Histochemistry (1982) 76:71–87

Histochemistry

Histochemical Evidence for Lysosomal Storage of Acid Glycosaminoglycans in Splenic Cells of Rats Treated with Tilorone

Renate Lüllmann-Rauch
Department of Anatomy, University of Kiel, Olshausenstrasse, D-2300 Kiel, Federal Republic of Germany

Summary. Tilorone, an agent with antiviral and antitumor activities, has previously been reported to produce clear cytoplasmic vacuoles in many cell types of the rat. The present study on rat spleen was planned to investigate the ultrastructural and histochemical features of the tilorone-induced vacuoles occurring in sinus endothelium, trabecular smooth muscle cells, and macrophages of the red pulp. Evidence was obtained that the vacuoles represent lysosomes overloaded with acid glycosaminoglycans (aGAG). The main purpose of the present study was to overcome the technical difficulties of preserving the intralysosomal storage materials which were highly water-soluble and non-fixable by aldehyde fixatives. Preservation, at least for the light microscopical level, was achieved by freeze drying and by means of cationic dyes which served also to characterize the storage materials on the basis of their acidities. Tissue slices were used to determine the critical $MgCl_2$ concentration necessary to abolish Alcian blue staining; cartilage and mast cells served as references. For the storage material in sinus endothelium, the critical $MgCl_2$ concentration was found to be >0.7 M, as compared to >0.5 M for cartilage and >0.9 M for mast cells. The storage materials in trabecular cells and macrophages were slightly less acidic than cartilagineous matrix and more heterogeneous than that in sinus endothelium. Ultrastructurally, positive staining with high iron diamine (HID) confirmed the presence of aGAG within the tilorone-induced vacuoles.

Introduction

Tilorone is an inducer of interferon production and has antiviral and antitumor activities (for review, Regelson 1981). Various cellular side effects of tilorone have been described in animals, and some also in humans: cytoplasmic vacuoles and basophilic inclusions in circulating lymphocytes and in splenic macrophages (Rohovsky et al. 1970; Zbinden and Emch 1972; Levine and Sowinski 1977); cytoplasmic vacuoles in the epithelia of plexus

0301-5564/82/0076/0071/$03.40

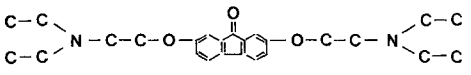


Fig. 1. Molecular structure of tilorone

choroideus and renal tubules (Levine and Sowinski 1977; Thelmo and Levine 1978; Wenk et al. 1979); lamellated cytoplasmic inclusions in liver, kidney (Leeson et al. 1976; Thelmo and Levine 1978) and corneal epithelium (Weiss et al. 1980). The lamellated inclusions occurring in distal tubules of the kidney were cytochemically identified as lysosomes (Thelmo and Levine 1978). The results indicate that in some cell types, the drug causes lysosomal storage of polar lipids. This is well compatible with the molecular structure of tilorone (Fig. 1); it is an amphiphilic cationic compound like many others that were shown to induce generalized lipidosis (for review, Lüllmann-Rauch 1979).

The reports on clear vacuoles, the occurrence of which cannot readily be explained on the basis of lipidosis, prompted us to examine the cytological effects of tilorone in several organs of the rat. Histochemical evidence was obtained that some types of cells develop lysosomal storage of acid glycosaminoglycans (aGAG). This effect can most clearly be demonstrated in the sinus endothelium of the spleen. The present report is focussed mainly on splenic red pulp. Other tissues will be described separately.

Materials and Methods

Young adult female Wistar rats were used in all experiments. Tilorone-HCl was a gift of Merrell Dow Pharmaceuticals (Cincinnati, Ohio). The following dyes and chemicals were used for histochemical work: Toluidine blue O (Merck, Darmstadt, F.R. Germany); Alcian Blue 8 GX (Gurr; Hopkin & Williams, Chadwell Heath, England); safranin O, acridine orange, N,N-dimethyl-m-phenylenediamine, N,N-dimethyl-p-phenylenediamine, cetylpyridinium chloride (CPC) (Sigma, München, F.R. Germany); $FeCl_3 \cdot 6H_2O$ and $MgCl_2 \cdot 6\ H_2O$ (Merck, Darmstadt).

Drug Treatment. In subchronic experiments (5–16 weeks; 16 animals) tilorone was administered as an additive to the ground chow at a concentration of 0.1%; the food consumption resulted in a daily drug dose of approximately 60 mg/kg of body weight. In some experiments, the drug was dissolved in water and administered by stomach tube (1 ml/100 g of body weight); 3 animals received 4 doses of 300 mg/kg within 8 days; with additional 3 animals this treatment was continued for 4–8 weeks with a reduced dosage (75 mg/kg every other day). Age-matched controls were kept under identical conditions but without receiving the drug. At the end of treatment, the animals were deeply anaesthetized with pentobarbital (i.p.-injection) and usually killed by vascular perfusion with fixative.

Routine Morphology. Animals were perfused with 3% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.2). Blocks of splenic tissue were postfixed with unbuffered 2% OsO_4 and embedded in Araldite. Semithin sections were stained with toluidine blue and pyronin G (Ito and Winchester 1963). Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with a Philips E 300 electron microscope.

Demonstration of Acid Phosphatase. Animals were perfused with 1% glutaraldehyde plus 1% paraformaldehyde (in 0.1 M cacodylate buffer, pH 7.2, containing 3% sucrose). 40 µm-tissue slices were cut with a Vibratome (Lancer, St. Louis, MO) and rinsed in cacodylate buffer

containing 7.5% sucrose. For enzyme demonstration Gomori's modified method (Barka and Anderson 1962) was applied using Na-β-glycerophosphate as substrate. Controls were run by omitting the substrate.

Cryostate Sections Stained with Cationic Dyes. Fresh splenic tissue was frozen in Arcton 12 (ICI, Cheshire, England) cooled with liquid nitrogen. Cryostate sections (10–15 µm) were air-dried and used without fixation. They were directly placed in 0.1% toluidine blue at pH 2 or pH 5 (Michaelis buffer) for 5 min, rinsed in the same buffer for 5 min, and mounted in DePeX. Alcian blue (1%) was used at pH 1 (0.1 N HCl) and at pH 2.5 (3% acetic acid); staining and mounting was performed according to Pearse (1968). Extractability of the Alcian blue positive material (pH 1) was tested by pretreating the sections for 2 h with 100% methanol, chloroform/methanol 2:1, chloroform/methanol/water 4:8:3, or with 0.9% NaCl solution.

Determination of the Critical Electrolyte Concentration. 40 µm-slices of splenic tissue (fixation as in paragraphs 3 or 4) were used to determine the critical electrolyte concentration at which Alcian blue staining would be abolished (Scott and Dorling 1965). The slices were incubated, not later than 3 h after tissue fixation, in 0.05% Alcian blue in 0.025 M acetate buffer (pH 5.8) containing various concentrations of $MgCl_2$ (from 0.3 M to 1.0 M). After incubation (20 h at 20° C) the slices were rinsed for 1 h with the same buffer plus $MgCl_2$ as used for staining, and were then dehydrated in graded ethanols, cleared in xylene, and mounted in DePeX for light microscopic examination. Some slices were osmicated, dehydrated and embedded in Araldite for preparing semithin and ultrathin sections. Mast cells and cartilage served as internal controls of the method. For this purpose small hand-cut pieces of aldehyde-fixed tracheal wall were included in each staining experiment. For a more rapid demonstration of the storage sites of polyanionic material additional tissue slices were incubated in 0.1% toluidine blue at pH 2 (Michaelis buffer) for 5–15 min, rinsed in the same buffer and mounted in DePeX.

