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Hydrocortisone Influences Developing Collaterals

2. A Cytochemical Study

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Summary. The effect of chronic administration of hydrocortisone on the topographic distribution of several hydrolases has been studied in fully grown coronary arteries and in coronary collaterals of the dog.

The response towards hydrocortisone appeared to be minimal in non-proliferating vessels, whereas significant enzyme adaptation was observed in growing vessels. New sites of activity were revealed for 5-nucleotidase and acid phosphatase. Strongly increased activity was noted in the vessel walls during the early stage of development for nucleoside diphosphatase and glucose 6-phosphatase. Well pronounced effects were observed equally for polyphosphatase and for thiamine pyrophosphatase. No modifications were induced in the case of alkaline phosphatase. An almost normal distribution pattern of these hydrolases was seen at the later stage of growth. The results were discussed in comparison with those obtained in untreated animals.

Introduction

Previous cytochemical studies on the experimentally induced growth of coronary collaterals in the dog indicated that the content and the topographic distribution of hydrolases in these vessels were strongly modified during this process (Borgers, 1971 a, 1971 b). A rather close relationship could be established between the morphologic alterations at different time periods after induction of growth and the behavior of some phosphatases (polyphosphatase, nucleoside diphosphatase, thiamine pyrophosphate, 5′-nucleotidase, acid phosphatase, glucose 6-phosphatase). The effect of hydrocortisone on the activation of enzyme systems during foetal and neonatal development has been intensively studied, i.e. in the intestinal tract (Moog, 1962, 1963, 1965).

The aim of this study was to determine whether chronic treatment with hydrocortisone would influence not only the mode of development but the enzyme pattern as well. Therefore the enzyme distribution during collateral growth after hydrocortisone treatment will be described in correlation with the distribution in the untreated animals to outline the specific effect of the corticosteroid on some metabolic events taking place at the early and at the late stages of collateral development.

Material and Methods

Experimental

In parallel with the morphologic experiments 16 beagles of either sex were prepared for cytochemical investigations.

The dogs were subdivided into the following groups:

2 dogs without any treatment (control).

1 dog with hydrocortisone treatment, 20 mg orally/kg/for 2 weeks (hydrocortisone 2 W).

1 dog with hydrocortisone treatment, 20 mg/orally/kg day, for 11 weeks (hydrocortisone 11 W).

2 dogs with constrictor[1] for 3 weeks, without hydrocortisone treatment (constrictor 3 W).

2 dogs with constrictor for 3 weeks, with hydrocortisone treatment, 20 mg/loraly/kg/day, for the last 2 weeks (constrictor + hydrocortisone 3 W).

2 dogs with constrictor for 8 weeks, without hydrocortisone treatment (constrictor 8 W).

2 dogs with constrictor for 8 weeks with hydrocortisone treatment 20 mg/orally/kg/day, for the last 7 weeks (constrictor + hydrocortisone 8 W).

2 dogs with constrictor for 12 weeks, without hydrocortisone treatment (constrictor 12 W).

2 dogs with constrictor for 12 weeks with hydrocortisone treatment, 20 mg/orally/kg/day, for the last 11 weeks (constrictor + hydrocortisone 12 W).

The hearts were perfused for 7 minutes through the cannulated aorta with an ice cold solution of freshly distilled 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. The excess fixative was washed out by a subsequent perfusion with the same buffer, containing 0.22 M sucrose. The hearts were quickly removed from the chest and small segments of normal arteries and (or) "midzone" pieces of collaterals (see morphologic study, Schaper, Borgers, Xhonneux, Schaper), were excised and stored overnight in the same buffer.

Cytochemistry

The distribution of the following phosphatases was investigated with the light and the electron microscope: alkaline phosphatase (AlPase), acid phosphatase (ACPase), nucleoside diphosphatase (NdiPase), thiamine pyrophosphatase (TPPase), polyphosphatase (PPase), 5′-nucleotidase (AMPase), adenosine triphosphatase (ATPase) and glucose 6-phosphatase (G-6-Pase).

The techniques for the preparation of vascular tissue for cytochemical use, the composition of the incubation media, incubation times, control incubation tests and further treatment of the sections have been outlined in a previous publication (Borgers, Schaper, Schaper, 1971).

