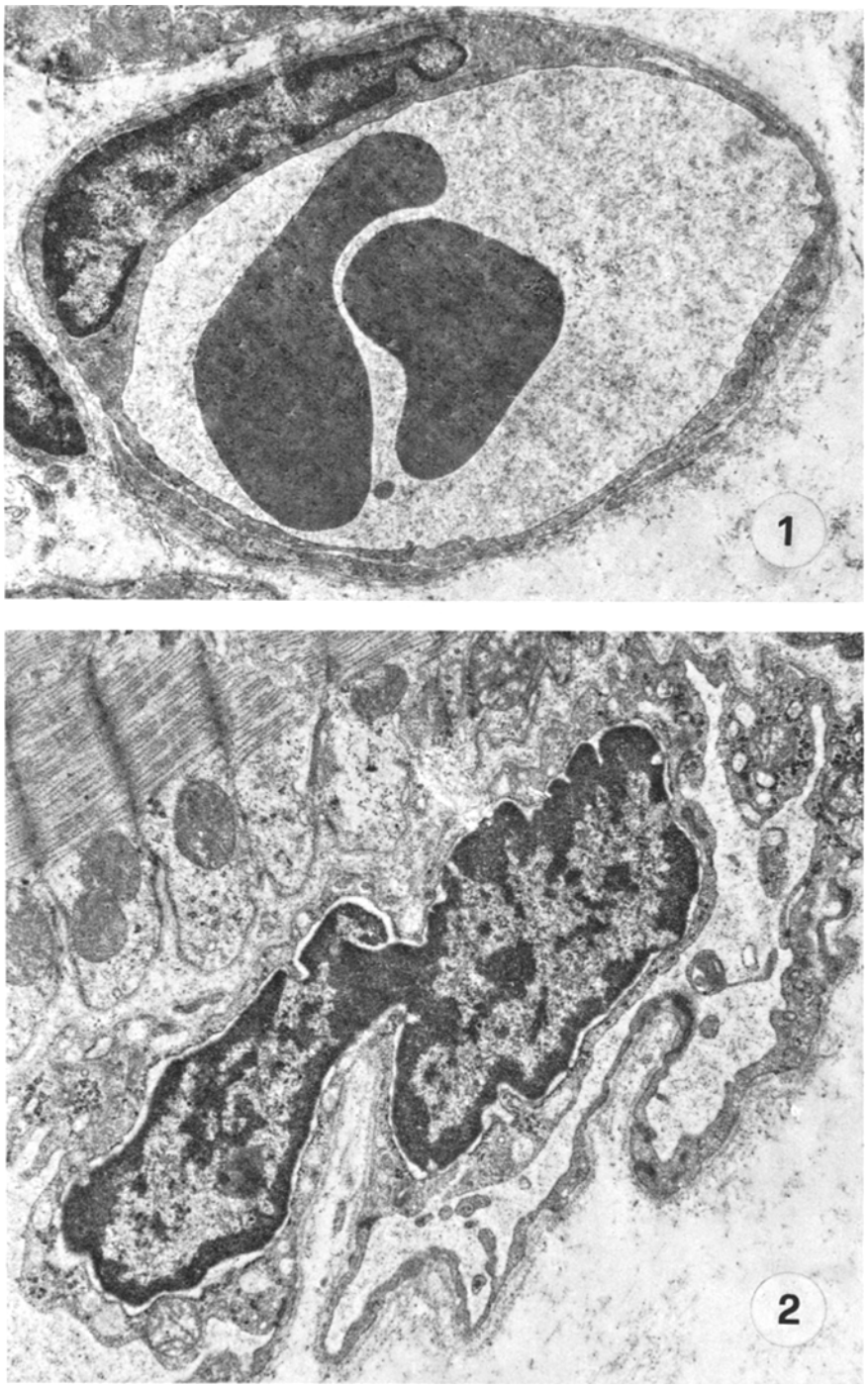


Fig. 1. Myocardium of control rat (× 12000). In the capillary wall two cross-sectioned endothelial cells can be observed; the nucleus is visible in one of them. The endothelium is rather thin and even. Pinocytosis is not especially pronounced: small vesicles are evenly