High Iron Diamine (HID) Stain. 40 µm-slices of aldehyde-fixed spleens (see paragraph 4) were stained for 20 h at room temperature in HID solution (Spicer et al. 1978b). Control slices were incubated in a medium where $FeCl_3$ was replaced by the same concentration of $MgCl_2$. The slices were then osmicated and processed for Araldite embedding. Mast cells in the tracheal wall and in the popliteal lymph node served as internal controls of the method.

Preservation of Anionic Storage Material. Toluidine blue (0.1%) was added to the fixation medium (3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2); the whole animal was perfused with this mixture. Osmication and embedding was done as for routine morphology. In another approach, 40 µm-tissue slices obtained as described above were incubated for 20 h at room temperature in 0.1% solutions of toluidine blue, acridine orange, or safranin O (in cacodylate buffer pH 7.2), or in 0.3% cetylpyridinium chloride (in cacodylate buffer pH 7.2). The slices were then processed for Araldite embedding. Semithin sections (1 µm) were examined without further staining.

Freeze-Drying. Fresh tissue was frozen in N_2-cooled Arcton. After freeze-drying the blocks were treated with OsO_4-vapour (2 h, 65° C) and embedded in Araldite. Semithin sections were stained with 0.1% toluidine blue at pH 2. PAS staining was performed on sections from which the resin had been removed by NaOH-ethanol as described previously (Lüllmann-Rauch 1982). Remarkably, the toluidine blue-positive material in sinus endothelium, trabecular cells, and macrophages, was not lost during the process of Araldite removal.

Results

Routine Morphology

The histology (Fig. 2a) and ultrastructure of the splenic red pulp of control animals was in accordance with descriptions in the literature (Pictet et al.

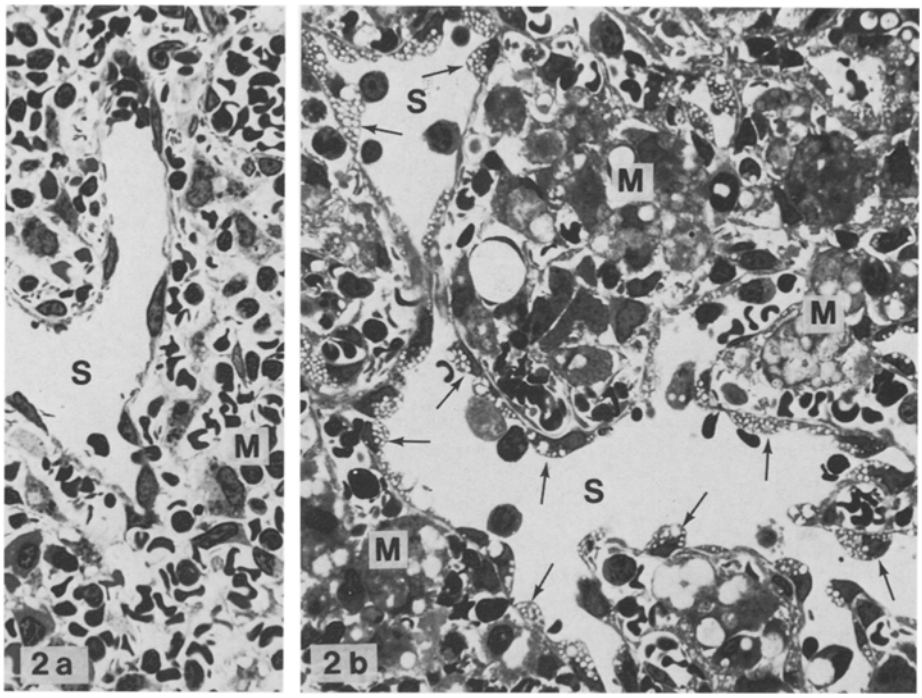


Fig. 2a, b. Splenic red pulp as seen in routine preparations. Araldite sections, stained with toluidine-pyronin. **a** Control rat. × 640. **b** Rat treated with tilorone (11 weeks, 60 mg/kg × day). The sinus endothelium shows clear cytoplasmic vacuoles (*arrows*). The macrophages in the pulpal cords are enlarged and contain clear vacuoles and weakly stained cytoplasmic inclusions. × 640. Abbreviations used throughout the figures: *S*, sinus lumen; *M*, macrophage; *B*, basal lamina; *T*, trabecula

1969). In the tilorone-treated rats (Fig. 2b), the main pathological feature of the sinus endothelium was the occurrence of numerous cytoplasmic vacuoles, which at low electron microscopic magnification appeared almost empty (Fig. 3a). The vacuoles were membrane-limited. Some electron-dense amorphous material was often attached to the inner surface of the membrane (Fig. 3b). The rest of the intravacuolar space was either empty or contained faint amounts of needle-like or of lamellated structures. Similar abnormal vacuoles, although more polymorphic and containing slightly larger

Figs. 3–5. Ultrastructure of splenic red pulp as seen in routine preparations

Fig. 3a, b. Sinus endothelium of a rat treated with tilorone (11 weeks, 60 mg/kg × day). **a** Numerous clear vacuoles are seen in the cytoplasm. × 7,000. **b** At higher magnification some vacuoles are seen to contain faint amounts of electron dense material and needle-like structures. × 43,000

Fig. 4a. Trabecular smooth muscle cells of the same rat as in Fig. 3. Numerous cytoplasmic vacuoles are seen. × 4,200. **b** Portion of **a**. The vacuoles are seen to contain small amounts of polymorphic electron-dense material. *mf*, myofilaments. × 34,000