Results

Light Microscopy

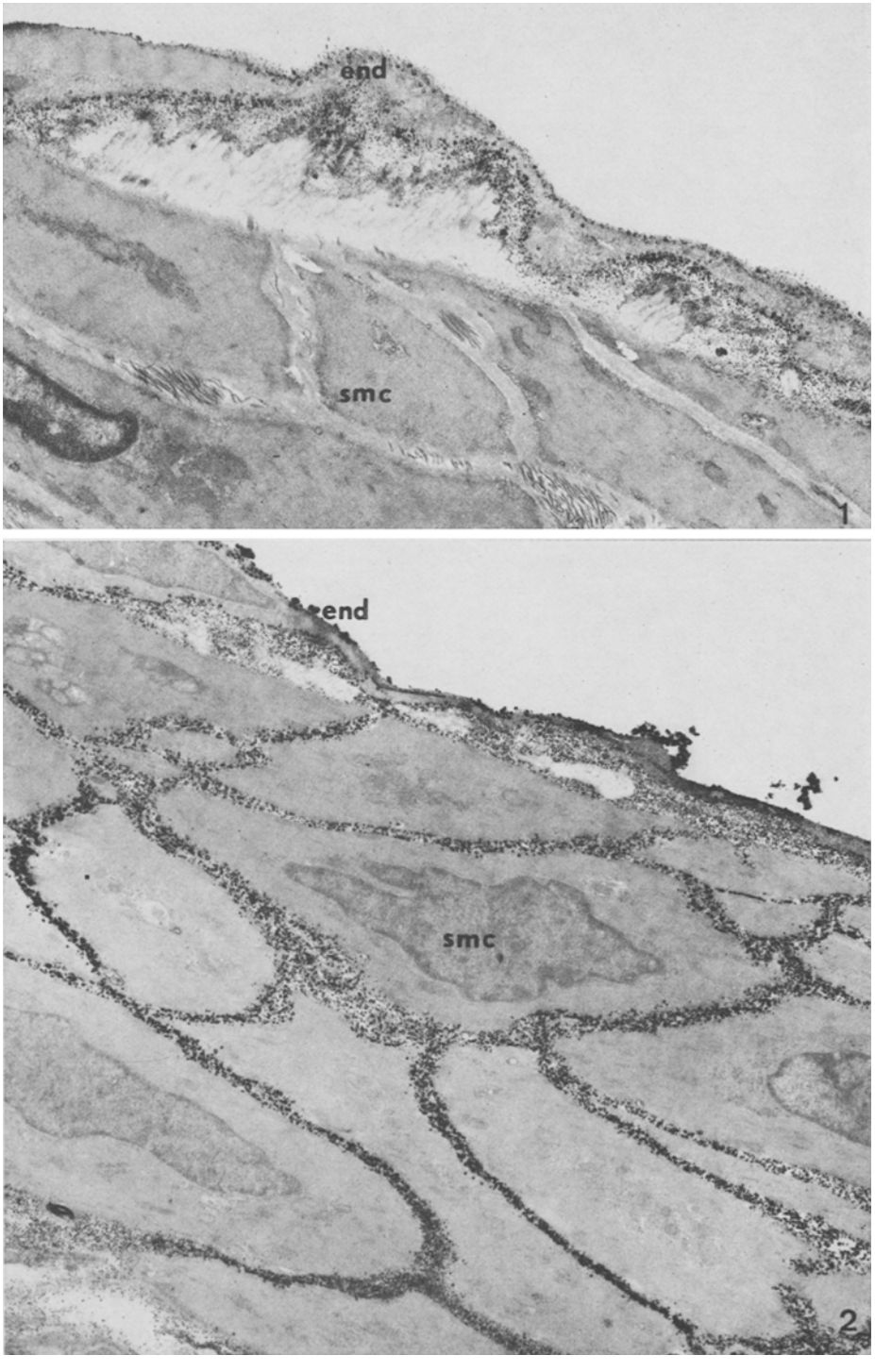
a) Control and Hydrocortisone Treated Groups

The first column of Table 1 summarizes the gross histochemical distribution of phosphatases in coronary arteries of hydrocortisone treated animals after 2,7 and 11 weeks. Proliferation of the vessel walls was never observed in the groups treated with hydrocortisone alone. With the exception of the presence of AMPase activity in the arterial endothelium in the hydrocortisone 11 week group, no difference in enzyme distribution between the control and hydrocortisone treated groups was noted.

Fig. 1. 5′-nucleotidase-Hydrocortisone 11 weeks. The reaction product is seen in multiple pinocytotic vesicles of the endothelial cells (*end*) of a small coronary artery. Medial smooth muscle cells (*smc*) lack the precipitate. (× 10800)

Fig. 2. Polyphosphatase-Hydrocortisone 2 weeks. Lead phosphate precipitate is present at the cell membranes of the arterial endothelium (*end*) and smooth muscle cells (*smc*). (× 6650)

1 Constrictor experiments were performed as described in the morphologic part of this study (Schaper, Borgers, Xhonneux, Schaper, 1973).



Figs. 1 and 2

Table 1. Distribution of phosphatase activities[a] in endothelial (end) subintimal (it), medial (med) and adventitial (adv) cells of coronary arteries and collaterials of control and treated dogs

Substrate	Site	Control	Hydrocortisone 2 W, 7 W, 11 W	Constrictor 3 W	Constrictor +hydrocortisone 3 W	Constrictor 8 and 12 W	Constrictor+ Hydrocortisone 8 and 12 W
b-GP	end	0	0	0	0	0	0
pH 9	it				0	0	0
	med	0	0	0	0	0	0
	adv	0	0	0	0	0	0
b-GP	end	1	1	3	2	1	1
pH 5	it				2	2	1
	med	1	1	3	2	1	1
	adv	1	1	3	2	1	1
AMP	end	0	1 (11 W only)	0	2	0	2
pH 7.2	it				0	0	0
	med	0	0	0	0	0	0
	adv	2	2	3	2	2	2
ADP	end	2	2	2	2	2	2
pH 7.2	it				1	0	1
	med	2	2	0	1	2	2
	adv	1	1	1	1	1	1
ATP	end	3	3	2	2	3	3
pH 7.2	it				1	0	2
	med	3	3	0	2	3	3
	adv	3	3	2	2	3	2
TPP	end	3	3	3	3	3	3
pH 7.2	it				2	0	1
	med	2	2	0	2	3	2
	adv	1	1	1	2	1	1
G-6-P	end	1	1	1	2	2	1
pH 6.7	it				2	2	1
	med	1	1	2	3	1	1
	adv	2	2	3	3	2	2

[a] Activities (amount of lead sulphide precipitate) are estimated on 1 micron thick epon-embedded sections; the reaction product is graded: 0 = no; 1 = weak; 2 = moderate, 3 = strong activity.

b) Constrictor and Constrictor + Hydrocortisone Treated Groups

The effect of hydrocortisone treatment on proliferating vessels (induced by implantation of an ameroid constrictor around the left circumflex artery) was more prominent and concerned most of the enzymes investigated in this study. A comparative evaluation of the activities between the constrictor groups and the constrictor + hydrocortisone groups is given in Table 1.

