

Light- and Electron-Microscopic Histochemistry of Fabry's Disease

T. FARAGGIANA, MD, J. CHURG, MD,
E. GRISHMAN, MD, L. STRAUSS, MD,
A. PRADO, HT (ASCP), D. F. BISHOP, PhD,
E. SCHUCHMAN, BS, and R. J. DESNICK, PhD, MD

From the Division of Renal Pathology, Department of Pathology and
Division of Medical Genetics, Mount Sinai School of Medicine,
New York, New York

A histochemical study was performed on light- and electron-microscopic level in a case of Fabry's disease. The patient underwent kidney transplantation for renal failure and died of heart failure 6 months later. Patient's tissues were studied at the light- and electron-microscopic levels with various embedding and staining techniques for lipids and carbohydrates. Two peroxidase-labeled lectins (from *Ricinus communis* and from *Bandeiraea simplicifolia*) known to have affinity for α - and β -D-galactose, were strongly reactive with the storage material on frozen sections. The ultrahistochemical

and extraction tests showed that the typical granules had a variable reactivity and morphologic characteristics in different cells, probably reflecting different composition. A small number of typical deposits were also observed in the transplanted kidney. This is the first reported case of recurrence of the storage disease in the allograft. Of interest was also the fact that the patient's blood inhibited normal α -galactosidase activity, suggesting a possible inhibitor-related mechanism in the pathogenesis of the recurrence. (Am J Pathol 1981, 103:247-262)

FABRY'S DISEASE is a storage disease characterized by lysosomal accumulation of specific neutral glycosphingolipids in most tissues and fluids of affected individuals.¹ The major accumulated glycosphingolipid has been identified as globoglycosylceramide ($\text{Gal}\alpha 1-4\text{Gal}\beta 1-4\text{Gluc}\beta 1-1'\text{cer}$),² and its accumulation ascribed to a defective activity of the lysosomal hydrolase, α -galactosidase A.^{2,3} These neutral glycosphingolipids are deposited in all areas of the body, occurring predominantly in the lysosomes of endothelial, perithelial and smooth muscle cells of blood vessels, and to a lesser extent in histocytic and reticular cells in connective tissue.^{1,4} The progressive accumulation of the spherocrystalline glycolipid deposits leads to clinicopathologic involvement of almost every system.^{1,4-7}

Early diagnosis is often elusive, and definite diagnosis requires demonstration of the enzymatic deficiency.⁴ Most frequently, however, the diagnosis is suspected from histologic, histochemical, or ultrastructural examination of biopsied renal tissue or skin. Identification of the stored material by histochemical techniques is plagued with difficulties.^{1,8} First, the glycolipid is almost entirely extracted by xylene; thus the use of paraffin sections is prevented. Second, there

are no absolutely specific histochemical tests for glycolipids. The most selective method is the periodic acid-Schiff (PAS) modification of Adams;⁸ it consists of blocking the ethylene bonds of unsaturated fatty acids and the 1-amino, 2-hydroxyl groups (which may also react with PAS) before the PAS staining. Parallel control sections after lipid extraction are also required. However, it may be impossible to distinguish the Fabry glycosphingolipid substrates from the glycolipids that accumulate in other glycosphingolipidoses and gangliosidoses. Moreover, frozen sections must be used, with consequent loss of fine cellular details.

Similarly, at the electron microscopic level, the typ-

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Address reprint requests to J. Churg, MD, Department of Pathology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029.

ical myelin-like structure, although suggestive, is not pathognomonic of Fabry's disease.^{2,9} Moreover, the need of previous fixation with osmium (a strong oxidative agent) greatly hampers the usefulness and specificity of many electron-microscopic histochemical techniques for carbohydrates, most of which are based on the use of periodic acid, which is also an oxidative agent.

In view of these limitations, we studied the use of alternative procedures to identify the glycosphingolipids in Fabry's disease. We report here the evaluation of methacrylate embedding mixtures for the preservation of glycolipids and the use of osmium postfixation for light- and electron-microscopic histochemistry of neutral glycolipids. In addition, we describe the use of two peroxidase-labeled lectins (from *Ricinus communis* and *Bandeiraea simplicifolia*) that bind specifically with β - and α -linked galactose moieties, respectively, and permit selective characterization of complex glycoconjugates in this and possibly other storage diseases. Finally, these techniques were used to examine the lesion in a case of considerable interest. The patient, who died 6 months after a renal allotransplantation, showed evidence for glycosphingolipid deposition in the donor kidney, suggesting a recurrence of the disease, an event that so far has not been reported.

Materials and Methods

Clinical History

The patient, a 36-year-old male, had acroparesthesias since childhood. He was admitted to the hospital with chronic renal failure, proteinuria, and hypertension. The diagnosis of Fabry's disease was suggested by the histochemical and ultrastructural studies of a renal biopsy. The diagnosis was confirmed by the demonstration of deficient α -galactosidase A activity in plasma and leukocytes.¹⁰ The patient was maintained on chronic dialysis until a renal cadaver allograft was performed. Two months after transplantation, the patient experienced graft rejection that responded to treatment. Six months later, the patient died with bronchopneumonia and heart failure. An autopsy was performed within 1 hour of death.