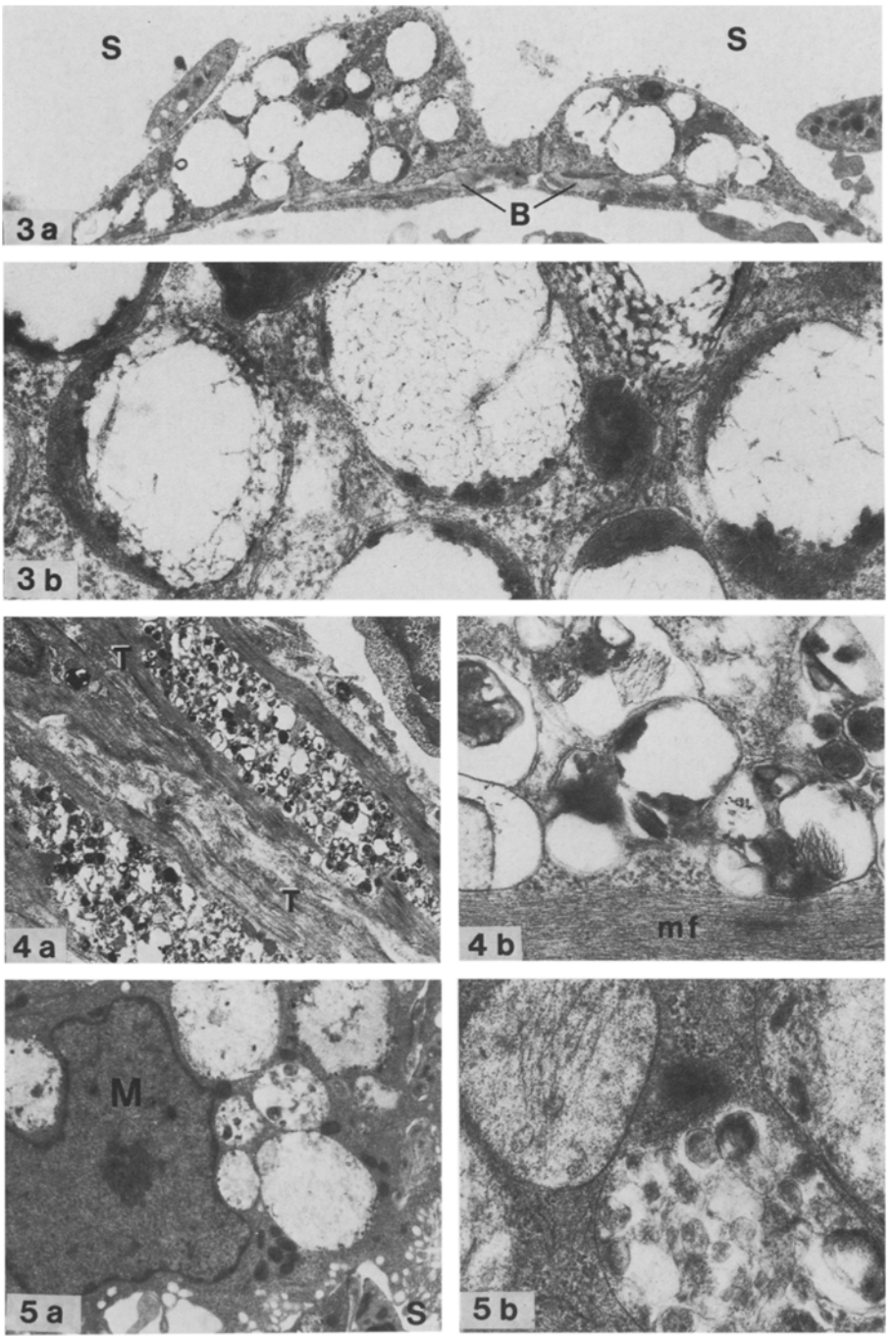


Fig. 5. **a** Macrophage in the red pulp of a rat treated with tilorone (8 days; 4 × 300 mg/kg). The cytoplasm is vacuolated. ×4,800. **b** Portion of the macrophage shown in (a). Flocculent and lamellar materials are seen the vacuoles. ×28,000

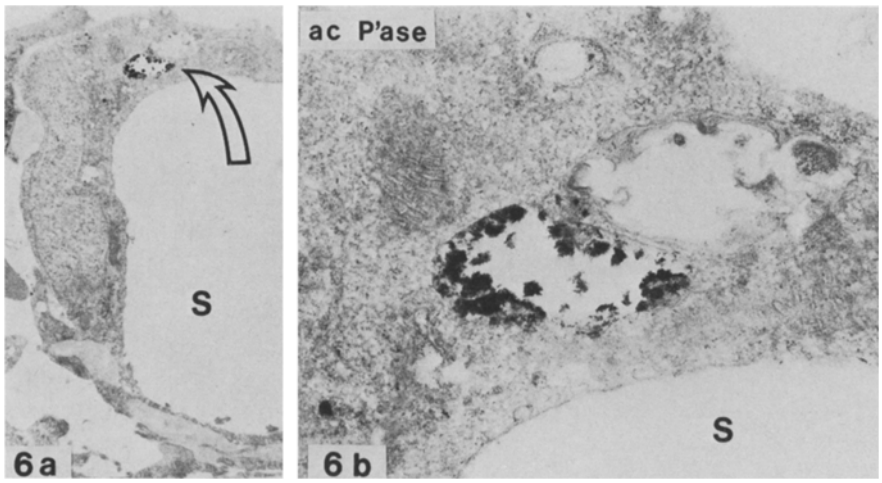


Fig. 6. a Sinus endothelium of a rat treated with tilorone (8 days, 4 × 300 mg/kg). Acid phosphatase preparation. The *arrow* points to an area which is shown in **b** at higher magnification. Unstained section. × 7,000. **b** One vacuole shows lead precipitates, while another vacuole appears devoid of enzyme reaction product. × 34,000

amounts of lamellated material, were regularly seen in the trabecular smooth muscle cells (Fig. 4). The macrophages in the cords of the red pulp were very much enlarged after chronic tilorone treatment and displayed huge cytoplasmic inclusion bodies which stained light blue or pink with toluidine blue-pyronin at alkaline pH as used for routine morphology. Under the electron microscope, the abnormal vacuoles usually contained flocculent material and scattered lamellated structures (Fig. 5). This pattern of cellular alterations was regularly found in the red pulp of all tilorone-treated animals regardless of the drug dosage and duration of treatment; the degree of alterations was dependent on the dosage and on the duration of drug treatment.

Acid Phosphatase

Acid phosphatase could be demonstrated in some though not in all abnormal vacuoles of the sinus endothelium (Fig. 6) and of the trabecular smooth muscle cells; in the abnormal vacuoles of the macrophages enzyme reaction product was observed much more regularly. In severely vacuolated sinus endothelia the lead precipitates were often diffusely distributed throughout the cytoplasmic bridges between the vacuoles rather than being confined to the vacuoles, in spite of well-confined precipitates in the vacuoles of immediately adjacent macrophages. This observation, which is similar to a cytochemical finding in cases of inherited mucopolysaccharidoses (Spicer et al. 1978a), indicates that lysosomal enzymes are difficult to preserve within these storage vacuoles; the enzymes seem to be lost from the vacuoles and redistributed within the cell during the process of fixation. The present cytochemical results are taken as evidence that the abnormal vacuoles under

study are lysosomal organelles. Those vacuoles in endothelium and smooth muscles that were free of reaction product are thought to have lost their acid phosphatase contents during tissue processing or in vivo already.

Histochemical Characterization of the Storage Material by Staining with Cationic Dyes

The observation of empty vacuoles suggested that the storage material might be water-soluble and non-fixable by glutaraldehyde nor by osmium thus being lost from the vacuoles during conventional tissue preparation. To draw a comparison between the tilorone-induced vacuoles and those seen in lysosomal storage diseases of man, mucopolysaccharidoses (Haust 1973; Van Hoof 1973a), and G_{M1}-gangliosidosis (Van Hoof 1973b) have to be considered in the first instance. Histochemical discrimination between the two possibilities should be obtained on the basis of stainability with cationic dyes and extractability of the stainable material.