Fig. 3. Acid phosphatase. Constrictor 3 weeks without hydrocortisone treatment. Multiple lysosomes (*ly*) and some Golgi cisternae (*g*) in endothelial (*end*) and in modified smooth muscle cells (*msmc*) show the precipitate. (× 10150)

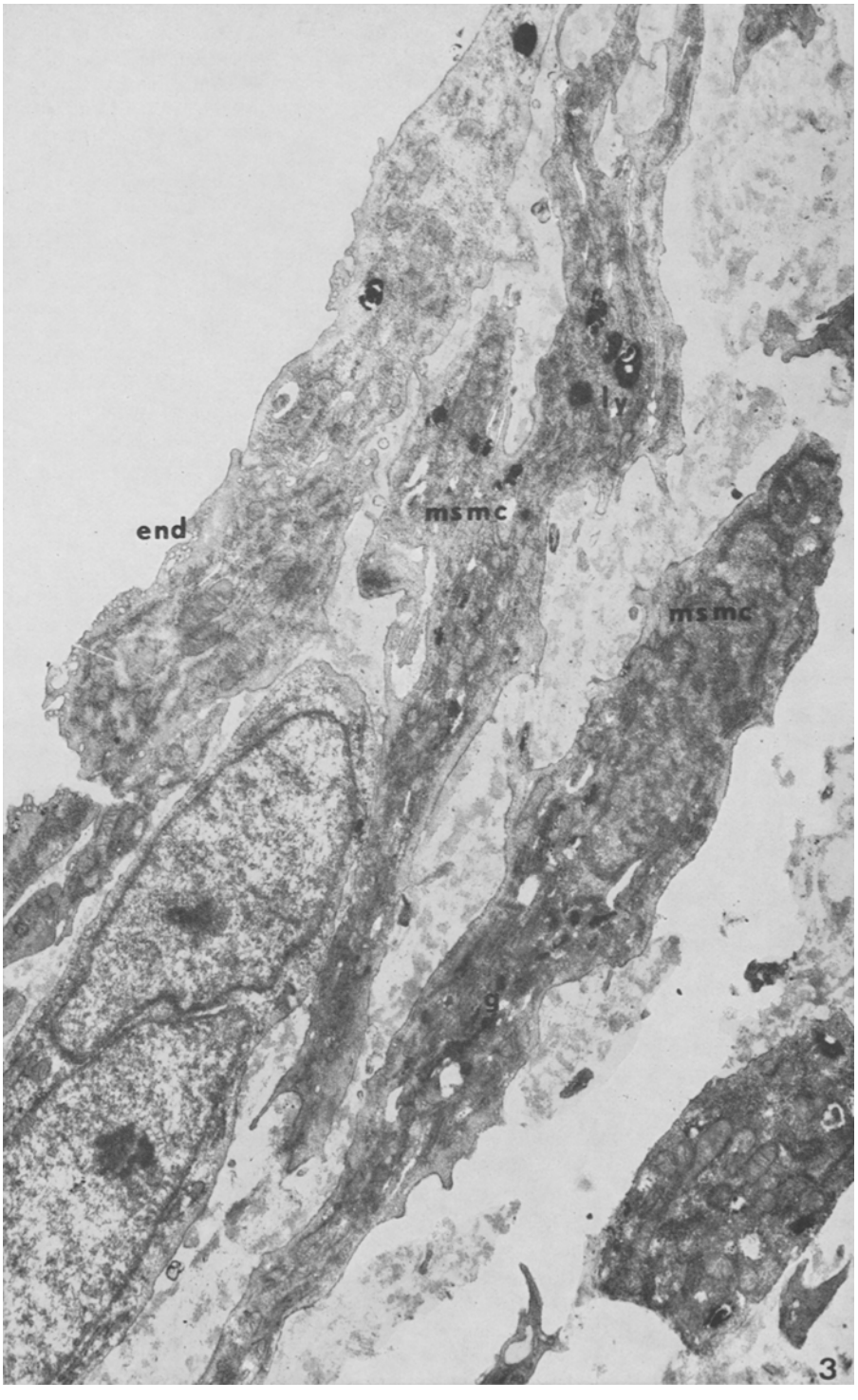


Fig. 3

Prominent changes in amount and topographic distribution of activities were seen at 3 weeks: i.e. lower levels in the hydrocortisone treated animals for ACPase in every layer of the vascular wall and for AMPase in the adventitia, whereas AMPase activity became visible in the endothelium. On the other hand, activity towards ATP, ADP and TTP in the media which was absent in the constrictor group became positive in the constrictor +hydrocortisone animals. G-6-Pase activity appeared very much enhanced after hydrocortisone treatment at this period.

A rather comparable pattern in localization of activities between the two groups was seen at 12 weeks, however, with two major exceptions: first, the persistence of AMPase on plasma membranes of the endothelial cells; and second, the presistence of PPase (ATP-, ADP-, and TPP-splitting enzyme) on the plasma membranes of cells belonging to the intimal thickening.

Electron Microscopy

The EM localization of phosphatase activities in coronary vessels of normal dogs and in developing coronary collaterals has been described previously (Borgers, 1971a; Borgers, Schaper, Schaper, 1971). Therefore, the results described herein concern only the hydrocortisone treated groups. No variation in the distribution pattern of enzymatic activities was noted among animals of the same group. For reasons of clarity the results will be presented for each enzyme separately:

Alkaline Phosphatase (AlPase)

Since terminal arterioles and large capillaries are the only vessels found reactive for this enzyme AlPase was expected to be of no importance in the collateral growth process. There was no induction of enzymatic activity seen in the hydrocortisone treated animals or in the constrictor + hydrocortisone treated animals.

5'-Nucleotidase (AMPase)

a) Hydrocortisone Treatment. Reactivity towards AMP on the plasma membrane of adventitial mesenchymal cells was similar to that in the control group. Only in the hydrocortisone 11 W animal activity was prominent in the pinocytic vesicles of the arterial endothelium, especially in those vesicles facing the media (Fig. 1).

b) Constrictor + Hydrocortisone Treatment. Whereas in the constrictor 3 W animals the activity was very intense on the plasma membrane of advential fibroblasts and fibrocytes, hydrocortisone treatment resulted in depletion of AMPase to normal levels in the adventitia. On the other hand, significant label was seen in the pinocytic vesicles lining the endothelial cell membrane at 3 weeks. A similar topographic distribution was present in the 8 and the 12 W groups (Fig. 10).