Tissue was obtained at the time of renal transplantation and at autopsy. The following procedures were used for light-microscopic study:

- 1) Frozen sections were cut from unfixed or formalin-fixed (24 hours) tissue.
- 2) Fixation in sodium acetate buffered formalin, or in phosphate-buffered (pH 7.2, 0.1 M) 4% paraformaldehyde.
- 3) Fixation as in 2. Embedding in glycol methacrylate-butoxyethanol mixture (JB4 mixture, Poly-

sciences) at 4 C. Dehydration was carried either in graded ethanols or in 97% pure glycol methacrylate (30 minutes) and then by 100% pure glycol methacrylate (30 minutes).

- 4) Fixation as in 2. Postfixation in 1% OsO₄ (50 minutes) in phosphate buffer (0.1 M, pH 7.2). Embedding in paraffin.
- 5) Fixation and postfixation as in 4. Dehydration in 70% and 95% ethanol. The pieces were left overnight in 100% ethanol, with several changes, and embedded in JB4 mixture. The long treatment in ethanol was necessary for the completion of the reduction of osmium linked to tissues in an intermediate state of oxidation. Failure to include such a step prevents the methacrylate polymerization, probably through interference of osmium oxides with the ethylene bond of the glycol methacrylate molecule.¹¹

Some of the sections from osmium-fixed blocks were bleached with a 5% solution of H₂O₂ for 15–20 minutes before staining. Other sections were treated with phenylhydrazine¹² to block free aldehyde groups, before procedures involving the use of Schiff reagent. From all the blocks, sections were stained according to the following methods: hematoxylin and eosin (H&E); PAS; modified PAS,^{1,8} PASM, oil red O, Sudan black, chloroform-ethanol extraction-PAS,⁸ performic acid-Schiff.⁸ The birefringence of various structures in stained or unstained section, as well as the presence of autofluorescence, was checked with the polarizing and fluorescence microscopes.

Frozen sections from unfixed tissues were stained with peroxidase-labeled lectin from *Ricinus communis* (RCA₁₂₀) (Sigma) according to Yamada et al.¹³ Fluorescein labeling proved unsuitable because of the native autofluorescence of the granules. Other sections were stained with peroxidase-labeled lectin from *Bandeiraea simplicifolia* (Sigma) for 90 minutes in phosphate-buffered saline (0.05 M, pH 7.4). After two washings in the same buffer and three washings in Tris buffer (0.05 M, pH 7.4), peroxidase activity was detected with the 3,3'-diaminobenzidine method of Graham and Karnovsky as described by Yamada.¹³

For electron microscopy, small blocks of tissue were fixed in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2). Some blocks were directly embedded in Epon or in glycol methacrylate (67 ml glycol-methacrylate, 27 ml butyl-methacrylate, 6 ml methyl-methacrylate, 2 g benzoyl peroxide [Lupercol]). A prepolymer for infiltration and embedding was obtained from this mixture by gentle heating.¹⁴ Other tissues were postfixated in osmium and embedded in Epon. Ultrathin sections were examined either unstained or after various treatments: uranyl acetate and

lead citrate, periodic acid–silver methenamine,¹⁵ periodic acid–thiocarbohydrazide–silver proteinate (PA-TCH-SP),¹⁶ hydrogen peroxide–phosphotungstic acid (H₂O₂-PTA).¹⁷

Assay of α -galactosidase A activity in tissues and fluids was carried out by the method of Desnick et al.¹⁰ The neutral glycosphingolipids in renal tissues was quantitated by gas chromatography according to the previously reported procedures.^{18,19} Electrophoretic analyses of purified normal α -galactosidase A²⁰ in heparinized plasma from normal individuals and hemizygotes with Fabry's disease were carried out as previously described.²¹

Results

The results of the light and electron microscopic observations with various procedures are summarized in Table 1.

Light-Microscopic Observations (Figures 1–12)

No major differences were observed between the histochemical behavior of formalin and paraformaldehyde fixed tissue. The aldehyde fixation had little influence on the reactivity of the storage material compared with that of unfixed tissues. The only exception seems to be the lectin affinity, which was much stronger in unfixed tissue. With these methods all the inclusions appeared strongly reactive, but other structures were also stained. In particular, red cells and some cell coats (brush borders in the kidney) reacted with the *Bandeiraea* lectin.

After formalin fixation and methacrylate embedding, the glycolipid droplets were almost completely lost from the vessel walls and glomerular podocytes

but were largely retained in kidney tubules and interstitial cells (Figures 1 and 7). Both types of droplets were removed by chloroform–methanol (Figure 8). This variation in behavior was dependent upon embedding procedures and was also reflected in other histochemical tests: vascular wall droplets (mainly within the myocytes) were the only ones faintly stained with the performic acid–Schiff sequence, which was otherwise negative (Figure 9). It must be stressed that all the droplets were completely extracted by chloroform–methanol (2:1, vol/vol) (Figure 11). A very few granules remained in some vascular walls after this procedure, but those were consistent with lipofuscin bodies on both the light- and electron-microscopic levels and will not be considered further.