(a) Cryostate Sections. In cryostate sections stained with toluidine blue at pH 2, the sinus endothelia and trabecular smooth musle cells showed up as structures that were filled with intensely metachromatic (purple) granules (Fig. 7b). Upon closer examination, trabecular cells were seen to contain a few blue-staining inclusions also, while the granules in the sinus endothelium appeared rather homogeneously metachromatic. The macrophages in the pulpal cords contained many large blue-staining inclusions and a few metachromatic (purple) granules. Similar results were obtained with toluidine blue at pH 5. In the spleen of control rats metachromatic structures were entirely absent (Fig. 7a), except an occasional mast cell. Alcian blue (AB) at pH 1 gave a brillant staining of granules in sinus endothelium and trabecular cells (Fig. 8); macrophages were also stained. Similar results were obtained with AB at pH 2.5. In the spleen of control rats no AB staining was observed at pH 1, apart from occasional mast cells (Fig. 8a). When air-dried, unfixed cryostate sections were pretreated with methanol (100%), with chloroform/methanol (2:1), or with chloroform/methanol/water (4:8:3) for 2 h, the staining pattern with AB (pH 1) was essentially the same as that obtained without pretreatment. In contrast, incubation in 0.9% NaCl solution (2 h) led to complete loss of the AB-stainable material (pH 1) from sinus endothelium, trabecular smooth muscle cells, and macrophages.

The histochemical results suggested intracellular storage of polyanionic material extractable by water but not by organic solvents. The most probable diagnosis seemed to be aGAG rather than gangliosides; the latter might be compatible with the metachromasia upon toluidine blue staining at pH 5 (Harris and Saifer 1960) but not with intense AB staining at pH 1 (Pearse 1968) nor with the resistance towards extraction with chloroform/methanol/water (Svennerholm and Fredman 1980).

An attempt to further characterize the storage material by determining the critical electrolyte concentration at which AB staining would be abol-

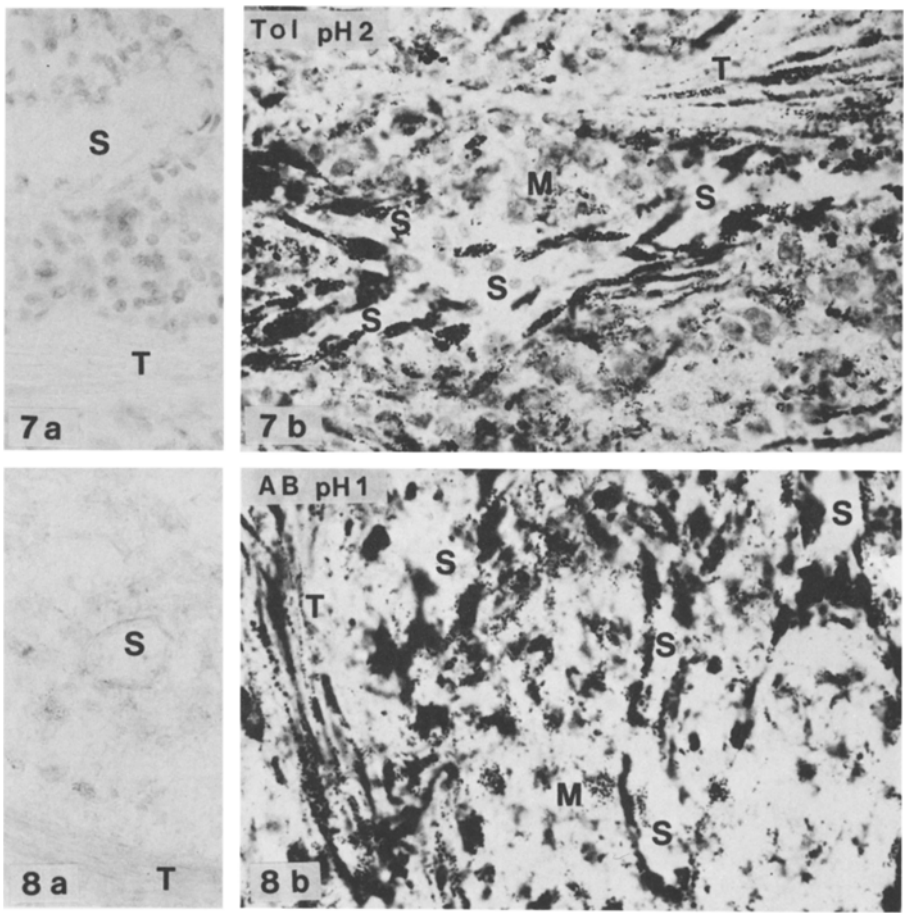


Fig. 7a, b. Cryostate sections stained with toluidine blue (pH 2). (Filter 589 nm). **a** Red pulp of a control rat. ×480. **b** Red pulp of a rat treated with tilorone (13 weeks; 60 mg/kg × day). The sinus endothelium and the trabecular cells contain numerous metachromatically staining (purple) inclusions. In the macrophages, most inclusions stain blue. ×480

Fig. 8a, b. Cryostate sections stained with Alcian blue (pH 1). (Filter 589 nm). **a** Red pulp of a control rat. ×480. **b** Red pulp of the same tilorone-treated rat as in Fig. 7b. The sinus endothelium and trabecular cells are intensely stained; macrophages are moderately stained. ×480

ished (Scott and Dorling 1965) was a failure, when cryostate sections were used. This was probably due to the rather low concentration of AB (0.05%) as recommended by Scott and Dorling (1965). Under this condition AB-staining material was irregularly distributed all over the section; the pattern obtained with 1% AB at pH 1 could not be reproduced. Since the cationic dye forms precipitates with aGAG (Scott et al. 1964), the application of 1% AB leads not only to staining but also to rapid fixation of the water-soluble material; the same explanation can account for the efficacy of tolui-

dine blue (Szirmai 1963). At an AB concentration of 0.05%, however, fixation seems to be too slow and insufficient thus leading to a redistribution of the stainable material.

(b) Tissue Slices. Glutaraldehyde applied in isotonic buffer solutions is thought to preserve biomembranes as barriers that are able to retain polar macromolecules (Arborgh et al. 1976; Ohtsuki et al. 1978). Therefore tissue slices (40 μm) of glutaraldehyde-perfused spleens were used for further histochemical experiments, assuming that appropriate fixation of the limiting membranes would retain the aGAG within the storage vacuoles, whereas cationic dyes might gradually penetrate into the vacuoles and might stain, and at the same time fix the aGAG. Brief incubation (5–15 min) of tissue slices in 0.1% toluidine blue at pH 2 indeed yielded intense metachromatic (purple) granules in sinus endothelia. Trabecular smooth muscle cells showed metachromatic granules and some blue inclusions. In the macrophages of the red pulp large blue and small metachromatic inclusions were seen. Sometimes, blue and metachromatic material appeared within the same cytoplasmic inclusion body.