The plasma membrane, facing the lumen of the vessel, exhibited a markedly sinuous profile, due to digitiform and foliaceous projections of variable length and considerable dimensions. On the other hand, the plasma membrane, facing the basal membrane, displayed numerous small infoldings which were less prominent than the former. The basal membrane was still complete and visible along the endothelium. The nuclei were irregular in shape, causing the endothelial cell to bulge into the lumen, which was, in fact, largely occupied by the cytoplasmic extensions and nuclei.

In the preparations obtained from the animals sacrificed on the 17th day after discontinuation of treatment, the same basic changes were noted. However, endothelial thickening was more uniform and a considerable increase in pinocytotic vesicles was noted. In addition, the infolding of the plasma membrane facing the lumen was more pronounced. The continuity of the endothelium was interrupted in some areas and extravasally localized red blood cells could be seen. In the surrounding spaces, minute bundles of collagen fibres were more visible (Fig. 4).

Cytoplasmatic edges, attributable to pericytes, were found only in the myocardial samples collected on the 17th day after discontinuation of treatment, whereas none were seen in those collected on the 3rd day.

In all preparations, independent of the time after discontinuation of treatment, the endothelium was found to be practically negative for ATPase activity, at least as regards the ultrastructural cytochemical demonstrability of this activity.

In the myocardial preparations from rats subjected simultaneously to the action of IDPN and HR and sacrificed on the 3rd day after discontinuation of treatment with IDPN, the endothelium was somewhat thickened and pinocytosis was considerable. Some of the vesicles were larger than usual. On the other hand, the irregularity in the profile of the plasma membrane facing the lumen was much less pronounced in the preparations from rats treated with IDPN alone; small protrusions into the lumen were occasionally noted. The outer boundary of the endothelium and the basal membrane appeared identical to the controls. The nuclei tended to bulge into the lumen (Figs. 5, 6).

On the 17th day after discontinuation of IDPN treatment, a distinct tendency to normal morphology was noted in the rats protected with HR. In many of the capillaries observed in the sections, the thickness of the endothelium had decreased. Pinocytosis was still considerable and was localized along the plasma membrane facing the lumen. Here and there, the plasma membrane facing the basal membrane and the basal membrane itself exhibited sinuous and corresponding profiles. The nuclei also tended to recover their normal configuration and appeared flat-

distributed. The plasma membrane facing the lumen is very even. The nucleus is arranged with the greater axis parallel to the vessel wall. On the left of the capillary, a pericyte with its nucleus is partially visible

Fig. 2. Myocardium of rat treated with IDPN for 8 days and sacrified on the 3rd day after discontinuation of treatment (× 13000). The capillary endothelium is infolded and thickened, with digitiform and foliaceous projections. The nucleus is also irregular in shape and bulges into the lumen