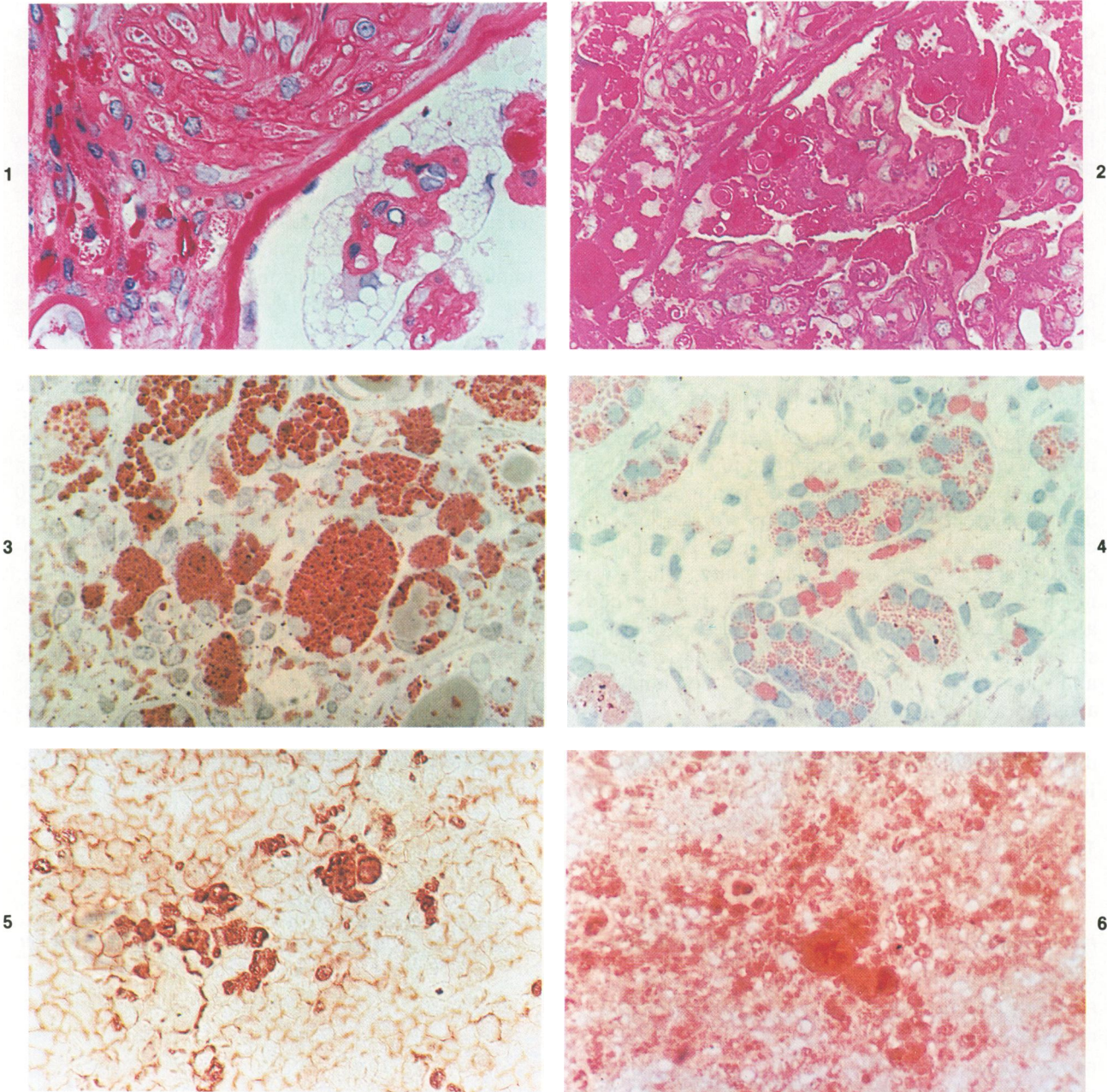
Embedding in methacrylate proved successful in retaining some of the inclusions, but only if dehydration was carried out in pure glycomethacrylate, avoiding any contact with ethanol or other alcohols. When the embedding mixture used for ultrastructural studies contained butyl as well as methyl-methacrylate, the storage material was not adequately preserved. Similar results were obtained with standard Epon. Osmium postfixation, as expected, had several effects on the preservation and reactivity of the lipids. After 50 minutes of postfixation, the inclusions appeared in unstained sections as pale brown bodies, when compared with the intense black of the adipose tissue. They were no longer extractable, even with hot chloroform–methanol or xylene.

Osmium induced Schiff positivity in many tissue structures and in particular in the storage inclusions, basement membrane, and collagen without pretreatment with periodic acid. Such Schiff positivity was completely abolished by 2-hour incubation with phenylhydrazine. After osmium fixation, the PAS reac-