Incubation of tissue slices with 0.05% AB (pH 5.8) in the presence of various concentrations of $MgCl_2$ enabled the polyanionic materials to be further characterized by the critical electrolyte concentration at which their stainability was abolished (Scott and Dorling 1965). As internal standards, mast cells and cartilage (tracheal wall) were used. Under the present condition, the critical electrolyte concentration for mast cell granules was above 0.9–1.0 M $MgCl_2$, that of cartilagineous matrix above 0.5 M $MgCl_2$; these values were obtained both in healthy control rats and in tilorone-treated rats. The splenic sinus endothelium of tilorone-treated rats was brillantly stained (Fig. 9) up to a $MgCl_2$ concentration of 0.7 M; at 0.9 M $MgCl_2$ staining was very weak and inconsistent. Trabecular smooth muscle cells showed many AB-positive granules at 0.3 M $MgCl_2$ which became less numerous at 0.5 M; at 0.7 M $MgCl_2$ staining was largely abolished. Macrophages of the pulpal cords showed large AB-positive inclusions at 0.3 M $MgCl_2$; at 0.5 M $MgCl_2$ a few small AB-positive granules were seen; at 0.7 M $MgCl_2$ staining was abolished.

In unstained semithin sections (1 μm) of Araldite-embedded tissue slices AB-positive material could be discovered within the abnormal vacuoles (Fig. 9). For electron microscopy, however, the preservation of the material was insufficient; the vacuoles appeared similar to those in tissues incubated without AB, or with AB in the presence of high $MgCl_2$ concentrations.

(c) High Iron Diamine Staining (HID). For demonstration of aGAG both at light microscopic and ultrastructural levels, the HID method (Spicer et al. 1978b) was applied. In unstained semithin Araldite sections examined with the light microscope, the abnormal vacuoles of splenic sinus endothelium showed dark peripheral rims; mast cell granules used as standard were intensely stained by HID. Ultrastructurally, most vacuoles of the sinus endothelium (Fig. 10) contained electron dense material due to HID staining

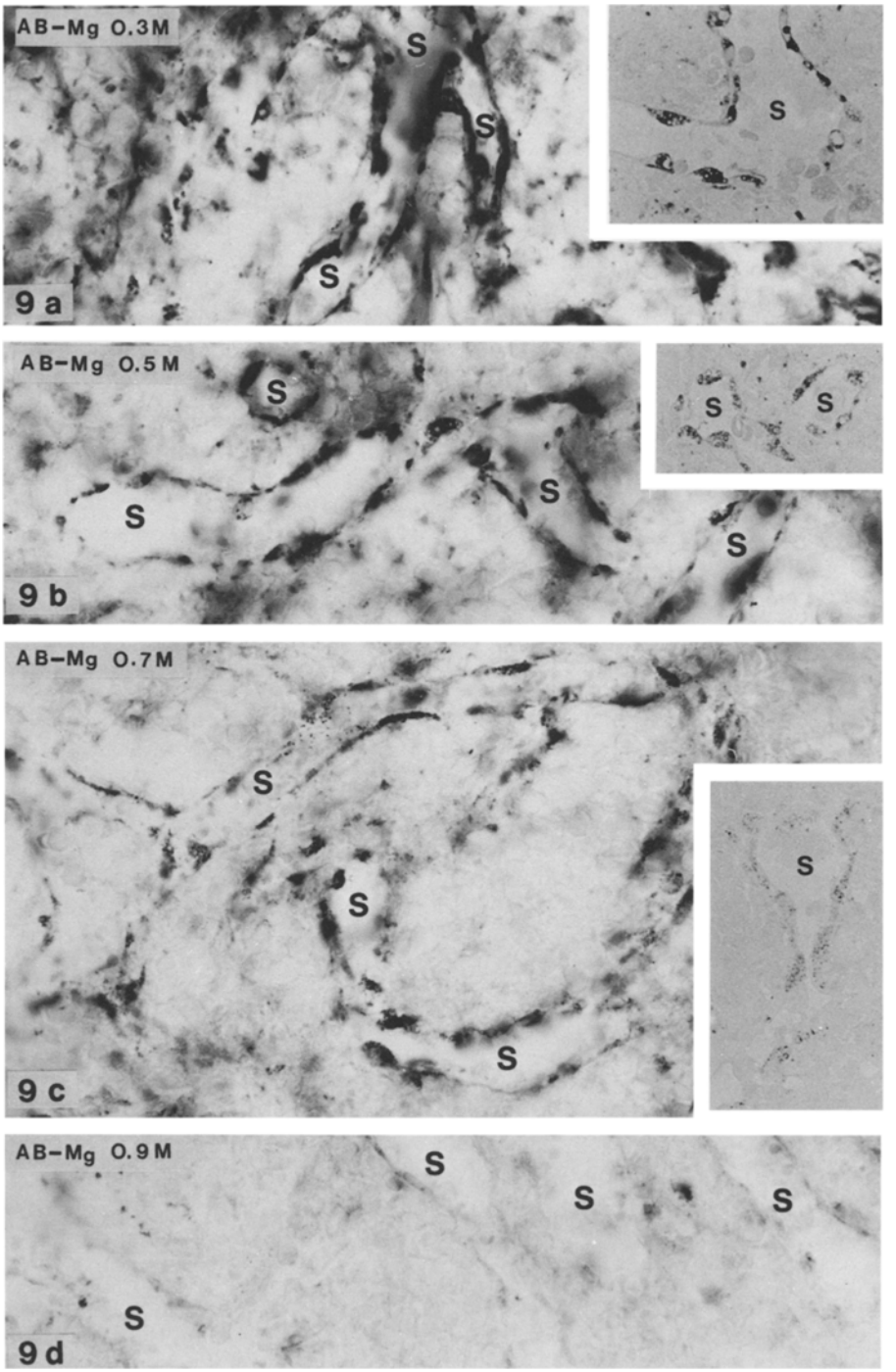


Fig. 9. Red pulp of a rat treated with tilorone (12 weeks, 60 mg/kg × day). Slices (40 μm) of aldehyde-fixed tissue were incubated with Alcian blue plus $MgCl_2$ (molar concentrations indicated). The sinus endothelium is clearly stained up to a $MgCl_2$ concentration of 0.7 M. At 0.9 M staining is abolished. The *insets* in Figs. a–c show sinus endothelia as seen in semithin Araldite sections prepared from the Alcian blue-incubated tissue slices. (Filter 589 nm). × 560