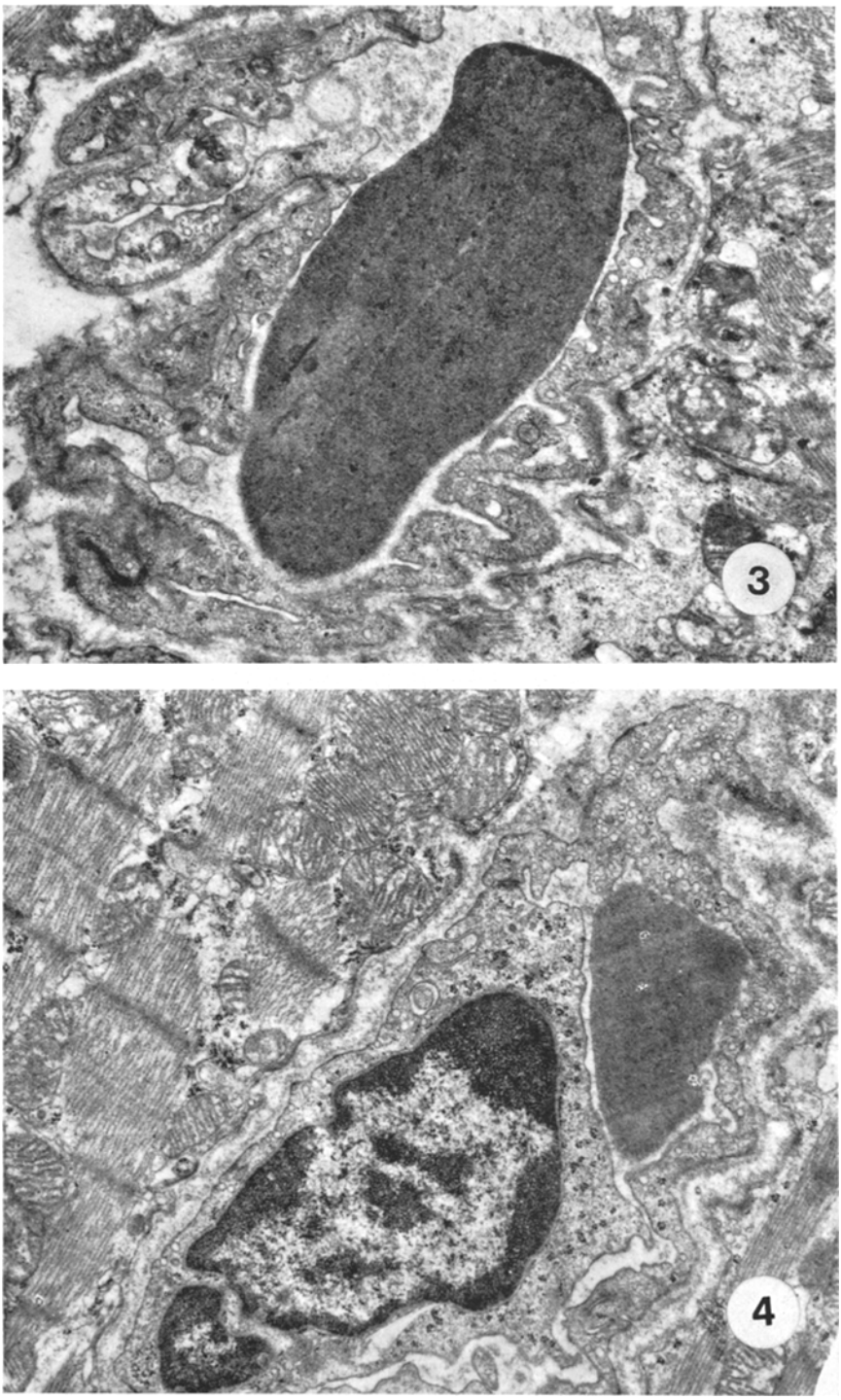
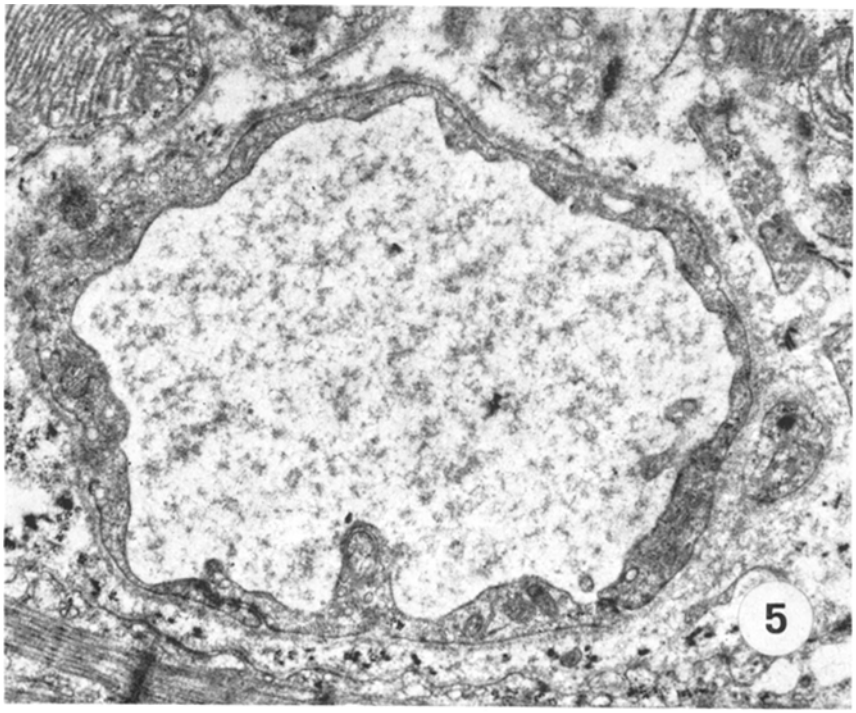