Table 1—Staining Procedures

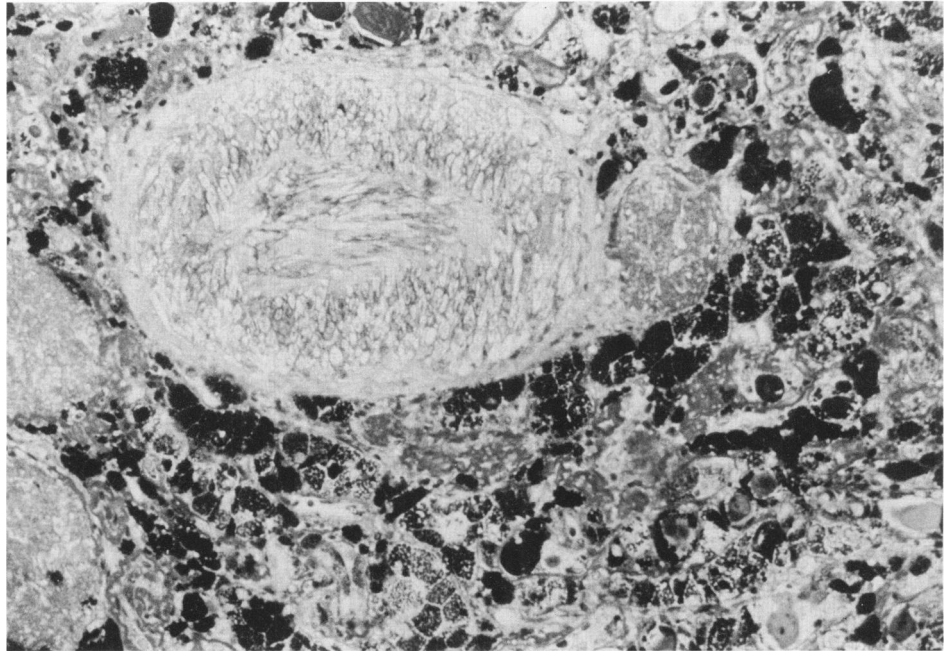
Fixation and Embedding	PAS	PAS modified	Chloroform-methanol-PAS	PASM	Oil red O	Sudan black	Oil red O-birefringence	Auto-fluorescence	Lectin 1 (<i>Ricinus</i>)	Lectin 2 (<i>Bandeiraea</i>)	PAT-CHSP	H ₂ O ₂ -PTA	Performic acid-Schiff
Unfixed—frozen sections	+	+	–	+	+	+	+	+	+	+	0	0	±
Formalin—frozen sections	+	+	–	+	+	+	+	+	–	–	0	0	±
Formalin—paraffin	–	–	–	–	–	–	–	–	–	–	0	0	–
Formalin—glycolmethacrylate	±	±	–	±	±	±	±	+	–	–	0	0	±
Formalin—osmium paraffin	+	0	+	+	+	+	+	–	–	–	0	0	0
Formalin—osmium glycolmethacrylate	+	0	+	+	+	+	+	–	–	–	0	0	0
Paraformaldehyde—Epon (EM)	0	0	0	–	0	0	0	0	0	0	–	–	0
Paraformaldehyde—osmium-Epon (EM)	0	0	0	+	0	0	0	0	0	0	+	+	0

Reactivity of storage granules with various fixation, embedding, and staining procedures: + = positive or strongly positive; ± = positive in some areas, negative in others (see text); – = negative; 0 = not performed.



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Figure 7—Kidney. Same treatment as in Figure 1. Note the loss of material in the vacuoles of the glomeruli and arterial wall and their preservation in tubular epithelium and interstitial cells. ($\times 100$)



tions, with or without previous phenylhydrazine treatment, were indistinguishable. Fat stains gave very strong reactions after osmium fixation: red with oil red O and bluish-black with Sudan black, against a brown background (Figures 3 and 10).

Hydrogen peroxide bleaching of the sections removed completely the reduced osmium, without reducing the stainability with oil red O, and apparently increasing its specificity (Figure 4). A beneficial effect was also observed with hematoxylin counterstain, which otherwise was rather faint. Hydrogen peroxide treatment also enhanced the Schiff positivity in the sections. The significance of this effect is not clear and is currently being investigated. The PAS modification of Adams (chloramine T-performic acid-phenylhydrazine-PAS) was virtually indistinguishable from

the standard PAS when performed after formalin fixation on either frozen sections or methacrylate embedded tissues.

The storage material was observed by light microscopy in almost all the organs examined. In the kidney, glomerular, tubular (mainly distal), and interstitial cells and vascular walls were involved. In the liver, the deposits were mostly in Kupffer cells, but occasionally also in the hepatocytes. Other organs with striking accumulations of the Fabry substrate were the intestines (both muscularis layers and ganglion cells), myocardium, vascular endothelium of skin, and the adeno- and neuro-hypophysis.

Electron Microscopy (Figures 13–19)

The inclusions showed heterogeneous composition

Figure 1—Kidney. Formalin fixation; glycolmethacrylate embedding; PAS stain. Note the loss of glycolipids from the arterial wall and glomerular podocytes, which appear vacuolated. Some interstitial cells retain these granules. ($\times 160$) (See also Figure 7.) **Figure 2**—Kidney. Formalin fixation; osmium postfixation; glycolmethacrylate embedding; PAS stain. The podocytes contain large PAS-positive vacuoles, sometimes showing a lamellar structure. After osmium fixation, the glycolipids are no longer extractable in lipid solvents. ($\times 160$) (See also Figure 8.) **Figure 3**—Kidney. Formalin fixation; osmium postfixation; glycolmethacrylate embedding; oil red O stain. The glycolipid deposits contained in the tubular and interstitial cells show a brown-reddish color due to the combination of reduced osmium (brown-black) and oil red O (red). ($\times 100$) (Compare with Figure 4.) **Figure 4**—Kidney. Formalin fixation; osmium postfixation; glycolmethacrylate embedding; hydrogen peroxide-oil red O stain. The vacuoles show the typical red color of the oil red O. The selectivity of the stain seems to be good despite the presence of the embedding resin. The H_2O_2 treatment has completely removed the reduced osmium and the brownish color of the stain. ($\times 100$) **Figure 5**—Spleen. Unfixed frozen section; peroxidase-labeled lectin from *Ricinus communis* (RCA_{120}); benzidine reaction. Many macrophages contain a strongly reactive material, extractable in lipid solvents. This lectin affinity technique demonstrates the presence of galactose residues in the storage vacuoles. ($\times 100$) **Figure 6**—Spleen. Unfixed frozen section; peroxidase-labeled lectin from *Bandeiraea simplicifolia*; benzidine reaction. The reaction pattern is similar to that in Figure 5. This lectin binds specifically to the α -galactose residues, known to be part of the substrate accumulated in Fabry's disease. ($\times 100$)