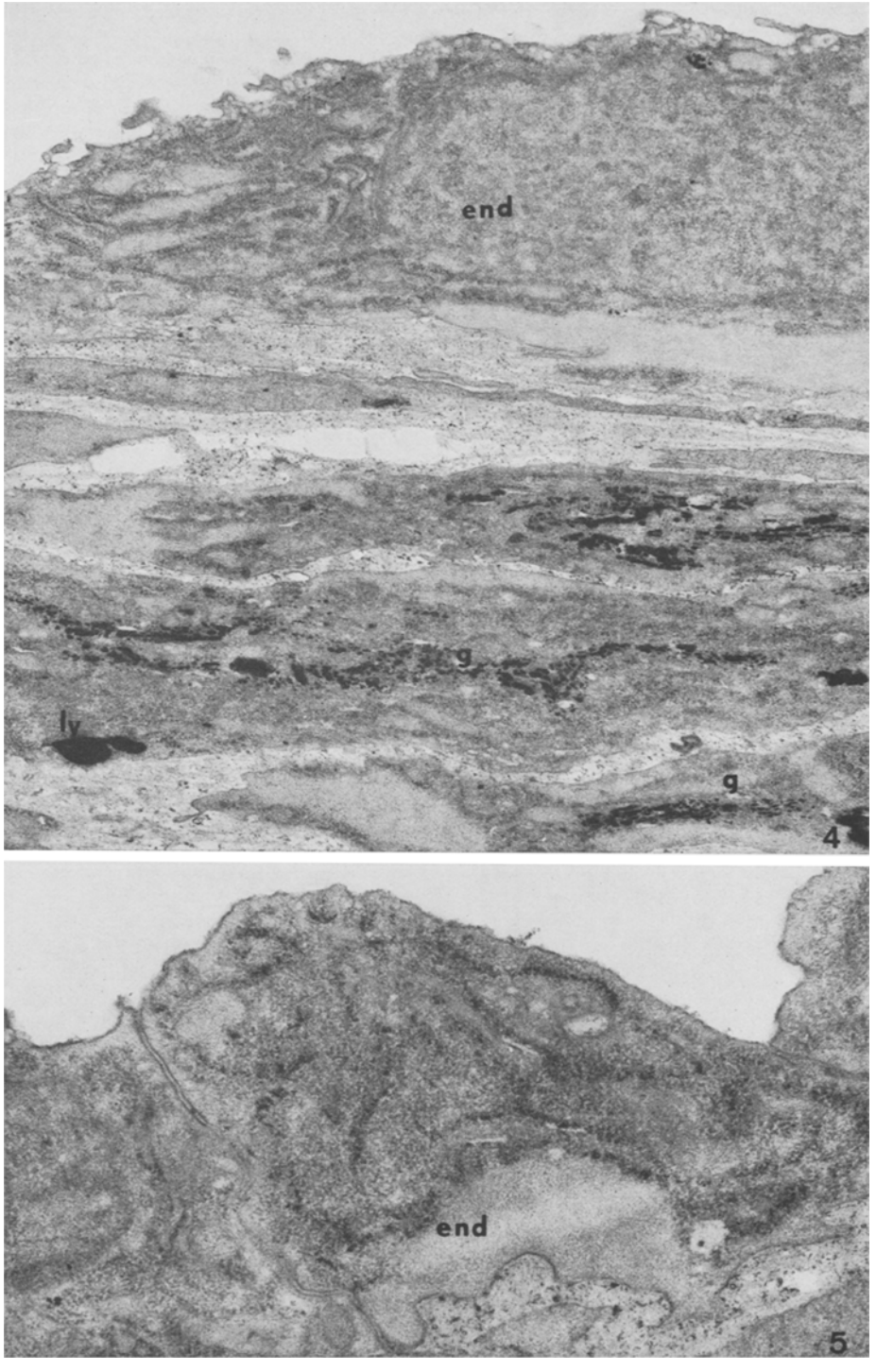
Polyphosphatase (PPase)

As PPase activity was considered the activity present at the plasma membranes and in the pinocytic vesicles of endothelial, smooth muscle and adventitial cells obtained after incubation with ATP, ADP and TPP as substrates.

a) Hydrocortisone Treatment. Treatment for 2, 7 and 11 weeks with hydrocortisone did not induce the slightest modification in content and topography of PPase. Plasma membranes and pinocytic vesicles of the above mentioned cells were uniformly stained (Fig. 2).

Fig. 4. Acid phosphatase. Constrictor +hydrocortisone 3 weeks. The very enlarged Golgi apparatus (*g*) of the modified smooth muscle cells situated in the intimal thickening, are strongly reactive. Note the paucity of lysosomes (*ly*). (× 16130)

Fig. 5. Acid phosphatase. Constrictor + hydrocortisone 3 weeks. The cisternae of endoplasmic reticiulum in an endothelial cell (end) are weakly stained with the reaction product. (× 24780)



Figs. 4 and 5

b) Constrictor + Hydrocortisone Treatment. The complete loss of reactivity in the media which was seen in the altered collaterals of the constrictor 3 W group, was not prominent after hydrocortisone treatment. Although a significant decrease was noted in cells belonging to the subendothelial thickening, every cell retained PPase activity to some degree. Activity on endothelial and adventitial cells seemed unaltered. At 8 and 12 weeks, a distribution pattern close to that in the control animals was obtained i.e. every cell of the vascular wall was reactive for PPase. This is in contrast again with the constrictor 8 W and 12 W groups, where the cells of the intimal thickening were devoid of PPase activity.

Acid Phosphatase (ACPase)

a) Hydrocortisone Treatment. As in the control dogs, the coronary arteries of the hydrocortisone treated animals (2, 7 and 11 weeks) showed very weak activity. Although more numerous in adventitial fibroblasts, most medial and intimal cells possessed very few lysosomes reactive for ACPase. Occasional labeling of one saccule and a few vesicles of the Golgi apparatus was noted in endothelial, smooth muscle and mesenchymal cells.

b) Constrictor + Hydrocortisone Treatment. A completely different picture of ACPase localization was seen at 3 weeks when compared to the corresponding constrictor 3 W group where a strong enhancement in all layers was observed (Fig. 3). This latter observation correlated well with the degree of morphologic alteration. Following hydrocortisone treatment such lesions were not observed. The number of ACPase rich lysosomes was significantly lower.