Fig. 3. Myocardium of rat treated with IDPN for 8 days and sacrificed on the 3rd day after discontinuation of treatment (× 11000). A great increase in pinocytotic vesicles is seen, many of which are small in size and intermingled with larger ones



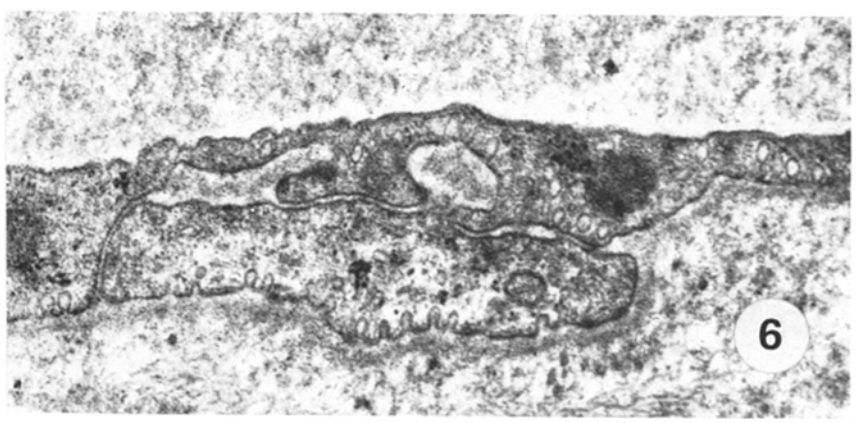
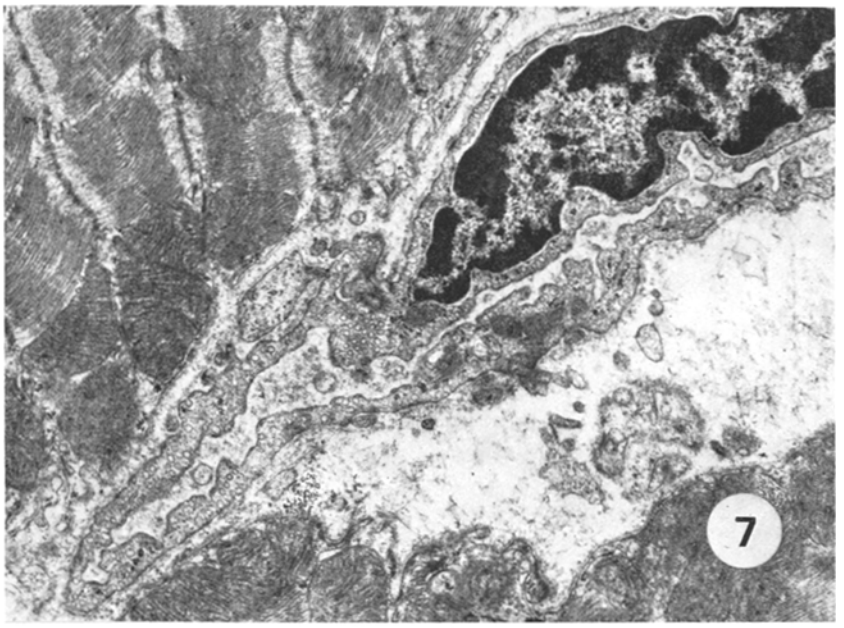