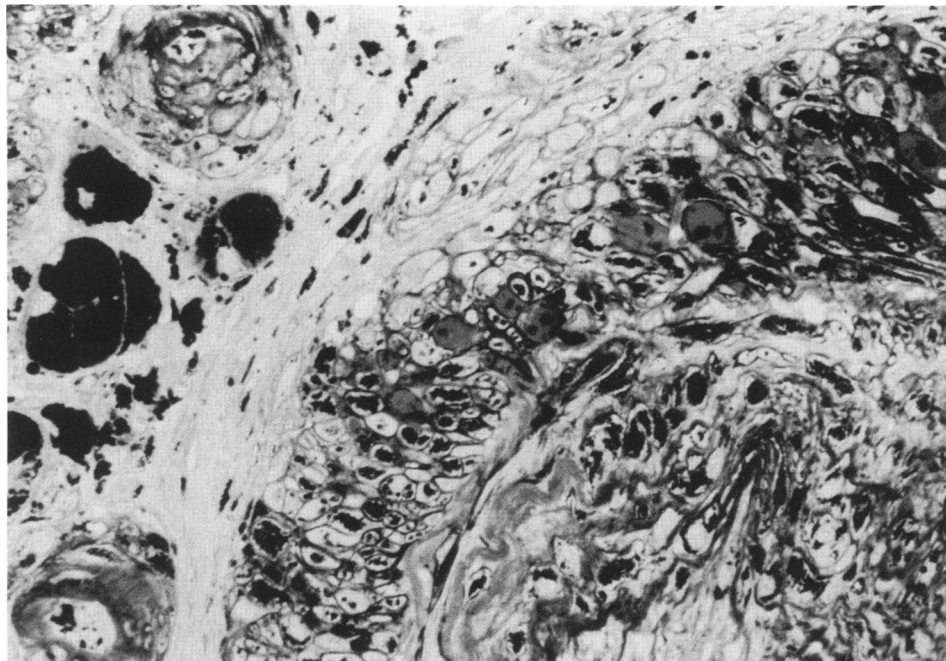


Figure 8—Kidney. Same treatment as in Figure 2. Note the preservation of material in vacuoles in every structure, including the arterial wall. ($\times 400$)

(Figure 13). With both the silver proteinate and phosphotungstic acid techniques, some areas were heavily stained, with occasional lamellar structures (Figure 16), while others were almost completely free of stain (Figures 14–16). A thin reactive layer was evident around some of the granules, suggesting the presence of a coated membrane. With the H_2O_2 -PTA sequence, only structures known to contain carbohydrates were stained. In addition to the inclusions, glycogen, collagen, and cell coats were stained (Figure 15). The

granules were also stained by the PASM procedure (Figure 17). The inclusions had a regular periodicity of about 60 \AA when examined at high magnification (Figure 18). Ultrastructural study revealed, after extensive search, a few inclusions in several endothelial cells of the intertubular capillaries in the transplanted kidney (Figure 19). These inclusions had the typical appearance of those found in patient's own organs, including the characteristic periodic structure (Figure 19). The inclusions were not visible by light microscopy.

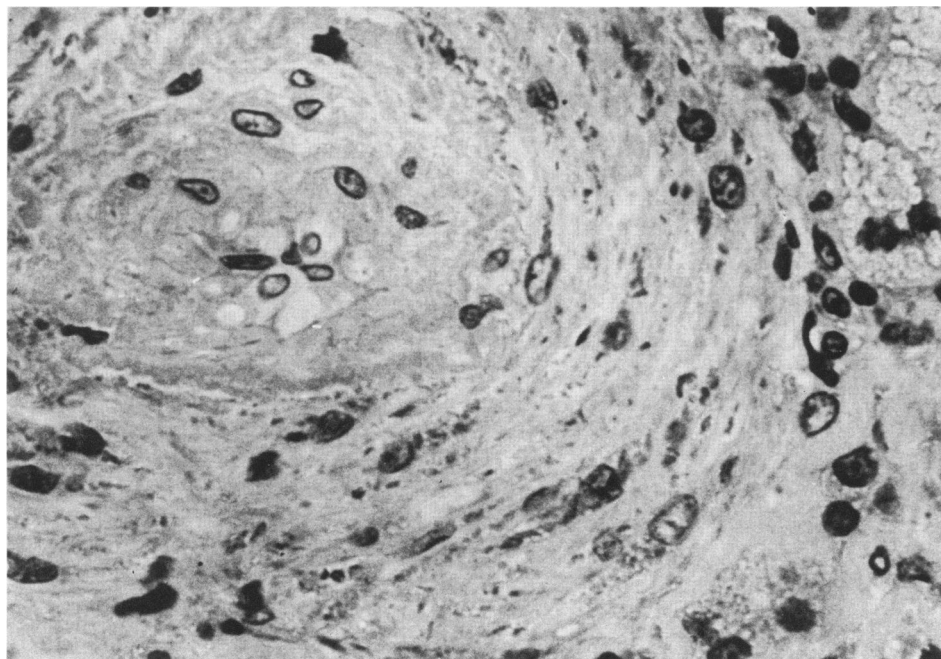
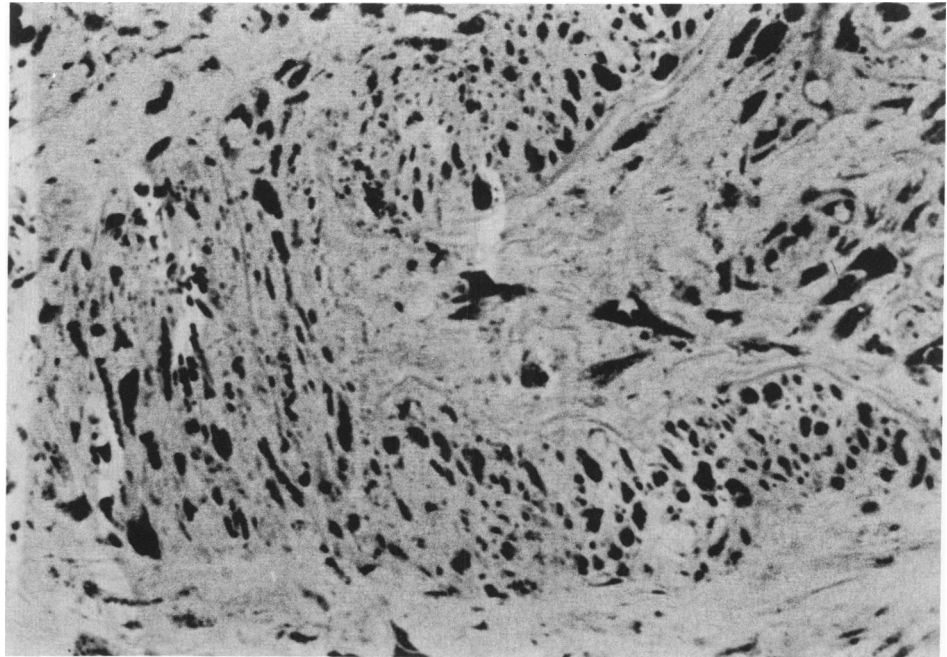