Cytolysomes, containing autologous or heterologous material were very seldon seen.

On the other hand, the very extended Golgi fields, especially prominent in the modified smooth muscle cells of the intimal thickening, were strongly and uniformly loaded with reaction product (Fig. 4). Moreover, weak labeling of the endoplasmic reticulum and the nuclear membrane was frequently observed in modified smooth muscle cells, endothelial cells (Fig. 5) and active fibroblasts. In the 8 and 12 week groups, the number of reactive bodies was again very low and the Golgi activity closely resembled the one observed in the corresponding constrictor groups. Endoplasmic reticulum and nuclear envelopes were completely devoid of precipitate.

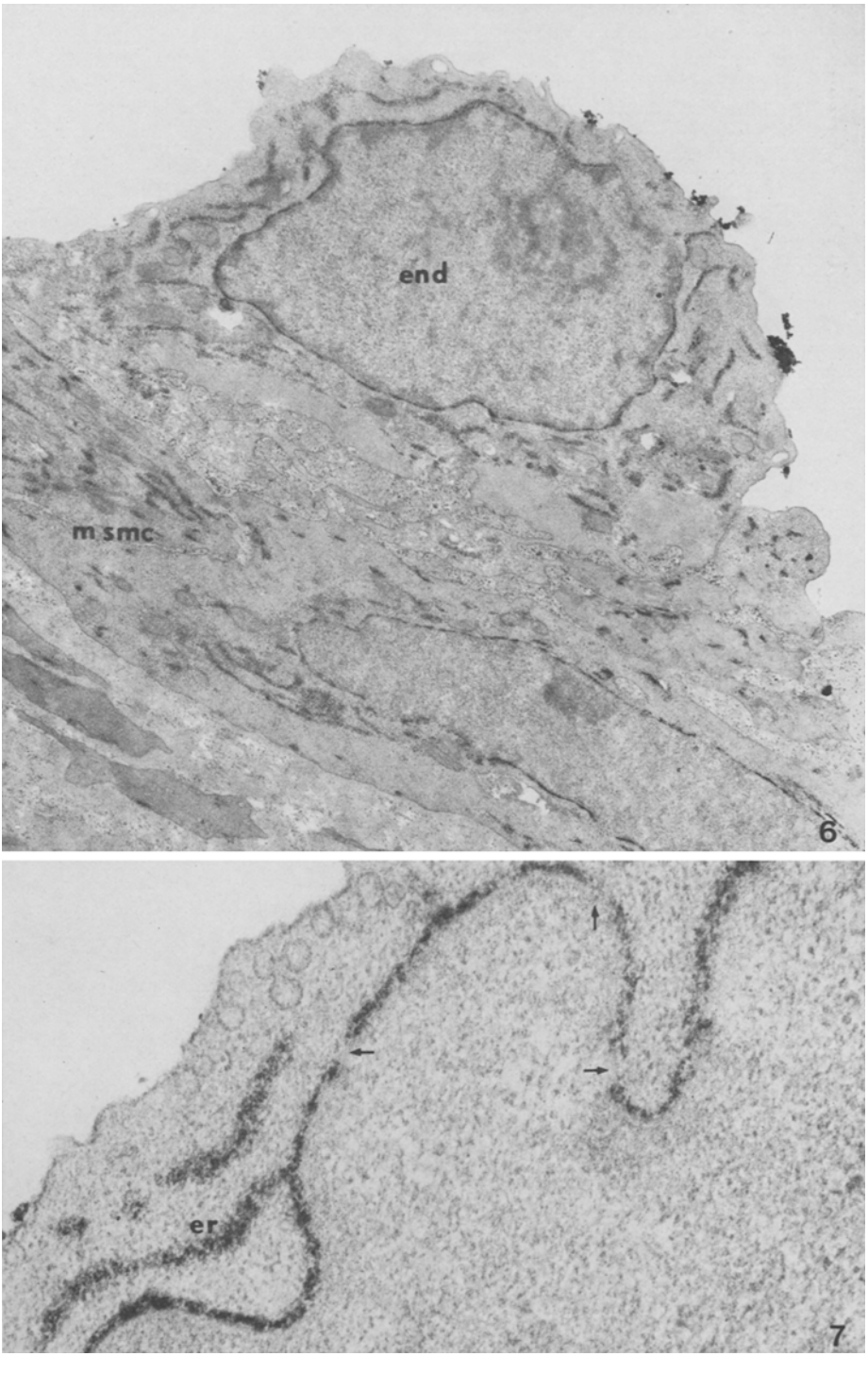
Glucose 6-Phosphatase (G-6-Pase)

a) Hydrocortisone Treatment. As in the control animal, a uniform distribution of G-6-Pase in the endoplasmic reticulum and in the nuclear membranes of the cells in every layer of the vascular wall was seen. The amount of precipitate varied between different cell types, i.e. moderate to weak activity in the endothelial cells; very weak activity in the inconspicuous short ER fragments in smooth muscle cells and moderate to strong activity in the multiple parallel strands of ER present in the adventitial fibroblasts. No obvious changes could be attributed to hydrocortisone treatment in these non-proliferating arteries.

b) Constrictor + Hydrocortisone. The changes in G-6-Pase content observed in the constrictor animals at 3 weeks i.e. a loss of G-6-Pase activity in necrotic endothelial and medial cells and the strong increase in active fibroblasts, differed completely from those observed after hydrocortisone treatment. In the latter group, a strongly increased reactivity was demonstrated in almost every cell type (Fig. 6). The well developed ER strands were stained thoroughly