Fig. 4. Myocardium of rat treated with IDPN for 8 days and sacrificed on the 17th day after discontinuation of treatment (× 16000). The endothelium still appears thickened. The presence of numerous pinocytotic vesicles is seen. The folds of the plasma membrane are broader. The nucleus is still rather roundish in shape

Fig. 5. Myocardium of rat treated with IDPN and HR sacrificed on the 3rd day after discontinuation of IDPN treatment (× 20000). The thickness of the endothelium is rather uniform. Pinocytosis is quite diffuse. The plasma membrane facing the lumen shows only few irregularities

Fig. 6. Myocardium of rat treated with IDPN and HR sacrificed on the 3rd day after discontinuation of IDPN treatment (× 52000). The presence of pinocytotic vesicles (500–600 Å) is observed. The vesicles are localized almost exclusively along the plasma membrane facing the basal lamina



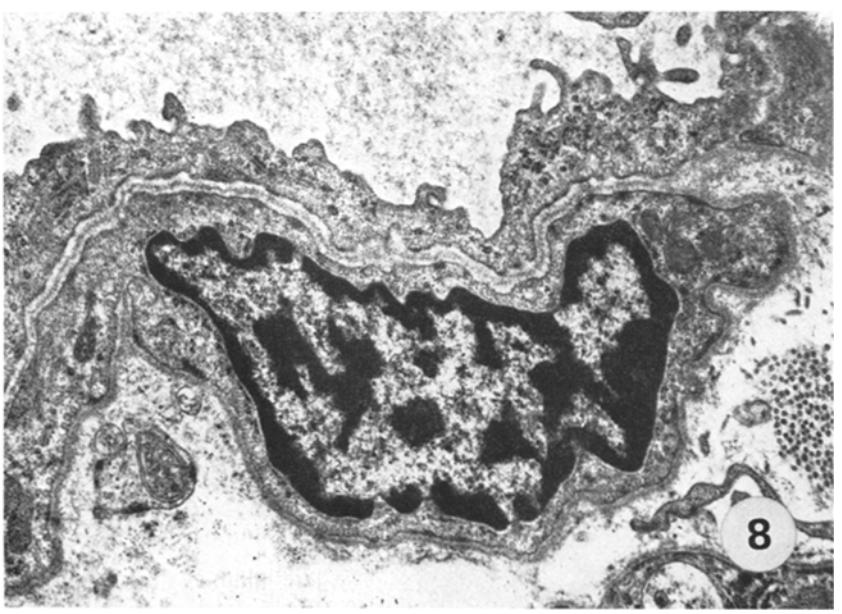


Fig. 7. Myocardium of rat treated with IDPN and HR sacrificed on the 17th day after discontinuation of IDPN treatment ($\times$ 13000). The endothelium displays a rather uniform thickness along with a very great increase in pinocytotic vesicles. The folds of the plasma membrane facing the lumen are broader. The nucleus is arranged with its greater axis parallel to the vessel wall

Fig. 8. Myocardium of rat treated with IDPN and HR sacrificed on the 17th day after discontinuation of IDPN treatment ($\times$ 16000). The endothelium is practically normal in appearance. The pericyte (a cell that generally disappeared after IDPN treatment) appears intact and along its plasma membrane pinocytosis is noted

tened along the vessel wall. Hence the profile of capillary lumen was regular, almost identical to that of the control sections. Pericytes were seen (Figs. 7, 8). However, there was no consistent indication of a recovery of ATPase activity (Fig. 11).

Likewise, the capillaries of the animals treated with IDPN and ES and killed on the 3rd day after discontinuation of treatment showed limited alterations. The changes did not, however, have the extreme appearance of those induced by treatment with IDPN alone. The endothelium had a turgescent appearance and showed an irregular profile. Pinocytosis was considerable and comparable to that observed in the IDPN animals. The vesicles opened particularly along the outer profile of the capillary section. The plasma membrane facing the lumen exhibited some infolding, the prominence of the latter varying with the area observed, while the plasma membrane facing the basal membrane still had a rather smooth profile. The nuclei appeared roundish in shape.