Figure 9—Formalin fixation; frozen section; performic acid-Schiff reaction. The reaction is mainly negative, especially in the tubular epithelial cells (*top right*). However, some vacuolar material in the vessel wall appears to react. ($\times 400$)

Figure 10—Formalin fixation; osmium postfixation; methylacrylate embedding; Sudan black stain. Note the strong reactivity of the vacuolar inclusion material in the vascular wall. After osmium postfixation, this lipid staining technique, like many histochemical procedures, loses its specificity. ($\times 400$)

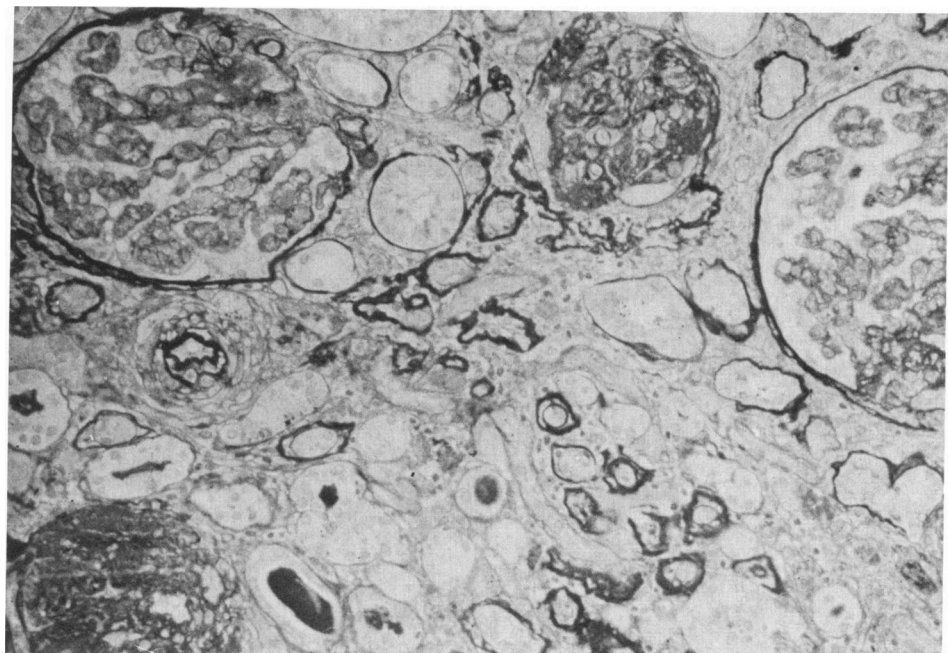


Biochemical Studies

Table 2 shows the levels of the individual neutral glycosphingolipids in the renal allograft and kidneys from normal individuals. In the allograft, the levels of globoglycosylceramide and galabiosylceramide, the substrates which accumulate in Fabry kidney, were not significantly different from their respective normal mean values. In an effort to find a circulating inhibitor of normal α -galactosidase A activity, mixtures

of purified plasma α -galactosidase A and heparinized plasma from the recipient as well as other Fabry patients with renal transplants and normal control subjects were assayed. As shown in Table 3, the recipient's plasma inhibited the purified activity by 43%, whereas 19–20%, 13–20%, and 12–20% inhibition were found in the mixtures containing plasma from other Fabry transplant recipients (RG, DG), other Fabry hemizygotes, and normal individuals, respectively. None of the medications which the recipients