Fig. 6. Glucose 6-phosphatase. Constrictor + hydrocortisone 3 weeks, The activity is visible in multiple profiles of endoplasmic reticulum and in the nuclear envelope of endothelial (*end*) and a modified smooth muscle cell (*msmc*). (× 22430)

Fig. 7. Glucose 6-phosphatase. Constrictor + hydrocortisone 3 weeks. Detailed localization of the precipitate in an endothelial cell of a coronary collateral. Note the intimate contact between the nuclear envelope and the endoplasmic reticulum (*er*). The arrows point to unreactive sites in the nuclear membrane. (× 50000)



Figs. 6 and 7

in endothelial cells, smooth muscle cells and especially in adventitial fibroblasts. Intimate contacts between nuclear envelope and profiles of ER, both loaded with lead phosphate precipitate, were very frequent (Fig. 7). The unusual high G-6-Pase load in the ER of modified smooth muscle cells was most prominent in those cells containing abundant glycogen deposits. Another unusual observation was the irregular deposition of the reaction product on the nuclear membrane, observed again in all cell types (Fig. 7).

At 8 and 12 weeks, the activity appeared depleted to almost normal levels, even in the subintimal proliferative area (Fig. 11). This contrasted very sharply with the increased load on the ER and or the nuclear envelope of subintimal cells in the corresponding untreated constrictor group.

Adenosine Tri-Phosphatase (ATPase)

Magnesium stimulated ATPase has not been found in mitochondria of either endothelial, smooth muscle or adventitial cells in all of the groups under study.

Thiamine Pyrophosphatase (TPPase)

a) Hydrocortisone Treatment. Hydrocortisone treatment for 2, 7 and 11 weeks did not seem to influence specific TPPase, a marker of the Golgi apparatus, as far as non-proliferating vessels are concerned.

b) Constrictor + Hydrocortisone Treatment. At 3 weeks increased TPPase activity was demonstrated in the extended Golgi zones of endothelial, smooth muscle, and mesenchymal cells of the collaterals (Fig. 9). However, this enhancement was not so pronounced as it was for ACPase in the same vessels. The degree of TPPase activity was considered normal at 8 and 12 weeks.

Nucleoside Diphosphatase (NdiPase)

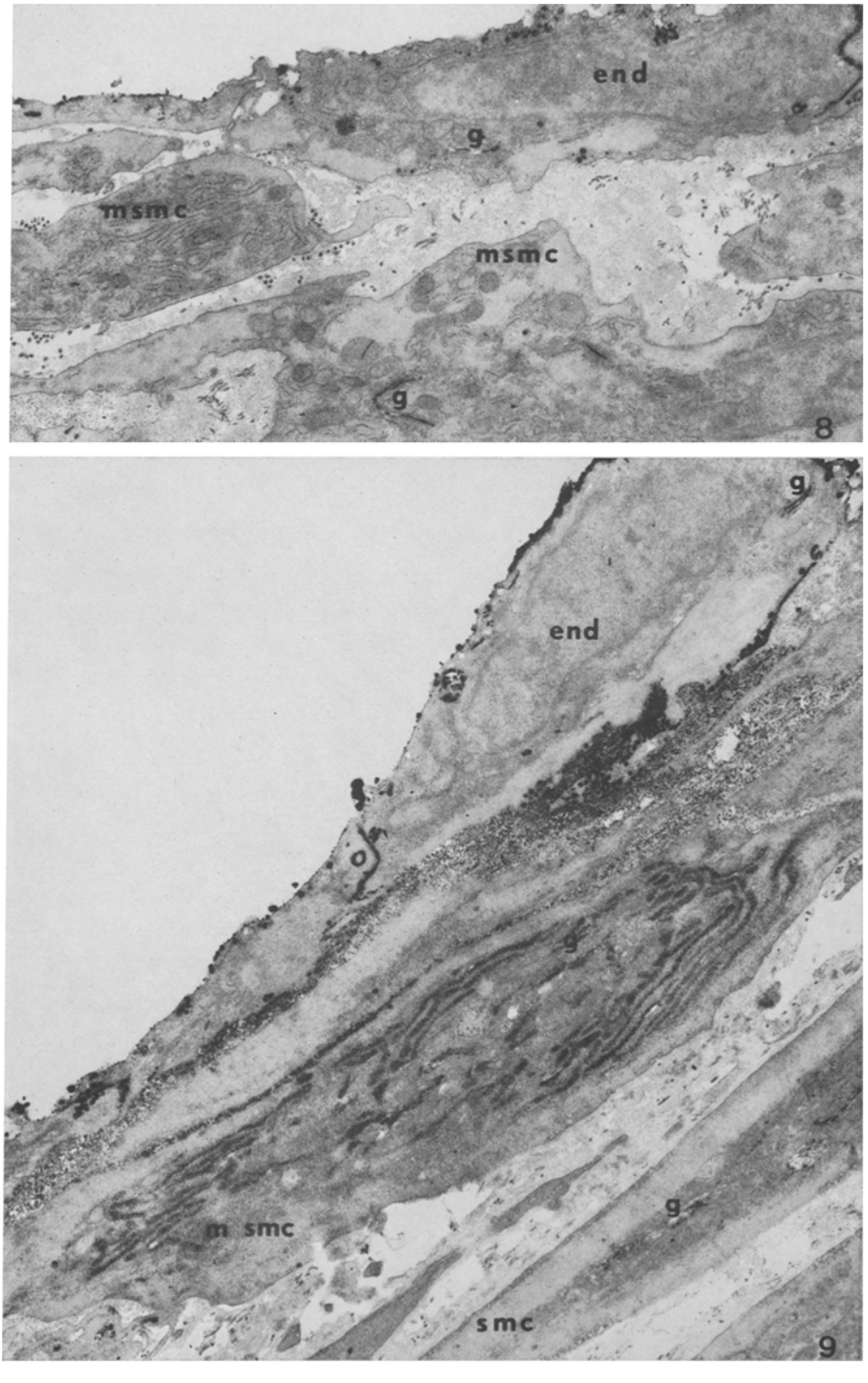
a) Hydrocortisone Treatment. Labeling of the endoplasmic reticulum and the nuclear envelope for NdiPase activity towards ADP and TPP was not observed in any of the hydrocortisone treated groups.

b) Constrictor + Hydrocortisone Treatment. A remarkable difference was noted between collaterals of hydrocortisone treated and untreated constrictor animals.