The rats protected with ES and sacrificed on the 17th day after discontinuation of IDPN treatment showed a tendency to morphological normality in many of the capillaries observed. In certain areas, the endothelium was found to be reduced in thickness and less irregular: invaginations and digitations of the plasma membrane were more rare. Pinocytosis was still diffuse and even more pronounced: the vesicles were small in size and evenly distributed throughout the cell, mainly along the outer plasma membrane, but also along the plasma membrane facing the lumen of the vessel. The nuclei tended, to a certain extent, to become flat. Pericytes were occasionally seen.

Comment

The reported ultrastructural observations confirm the interest of the study of the vascular membrane in the course of experimental lathyrism.

Previous histochemical and histoenzymatic investigations (Gerzeli, 1971; Gerzeli *et al.*, 1971) had already placed emphasis on alterations of the walls of the microvessels. These were alterations of the endothelium which did not appear to be directly attributable to the wellknown action of lathyrogenic agents on collagen (Tanzer, 1965) and on lysyl oxidase (Nayaranan *et al.*, 1972). Proliferation and swelling of endothelial cells were previously studied at ultrastructural level only in the aorta (Keech, 1960; Simpson *et al.*, 1962).

The greatest emphasis should now be placed on the considerable hypertrophy of the endothelial cells, which is accompanied by a very sinuous profile of the cell membrane, on the consistent increase in pinocytotic vesicles occurring mostly along the plasma membrane facing the basal lamina and on the insufficiently demonstrable ATPase activity. This suggests that various and well-distinct structural endothelial mechanisms are primarily involved: they have the effect of altering the dynamics of transmembranal exchanges, thereby modifying the permeability of the vessel wall as well.

Studies on the ultrastructural localization of Na-activated ATPase activity corroborate previous light microscopy histochemical and histo-enzymatic data. The inhibition of ATPase persists for several days, even after discontinuation of IDPN treatment. It is noteworthy that this enzyme, as is generally agreed, is membrane bound and intervenes in the active transport of electrolytes.