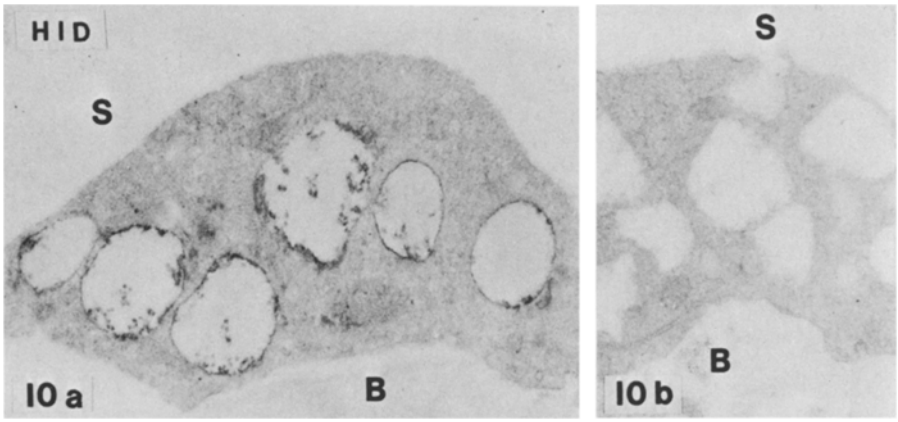


Fig. 10. a Sinus endothelium of a rat treated with tilorone (13 weeks, 60 mg/kg × day). High iron diamine (HID) procedure. Positively staining material is attached to the inside of the limiting membrane of the cytoplasmic vacuoles. × 19,000. **b** Control preparation incubated in a medium containing $MgCl_2$ instead of Fe Cl_3. No staining is obtained. × 24,000

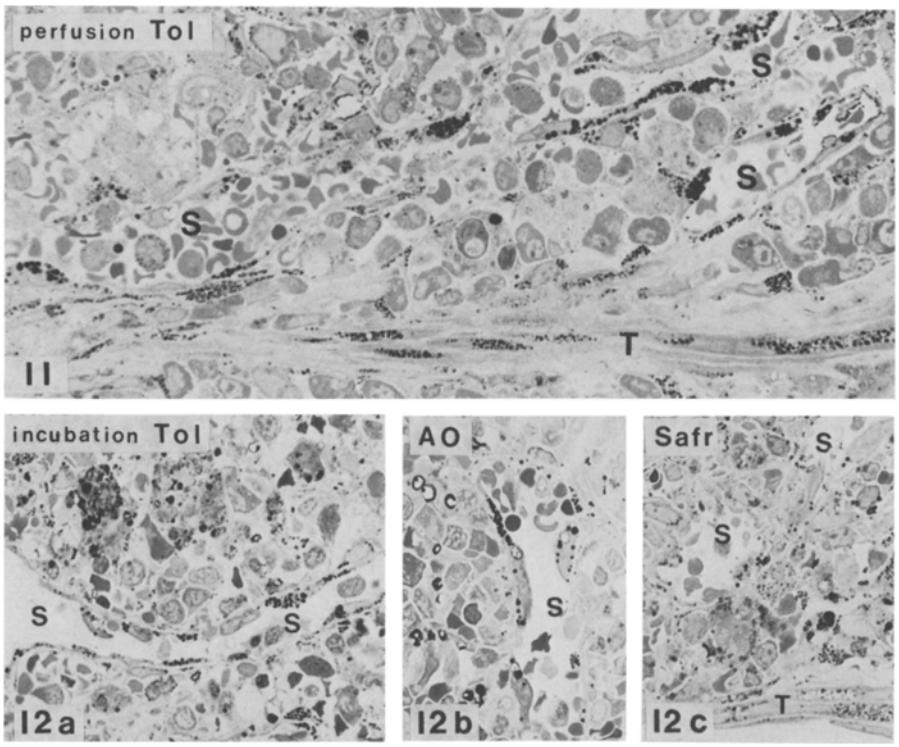
which was attached to the inside of the limiting membrane. In trabecular smooth muscle cells and in the macrophages of the red pulp, some vacuoles stained positive with HID while others did not.

Attempts to Preserve the Storage Material

Two approaches were successful to preserve the storage material at least for the light microscopical level, i.e., fixation with cationic dyes and freeze drying.

(a) Cationic Dyes as Fixatives. Several cationic dyes such as toluidine blue, safranin O, acridine orange (Shepard and Mitchell 1976a, b, 1981), AB (Ruggeri et al. 1975; Schofield et al. 1975), and the long-chain quaternary amonium salt cetylpyridinium chloride (CPC) (Engfeldt and Hjertquist 1968) have been successfully used for improved preservation of aGAG in the extracellular matrix. The present findings on cryostate sections and tissue slices treated with toluidine blue or AB indicated that cationic dyes are effective also in the fixation of intracellularly stored aGAG.

Toluidine blue (0.1%) was added to the conventional aldehyde fixative which was applied by vascular perfusion. In other experiments, tissue slices of aldehyde-perfused spleens were incubated at pH 7.2 with toluidine blue, safranin, acridine orange, or CPC. In unstained semithin Araldite sections the sinus endothelium and the trabecular smooth muscle cells were seen to contain cytoplasmic inclusions intensely stained with the respective dye (Figs. 11 and 12); clear vacuoles were much less frequent than in spleens processed for routine morphology (Fig. 2b), or in tissue slices incubated in buffer only. After CPC incubation only clear vacuoles were seen in sinus endothelium and trabecular cells suggesting that the compound was not



Figs. 11 and 12. Preservation of the storage materials by means of cationic dyes

Fig. 11. Red pulp of a rat treated with tilorone (11 weeks, 60 mg/kg × day). Perfusion fixation with glutaraldehyde plus toluidine blue. Araldite section without further staining (Filter 589 nm). The sinus endothelium and the trabecular cells contain intensely stained inclusions rather than clear vacuoles as seen in routine preparations. × 640

Fig. 12a–c. Red pulp of a rat treated with tilorone (8 weeks, 4 × 300 mg/kg and 20 × 75 mg/kg). Aldehyde-fixed tissue slices were incubated with toluidine blue (**a**), with acridine orange (**b**), or with safranin O (**c**). Araldite sections without further staining. (Filters: **a**, 589 nm; **b**, 520 nm; **c**, 560 nm). In sinus endothelia, in trabecular cells and in macrophages inclusions are stained with the respective dye. Clear vacuoles are less frequent than in routine preparations. × 640

effective to preserve the intracellularly stored aGAG. At the ultrastructural level, the preserving effect of the cationic dyes was not readily apparent. The vacuoles in the sinus endothelium and trabecular cells did not significantly differ from those seen after conventional tissue preparation.

(b) Freeze Drying. When semithin Araldite sections of freeze-dried splenic tissue were treated with toluidine blue at pH 2, the sinus endothelium (Fig. 13) and trabecular smooth muscle cells displayed intensely stained (blue) cytoplasmic inclusions. The absence of empty vacuoles indicated that their occurrence with conventional tissue preparation was due to artificial loss of storage material