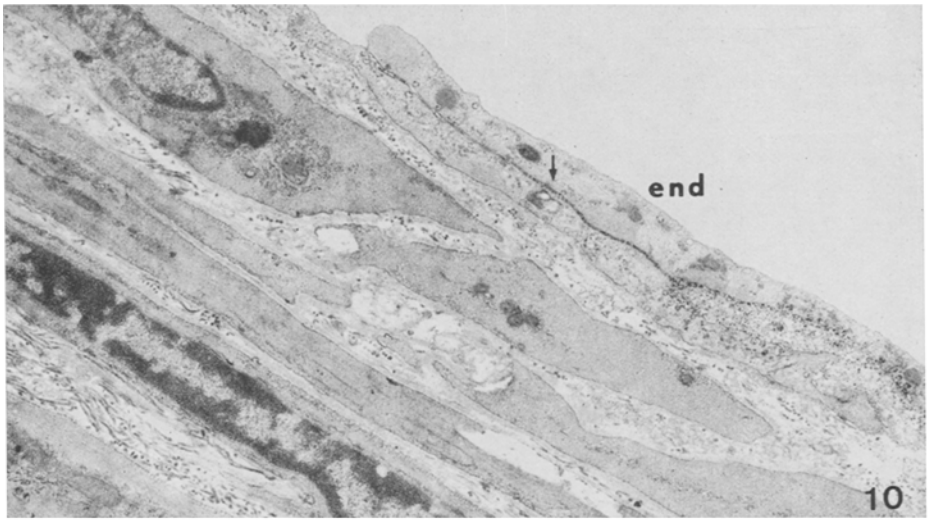
At 3 weeks, in the hydrocortisone treated group, the strongly developed endoplasmic reticulum, prominent in almost all endothelial, modified smooth muscle and adventitial cells, showed a positive reaction, faint towards ADP but rather strong towards TPP. The activity was far more pronounced in the modified smooth muscle cells present in the initimal thickening (Fig. 9). The nuclear envelope was reactive in a similar way. In the corresponding constrictor group without hydrocortisone treatment no reaction product was found in the endoplasmic

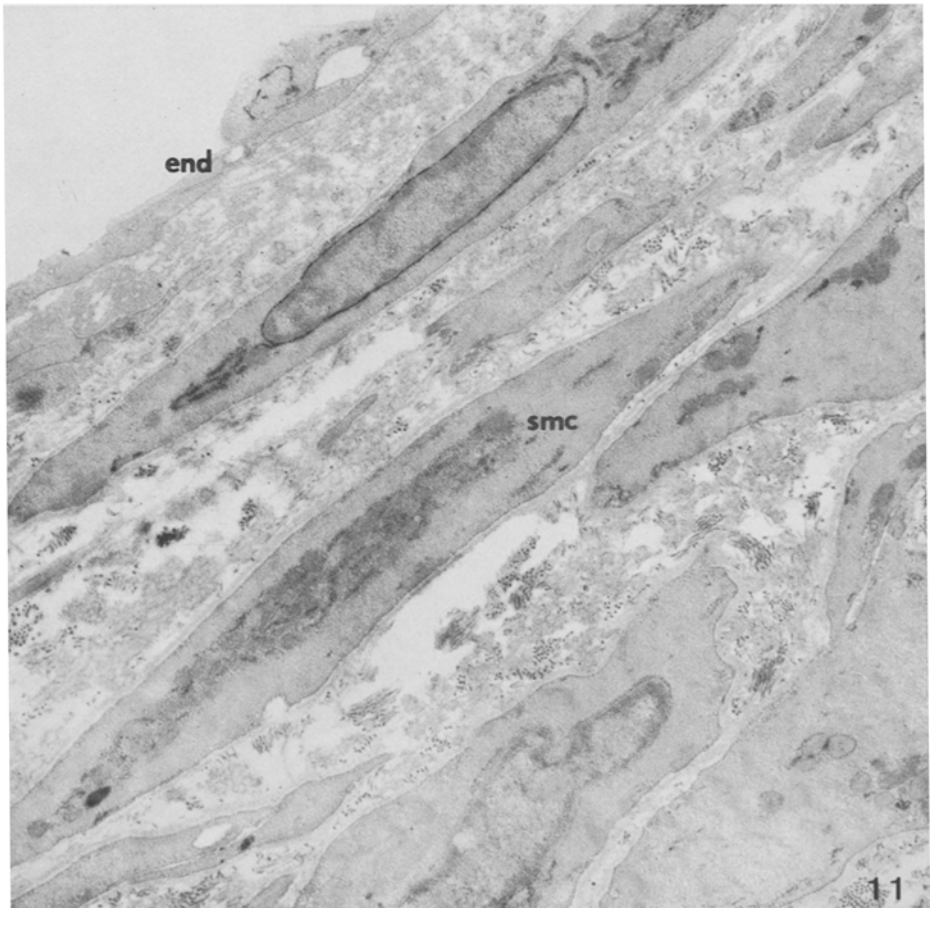
Fig. 8. Enzymes splitting thiamine phosphatase. Constrictor 3 weeks without hydrocortisone treatment. The plasma membranes and pinocytic vesicles of endothelial (*end*) and modified smooth muscle cells (*msmc*) are stained for polyphosphatase activity. The Golgi apparatus (*g*) shows thiamine pyrophosphatase activity. Note the absence of nucleoside diphosphatase activity in the ER of the modified smooth muscle cells. (× 11840)

Fig. 9. Enzymes splitting thiamine pyrophosphate. Constrictor + hydrocortisone 3 weeks. Polyphosphatase activity is seen on plasma membranes and in pinocytic vesicles of endothelial (*end*) and modified smooth muscle cells (*msmc*); thiamine pyrophosphatase is present in the Golgi apparatus (*g*) of the modified and the medial smooth muscle cells (*smc*) and nucleoside diphosphatase is present in numerous strands of ER of the modified smooth muscle cell. (× 10970)



Figs. 8 and 9





Figs. 10 and 11

reticulum and the nuclear membranes (Fig. 8). At 8 and 12 weeks, a completely opposite picture was obtained, i.e. cells belonging to the intimal thickening were reactive in the collaterals of the constrictor group, whereas in the hydrocortisone treated group, these cells were completely devoid of NdiPase activity.