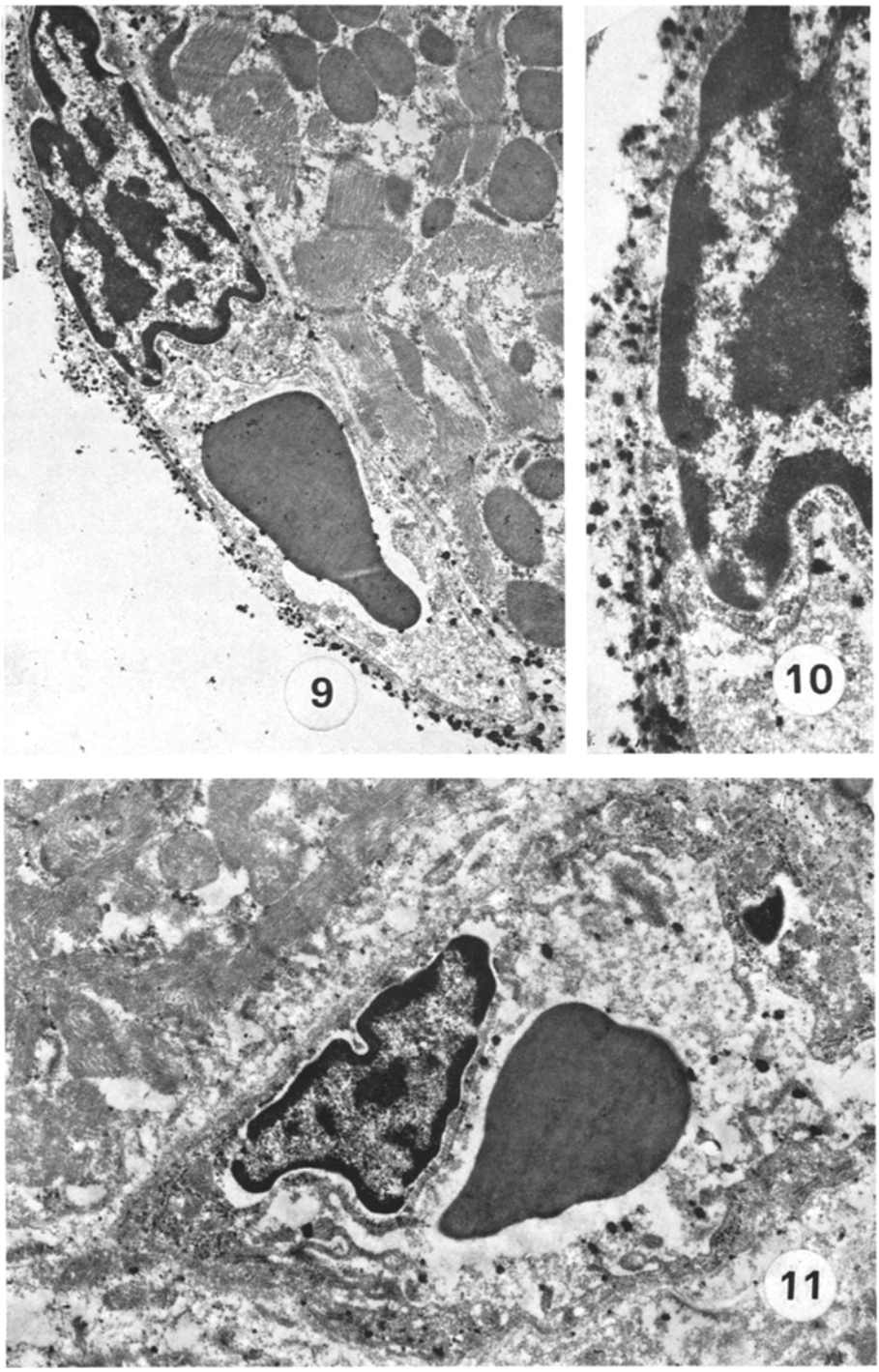


Fig. 9. Myocardium of control rat. Reaction for localization of ATPase activity after White and Krivit, 1965 (× 10000). Precipitates are recognized along the endothelium and are distributed prevalently along the plasma membrane facing the basal lamina

In concomitance and, to certain extent, in opposition to this process, there is an increase in pinocytosis vesicles, which may be linked to the transcellular transport of other solutes, macromolecules in particular.

The possible direct action of lathyrogenic substances on fine structural and metabolic mechanisms offers new research prospects hitherto insufficiently considered. In fact, most of the vascular alterations have been studied in larger vessels, the aorta in particular. In this case, the very marked action of lathyrogens on the formation of elastin and collagen fibres, and consequently the severe injury to the connective tissue coat tends to mask other phenomena, e.g. the changes of the endothelial membrane.

The second noteworthy finding of the present study is the marked protective action of certain flavonoids. There was a less pronounced thickening and infolding of vessel wall, with pinocytotic vesicle proliferation. In addition, there was a more rapid recovery of normal vessel morphology.

The suitability of a study on this model of substances possessing a possible vasoprotective effect is thus confirmed. The question proves to be of especial interest here, since previous studies have provided data on the beneficial effect of flavonoids in the maintenance of vascular integrality and permeability (Böhm, 1968); however, it has not been possible to determine its molecular mechanism of action. Since the protective effect of these substances against lathyrism is displayed at two clearly different levels at least (*viz.* formation of collagen fibres, integrity of endothelial membrane), the existence of a single and common site of action of lathyrogens and flavonoids is subject to discussion, with effect on more than one metabolic pathway. Recent research has suggested that flavonoids counteract lathyrogens via an effect combining certain metallic ions essential to the activity of these enzymes (Cetta *et al.*, 1971, 1972).

Hence this may account for the variety of effects and possible organ specificity of lathyrogens as well as the extensive protective effect of flavonoids, which is subject to further confirmation.