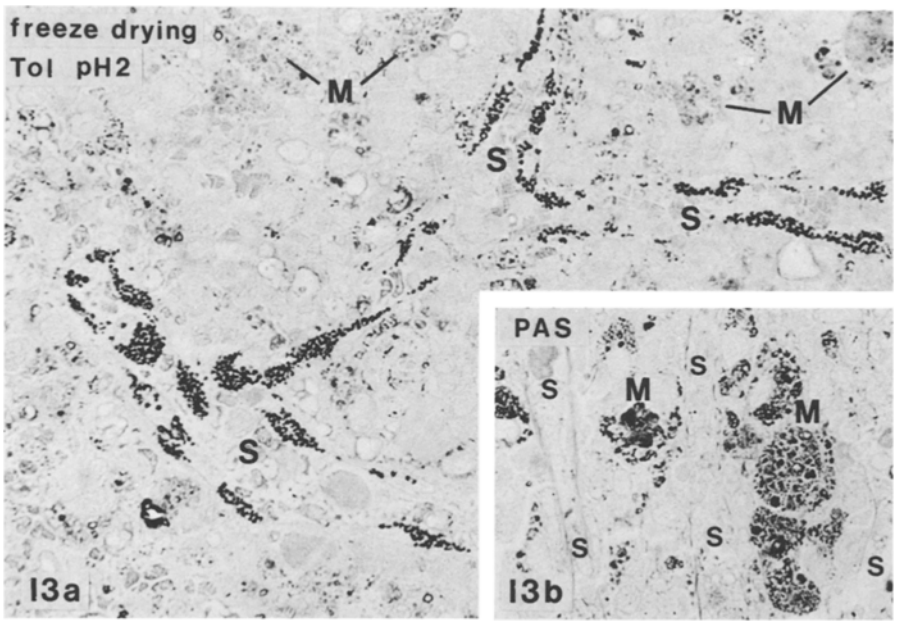


Fig. 13a, b. Red pulp of a rat treated with tilorone (12 weeks, 60 mg/kg × day). Araldite sections of freeze-dried tissue. **a** Toluidine blue stain (pH 2) (Filter 589 nm). The sinus endothelium contains brillantly stained inclusions instead of clear vacuoles. In the macrophages, a few intensely stained and many weakly stained inclusions are seen. × 640. **b** PAS stain (Filter 540 nm). The macrophages contain PAS-positive material. The sinus can be recognized by the discontinuous basal lamina which is PAS-positive. The sinus endothelium is unstained. × 640

Semithin sections of freeze-dried material were used also to search for PAS-positive material. The macrophages of the pulpal cords contained PAS-stained inclusions (Fig. 13b). Some trabecular cells were weakly stained, most of them were negative. The sinus endothelium was entirely negative.

Discussion

The present study reveals abnormal lysosomal storage of water-soluble materials which have staining characteristics of partly sulphated aGAG (Table 1). On the basis of the critical electrolyte concentrations necessary to abolish AB-staining, the three splenic cell types under study and the structures used as internal standards can be placed in decreasing order of acidity of the aGAG present therein: mast cell granules > storage material in sinus endothelium > cartilagineous matrix > storage material in trabecular cells > storage material in macrophages of the red pulp. The staining patterns obtained with AB, toluidine blue (pH 2), and with the HID-method suggest that the polyanionic material stored in sinus endothelium is rather homogeneous as far as its acidity is concerned, whereas the materials in trabecular cells, and even more so in the inclusions of macrophages are rather heteroge-

Table 1. Staining behaviour of the abnormal materials stored in splenic cells as compared with natural standards

	Tol pH 2	AB pH 1	AB Mg 0.3	AB Mg 0.5	AB Mg 0.7	AB Mg 0.9
Sinus endothelium	++ (P)	++	++	++	+	–
Trabecular smooth muscle	++ (P>B)	++	++	+	–	–
Macrophages in pulpal cords	++ (B>P)	+	+	(+)	–	–
Mast cells	++ (R or P)	++	++	++	++	+
Cartilage	++ (P)	n.e.	++	++	–	–

Symbols: Tol pH 2=0.1% toluidine blue at pH 2 (cryostate sections and tissue slices); AB pH 1=1% Alcian blue at pH 1 (cryostate sections); AB–Mg 0.3=0.05% Alcian blue (pH 5.8) plus 0.3 M $MgCl_2$ (tissue slices). AB-Mg 0.5 to 0.9, analogous to AB-Mg 0.3; ++=intense staining; +=moderate staining; (+)=moderate staining of a few inclusions; –=weak or no staining; P=purple inclusions; P>B=many purple and some blue inclusions; B>P= many blue and some purple inclusions; R=red granules; n.e.=not examined

neous; this is reflected also by the ultrastructure,. Identification of the storage materials must await histochemical analysis using degrading enzymes.

Storage phaenomena similar to those in splenic red pulp were observed also in other tissues of tilorone-treated rats as will be described in separate communications. It appears that tilorone induces mucopolysaccharidosis in several cell types, in addition to inducing lipidosis in certain cells (see introduction). Obviously biochemical identification of the storage materials is necessary to corroborate the histochemical diagnosis. On the other hand, histochemistry is indispensible for a description of the cellular and subcellular patterns of aGAG storage.

The main purpose of the present investigation was to overcome the technical problems of preserving the water-soluble materials for histochemical analysis. This technical difficulty is encountered also with the inherited mucopolysaccharidoses as pointed out by Haust (1973), and it may explain why there are relatively few detailed histochemical studies (for references, Haust 1973; Spicer et al. 1978a), in contrast to the great number of biochemical investigations in this field. The methodical situation with lysosomal storage of aGAG is, for two reasons, more complicated than any situation where aGAG are to be studied histochemically at sites of their natural occurrence, i.e., in the extracellular matrix (Wight and Ross 1975; Thyberg 1977; Hay 1981), on cell surfaces (Simionescu et al. 1981; Spicer et al. 1981), or in normal intracellular storage organelles (Jaques 1980): (a) At all extracellular sites, the aGAG are readily accessible for cationic reagents used as fixatives, dyes or electron-dense markers; and they are accessible also for exogenous degrading enzymes applied before or after fixation. (b) Natu-

ral aGAG are either covalently bound to proteins (Lindahl and Höök 1978) or, in the case of mast cell granules, they form tight complexes with proteins (Jaques 1980); their association with proteins renders them fixable by aldehydes at least to the extent that they are preserved for qualitative histochemical analysis. In inherited mucopolysaccharidoses, however, the intralysosomally stored aGAG are of low molecular weights and probably not bound to fixable proteins (Haust 1973); therefore they are easily lost from the storage sites during the process of routine tissue preparation. This appears to be true also for the tilorone-induced mucopolysaccharidosis.

Our experiments with cryostate sections took advantage of both the fixating and the staining properties of cationic dyes as proposed by Szirmai (1963); furthermore we applied classical histochemical methods to aldehyde-fixed tissue slices, i.e., at a stage of tissue preparation where the storage material was, to some extent, still retained by biomembranes which prevented redistribution and loss. These approaches permitted coarse histochemical characterization of the storage materials on the basis of their acidities, and they yielded satisfactory preservation for light microscopy, yet not for electron microscopy. Ultrastructural demonstration was so far achieved with the HID method only (Fig. 10). The tissue slices appear unsuitable for the application of degrading enzymes which would usually be employed for histochemical identification of unknown substrates; the aGAG are not accessible for the enzymes as long as the lysosomal membranes serve as a barrier. Experiments with freeze-dried tissue material are now in progress to test the susceptibilities towards enzyme digestion.

The present findings on sinus endothelia and trabecular cells are closely reminiscent of the splenic alterations observed in several types of inherited mucopolysaccharidoses (Dawson 1954; Haskins et al. 1980). This further supports the notion that tilorone induces mucopolysaccharidosis. The molecular mechanisms responsible for this drug side effect are entirely open to speculation at present.