Discussion

Chronic hydrocortisone treatment which has been shown to induce major modifications in the morphologic pattern of collateral development (Schaper, Borgers, Xhonneux, Schaper (1973), does evoke equally a modified pattern in phosphatase distribution. It was generally observed that most changes follow rather closely the degree of morphologic alteration as noted already in the collaterals of the untreated constrictor groups (Borgers, 1971 a). However, some peculiar changes in enzymatic distribution may reflect a specific response to hydrocortisone.

Alkaline phosphatase, an enzyme which is known to be stimulated by hydrocortisone in embryonic tissue (Moog, 1962, 1963, 1965) was not affected during this growth process. This was not astonishing since this enzyme is normally located in the endothelium of smaller sized vessels.

On the other hand, the specific phosphomonoesterase "5′-nucleotidase" was present at an unusual site, namely in the pinocytic vesicles lining the endothelial cell membranes. This site was never found positive by us, either in normal arteries or in collaterals of dogs in the constrictor group. Whether this significant induction of 5′-nucleotidase is related to an induced production of a vasoactive substance at a particular site in the vascular wall, necessary for relaxation of vascular smooth muscle, remains unsolved.

The effect of hydrocortisone on lysosomes, particularly on the stabilization of the lysosomal membrane, is a well documented phenomenon (Weissman, 1966). The level of lysosomal ACPase, which is supressed when compared to the vessels of the untreated constrictor animals during the early period of development, is probably related in a fundamental way to the anti-inflammatory action of hydrocortisone.

On the other hand ,the strongly enhanced activity of this enzyme in the enlarged Golgi fields and the occurrence in the ER and the nuclear envelopes deserves special attention. This suggests that the de novo synthesis of enzyme in actively proliferating subintimal and intimal cells is induced or at least not inhibited by hydrocortisone. This peculiar localization in cells, possessing only a limited number of secondary lysosomes, may be interpreted in various ways. The most likely, in our opinion, is that there exists an anachronism between the de novo synthesis of ACPase and the utilization of the enzyme, one or both processes being influenced by the drug. This emphasizes that the biogenesis of this lysosomal enzyme is not directly correlated with the endocytic activity of these cells.

Fig. 10. 5′-nucleotidase. Constrictor + hydrocortisone 12 weeks. Weak activity is visible in the interspace between endothelial cells. (× 12360)

Fig. 11. Glucose 6-phosphatase. Constrictor + hydrocortisone 12 weeks. The reaction product is only seen in moderate amounts in ER profiles and in the nuclear envelopes of endothelial (*end*) and smooth muscle cells (*smc*) (× 13220)

The behavior of polyphosphatase during early and late stages of development reflects rather well the general pattern of development. Although the exact role of this enzyme is not entirely understood, its uniform distribution on the plasma membrane and on pinocytic vesicles of every cell constituting the vascular wall suggest that its activity is related to cell membrane integrity (as an energy source) or involved in the transport of metabolities across the cell membrane. The preservation of the activity in subintimal cells during hydrocortisone treatment (in contrast to the complete loss in the constrictor group) strongly suggests that these cells, which also appeared to be better organized than the cells of the corresponding constrictor group, apparently retain also a more normal functional aspect.

A specific response towards hydrocortisone treatment came out very clearly in the case of nucleoside diphosphatase, localized in the ER and the nuclear membrane. Since this enzyme is involved in synthetic processes it becomes a useful marker, reflecting the metabolic events which take place in the cell cytoplasm. Its activity, which was not detected under normal conditions in coronary arteries and which was present in the constrictor group only at 12 weeks, was revealed after the hydrocortisone treatment as early as 3 weeks.

The influence of glucocorticoids on the activity of glucose 6-phosphatase was studied by Goodman, Terner, Spiro (1968); Weber, Singhal, Stamm (1963); Williams (1960); De Bodo and Altszuler (1958); Nordlie, Arion, Glende (1965). Enhanced glyconeogenesis was reported together with increased de novo synthesis of G-6-Pase, which plays a key role in this metabolic pathway. Sea and Fishman (1964) have shown that hydrocortisone has a stimulating effect on glycogen synthetase. In our study we found a concomitant increase in glycogen deposition and a marked increase in G-6-Pase.

Quite similar to nucleoside diphosphatase. the high level of ACPase activity obtained in the modified smooth muscle cells of collaterals from the constrictor group at 12 weeks was already reached in the hydrocortisone treated group at 3 weeks. Thus, together with prominent development of the ER system in the modified smooth muscle cells, at least two hydrolases are found to be strongly enhanced.

The fact that major changes in enzyme distribution were prominent only in the hydrocortisone treated constrictor animals and not in the coronaries of dogs without constrictor, indicates that the drug influences enzyme systems only in cells that participate actively in the process of growth.

Most of the reported metabolic changes involving enzymatic adaptation, induced by corticosteroids are related to the liver, lymphoid tissue, kidney, intestine and in vitro assays on cell cultures (Rosen and Nichol, 1963). To our knowledge, vascular tissue has never been the subject for studying variations in enzyme topochemistry after chronic hydrocortisone treatment.

Although we are aware that no comparative conclusions can be drawn from totally different organs and experimental systems, we think that these results indicate to some extent that chronic hydrocortisone administration induces enzymatic adaptations in the vascular wall but, and this is a major point, almost exclusively during developement of this tissue.

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