References

Böhm, K.: The flavonoids. Aulendorf/Württ.: Cantor KG 1968

Cetta, G., Gerzeli, G., Castellani, A. A.: Effet des flavonoides sur le collagène dans le lathyrisme. Angiologica **9**, 235–247 (1972)

Cetta, G., Gerzeli, G., Quartieri, A., Castellani, A. A.: Protective effect of flavonoids on the collagen of lathyritic rats. Experientia (Basel) **27**, 1046–1048 (1971)

Gerzeli, G.: Studio istoenzimatico dell'effetto protettivo dei flavonoidi verso l'angiolatirismo. Arch. ital. Anat. Embriol., Suppl. **76**, 44–45 (1971)

Gerzeli, G., De Piceis Polver, P., Prenna, G.: Ricerche enzimoistochimiche sui capillari miocardici di cavia. Minerva med. **62**, 445–449 (1971)

Fig. 10. Myocardium of control rat. Reaction for localization of ATPase activity after White and Krivit, 1965 (× 38000). The presence of precipitates is seen in greater detail, owing to the reaction along the capillary wall

Fig. 11. Myocardium of rat treated with IDPN for 8 days sacrificed on the 3rd day after discontinuation of treatment. Reaction for ATPase after White and Krivit, 1965 (× 13000). The reaction is practically negative

Heath, H., Rutter, A. C.: Retinal angiopathy in the iminodipropionitrile treated-alloxan-diabetic rat. Brit. J. exp. Path. **47**, 116–120 (1966)

Hoff, H. F.: A comparison of the fine-structural localization of nucleoside phosphatase activity in large intracranial blood vessels and the thoracic aorta of rabbits. Histochemie **13**, 183–191 (1968)

Hoff, H. F.: Further studies on the ultrastructural localization of a magnesium dependant neutral ATPase in arteries. Histochemie **18**, 210–216 (1969)

Hosoda, Y., Iri, H.: "Antiolathyrism". Angiology **18**, 616–627 (1967)

Keech, M. K.: Electron microscope study of the lathyritic rat aorta. J. biophys. biochem. Cytol. **7**, 539–545 (1960)

Majno, G.: Ultrastructure of the vascular membrane. In: Handbook of physiology, section 2: Circulation, vol. **3**, p. 2293–2375. Washington: American Physiological Society 1965

Nayaranan, A. S., Siegel, R. C., Martin, G. R.: On the inhibition of lysyl oxidase by β-aminopropionitrile. Biochem. biophys. Res. Commun. **46**, 745–752 (1972)

Paterson, R. A., Heath, H.: The effect of hydroxyethyl-substituted rutosides on the $\beta.\beta'$-iminodipropionitrile induced retinopathy in the rat. Brit. J. exp. Path. **49**, 283–287 (1968)

Selye, H.: Lathyrism. Rev. canad. Biol. **16**, 1–82 (1957)

Siegel, R. C., Pinnel, S. R., Martin, G. R.: Cross-linking of collagen and elastin. Properties of lysyl oxidase. Biochemistry (Wash.) **9**, 4486–4492 (1970)

Simpson, C. F., Pritchard, W. R., Harms, R. H., Sautter, J. H.: Electron microscopy of the cardiovascular system of the normal and beta-aminopropionitrile fed turkey. Exp. mol. Pathol. **1**, 321–343 (1962)

Tanzer, M.: Experimental lathyrism. Int. Rev. connect. Tissue Res. **3**, 91–112 (1965)

Venable, H. J., Coggeshall, R.: A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. **25**, 407–408 (1965)

Wachstein, M., Meisel, E.: Histochemistry of hepatic phosphatase of a phisiologic pH. Amer. J. clin. Path. **27**, 11–23 (1957)

White, J. G., Krivit, W.: Fine structural localisation of adenosine-triphosphatase in human platelets and other blood cells. Blood **27**, 554–567 (1965)

Williams, S., Paterson, R. A., Heath, H.: The effect of $\beta.\beta'$-iminodipropionitrile and anaesthesia on some adenine nucleotides of rat brain and retina. J. Neurochem. **15**, 227–233 (1968)

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