Acknowledgement. The excellent technical assistance of Mrs. Dagmar Niemeier is gratefully acknowledged.

References

Arborgh B, Bell P, Brunk U, Collins VP (1976) The osmotic effect of glutaraldehyde during fixation. A transmission electron microscopy, scanning electron microscopy and cytochemical study. J Ultrastruct Res 56:339–350

Barka T, Anderson PJ (1962) Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler. J Histochem Cytochem 10:741–753

Dawson IMP (1954) The histology and histochemistry of gargoylism. J Pathol 67:587–604

Engfeldt B, Hjertquist SO (1968) Studies on the epiphysial growth zone. I. The preservation of acid glycosaminoglycans in tissues in some histotechnical procedures for electron microscopy. Virchows Arch (Cell Pathol) 1:222–229

Harris AF, Saifer A (1960) The metachromasy of strandin. J Neurochem 5:218–230

Haskins ME, Aguirre GD, Jezyk PF, Patterson DF (1980) The pathology of the feline model of mucopolysaccharidosis VI. Am J Pathol 101:657–674

Haust MD (1973) The genetic mucopolysaccharidoses. Int Rev Exp Pathol 12:251–314

Hay ED (1981) Extracellular matrix. J Cell Biol 91:205s–223s

Ito S, Winchester RJ (1963) The fine structure of the gastric mucosa in the bat. J Cell Biol 16:541–577

Jaques LB (1980) Heparins-anionic polyelectrolyte drugs. Pharmacol Rev 31:99–166

Leeson GA, Biedenbach SA, Chan KY, Gibson JP, Wright GJ (1976) Decrease in the activity of the drug-metabolizing enzymes of rat liver following the administration of tilorone hydrochloride. Drug Metab Dispos 4:232–238

Levine S, Sowinski R (1977) T-lymphocyte depletion and lesions of choroid plexus and kidney induced by tertiary amines in rats. Toxicol Appl Pharmacol 40:147–159

Lindahl U, Höök M (1978) Glycosaminoglycans and their binding to biological macromolecules. Annu Rev Biochem 47:385–417

Lüllmann-Rauch R (1979) Drug-induced lysosomal storage disorders. In: Dingle JT, Jacques PJ, Shaw IH (eds) Lysosomes in applied biology and therapeutics, vol 6. North Holland, Amsterdam, pp 49–130

Lüllmann-Rauch R (1982) Lysosomal glycogen storage mimicking the cytological picture of Pompe's disease as induced in rats by injection of an α-glucosidase inhibitor. II. Alterations in kidney, adrenal gland, spleen and soleus muscle. Virchows Arch (Cell Pathol) 39:187–202

Ohtsuki I, Manzi RM, Palade GE, Jamieson JD (1978) Entry of macromolecular tracers into cells fixed with low concentrations of aldehydes. Biol Cell 31:119–126

Pearse AGE (1968) Histochemistry-theoretical and applied. 3ed Vol 1. Little, Brown & Co, Boston

Pictet R, Orci L, Forssmann WG, Girardier L (1969) An electron microscope study of the perfusion-fixed spleen. I The splenic circulation and the RES concept. Z Zellforsch 96:372–399

Regelson W (1981) The biological activity of the synthetic polyanion, pyran copolymer (diveema, MVE, 46015) and the heterocyclic bis DEAE fluorenone derivative, tilorone and congeners: clinical and laboratory effects of these agents as modulators of host resistance. Pharmacol Ther 15:1–44

Rohovsky MW, Newberne JW, Gibson JP (1970) Effects of an oral interferon-inducer on the hematopoietic and reticuloendothelial systems. Toxicol Appl Pharmacol 17:556–558

Ruggeri A, Dell'Orbo C, Quacci D (1975) Electron microscopic visualization of proteoglycans with Alcian blue. Histochem J 7:187–197

Schofield BH, Williams BR, Doty SB (1975) Alcian blue staining of cartilage for electron microscopy. Application of the critical electrolyte concentration principle. Histochem J 7:139–149

Scott JE, Dorling J (1965) Differential staining of acid glycosaminoglycans (mucopolysaccharides) by Alcian blue in salt solutions. Histochemie 5:221–233

Scott JE, Quintarelli G, Dellovo MC (1964) The chemical and histochemical properties of Alcian blue. I. The mechanism of Alcian blue staining. Histochemie 4:73–85

Shepard N, Mitchell N (1976a) Simultaneous localization of proteoglycan by light and electron microscopy using toluidine blue O. A study of epiphyseal cartilage. J Histochem Cytochem 24:621–629

Shepard N, Mitchell N (1976b) The localization of proteoglycan by light and electron microscopy using safranin O. J Ultrastruct Res 54:451–460

Shepard N, Mitchell N (1981) Acridine orange stabilization of glycosaminoglycans in beginning endochondral ossification. Histochemistry 70:107–114

Simionescu M, Simionescu N, Silbert JE, Palade GE (1981) Differentiated microdomains on the luminal surface of the capillary endothelium. II. Partial characterization of their anionic sites. J Cell Biol 90:614–621

Spicer SS, Garvin AJ, Simson JAV, Wertelecki V (1978a) Cytochemistry of the skin of patients with mucopolysaccharidoses. Histochem J 10:137–150

Spicer SS, Hardin JH, Setser ME (1978b) Ultrastructural visualization of sulphated complex carbohydrates in blood and epithelial cells with the high iron diamine procedure. Histochem J 10:435–452

Spicer SS, Baron DA, Sato A, Schulte BA (1981) Variability of cell surface glycoconjugates. Relation to differences in cell function. J Histochem Cytochem 29:994–1002

Svennerholm L, Fredman P (1980) A procedure for the quantitative isolation of brain gangliosides. Biochim Biophys Acta 617:97–109

Szirmai JA (1963) Quantitative approaches in the histochemistry of mucopolysaccharides. J Histochem Cytochem 11:24–34

Thelmo WL, Levine S (1978) Renal lesions induced by tilorone and an analog. Am J Pathol 91:355–360

Thyberg J (1977) Electron microscopy of cartilage proteoglycans. Histochem J 9:259–266

Van Hoof F (1973a) Mucopolysaccharidoses. In: Hers GH, Van Hoof F (eds) Lysosomes and storage diseases. Academic Press, New York, pp 217–259

Van Hoof F (1973b) G_{M1}-gangliosidosis. In: Hers GH, Van Hoof F (eds) Lysosomes and storage diseases. Academic Press, New York, pp 305–321

Weiss JN, Weinberg RS, Regelson W (1980) Keratopathy after oral administration of tilorone hydrochloride. Am J Ophthalmol 89:46–53

Wenk EJ, Levine S, Hoenig EM (1979) Fine structure of contrasting choroid plexus lesions caused by tertiary amines or cyclophosphamide. J Neuropathol Exp Neurol 38:1–9

Wight TN, Ross R (1975) Proteoglycans in primate arteries. I. Ultrastructural localization and distribution in the intima. J Cell Biol 67:660–674

Zbinden G, Emch E (1972) Effect of tilorone HCl, an oral interferon-inducer, on leukopoiesis in rats. Acta Haematol 47:49–58

Received July 15, 1982