Figure 11—Formalin fixation; methylacrylate embedding; chloroform-methanol extraction; PAS stain. Note the complete loss of storage material in vacuoles. ($\times 160$)



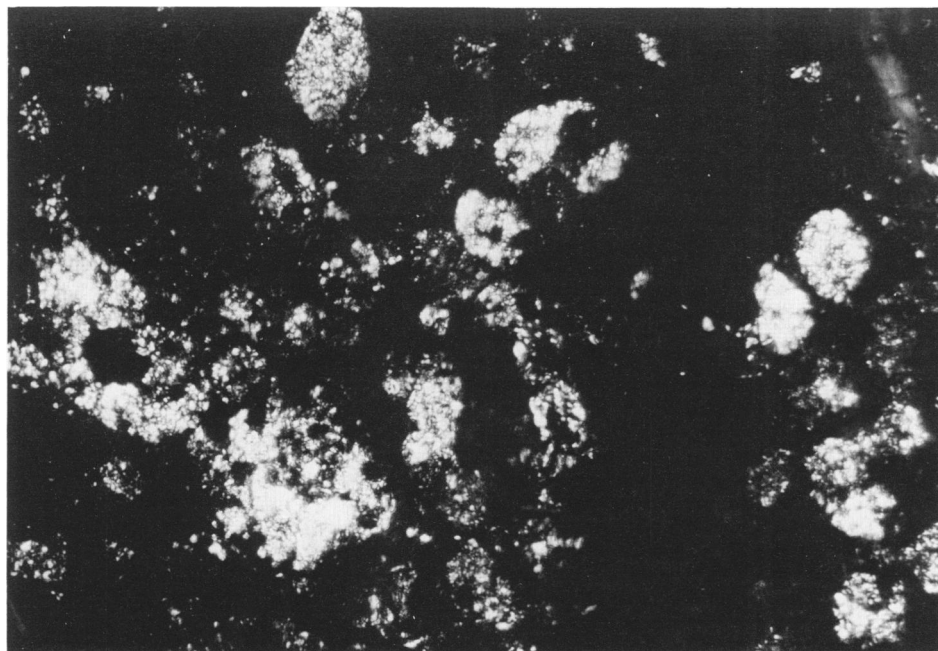


Figure 12—Formalin fixation; methacrylate embedding; oil red O stain; polarized light. Note the strong birefringence of the vacuolar inclusions. The presence of Maltese crosses shows that many glycolipids are present in the form of spherocrystals. ($\times 400$)

received inhibited α -galactosidase A activity. Therefore, the possibility that the recipient had produced antibodies to the normal enzyme was evaluated. As demonstrated previously,²⁰ when purified α -galactosidase A from normal human spleen was electrophoresed in the presence of rabbit antihuman splenic or plasma α -galactosidase A, the mobility of the enzyme was retarded. Analogously, when the purified plasma enzyme was mixed with the recipient's plasma, it was also retarded (Figure 20; lane 3). In addition, the apparent activity was reduced, as assessed by the brightness of the activity band (lane 3), compared with the activity observed in the other lanes, consistent with the results of the *in vitro* assays (Table 2). In contrast, neither normal plasma (lane 2) nor plasma from a nontransplanted Fabry hemizygote had any effect on the mobility or apparent activity of the added enzyme (lane 4). Treatment with antibody (lane 5) also retarded the mobility of a portion of the added enzyme, while treatment with 10 times this amount of antibody yielded a complex that was immobile (lane 6) and probably a precipitate. In each case, the more antibody (or recipient plasma) present in the mixture, the greater the retardation of the enzymes' electrophoretic mobility.

Discussion

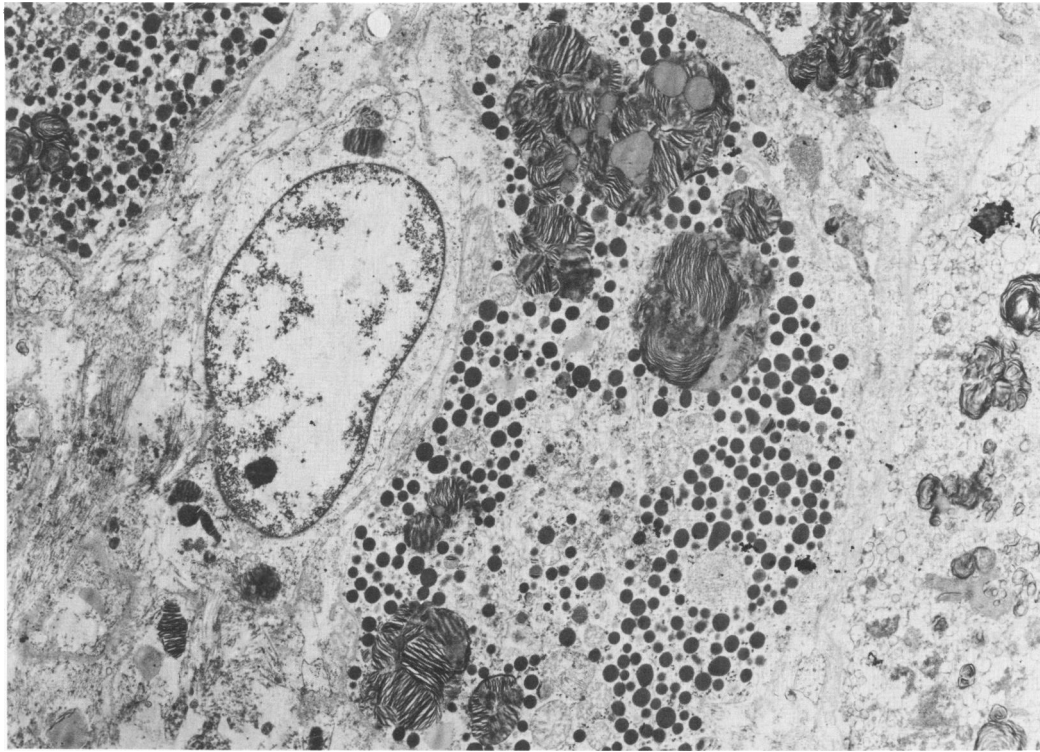
The purpose of this study is to characterize the storage material in Fabry disease by means of light- and electron-microscopic histochemistry. The Fabry substrates are complex glycosphingolipids with terminal

α -galactosyl moieties. From a histochemical point of view, these substrates are therefore expected to display (and indeed do display) the staining characteristics of both lipids and carbohydrates.

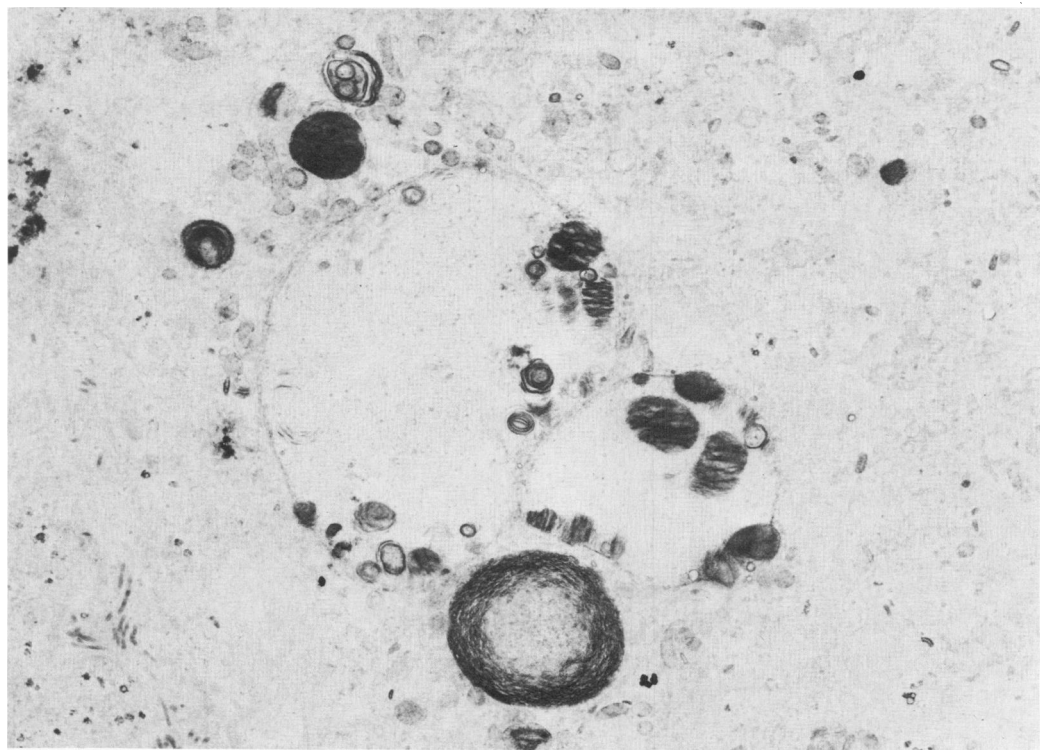
Since in formalin-fixed paraffin-embedded tissue the glycolipids are completely extracted, we use alternate fixation and embedding procedures.

Osmium tetroxide has long been known to be an excellent fixative for lipids in general and for the polyunsaturated forms in particular. Similar observations have been made with the use of potassium dichromate as a fixative for preservation of lipids, including those of Fabry disease.²² Our results are in agreement with these data. Osmium, both in methacrylate and in paraffin sections, preserves material that otherwise would be extracted by solvents used for the processing of tissue (alcohol, xylene, methacrylate itself). After osmium fixation, lipids are no longer extractable, even in hot chloroform-methanol. Thus, after osmium postfixation, the distribution of storage granules can be studied in paraffin or methacrylate sections with better resolution than in frozen sections. However, the specificity of almost all subsequent histochemical tests is considerably reduced. In fact, osmium induces a diffuse Schiff positivity, acting on vic-glycol groups²³ and on double bonds of unsaturated lipids;²⁴ it also induces a Sudan black stainability of tissues, even when lipids have been previously extracted.²⁵

We attempted to restore some of the specificity of the PAS reaction after osmium fixation, using a phenylhydrazine blockade or carbonyl groups. Since this



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Figure 13—Pituitary gland. Paraformaldehyde fixation; osmium postfixation; Epon embedding; uranyl and lead "stain." Note the heavy accumulation of storage material in the parenchymal cells. Note also the nonhomogeneous morphologic appearance of the granules ($\times 6900$) (Compare with Figure 14.) **Figure 14**—Liver. Kupffer cell; paraformaldehyde fixation; osmium postfixation; Epon embedding; hydrogen peroxide-phosphotungstic acid "stain." Note the nonhomogeneous reactivity of the vacuolar material with this staining procedure, considered selective for carbohydrates. Note also the thin-layer (coat) lining the lysosomes. ($\times 18,000$)

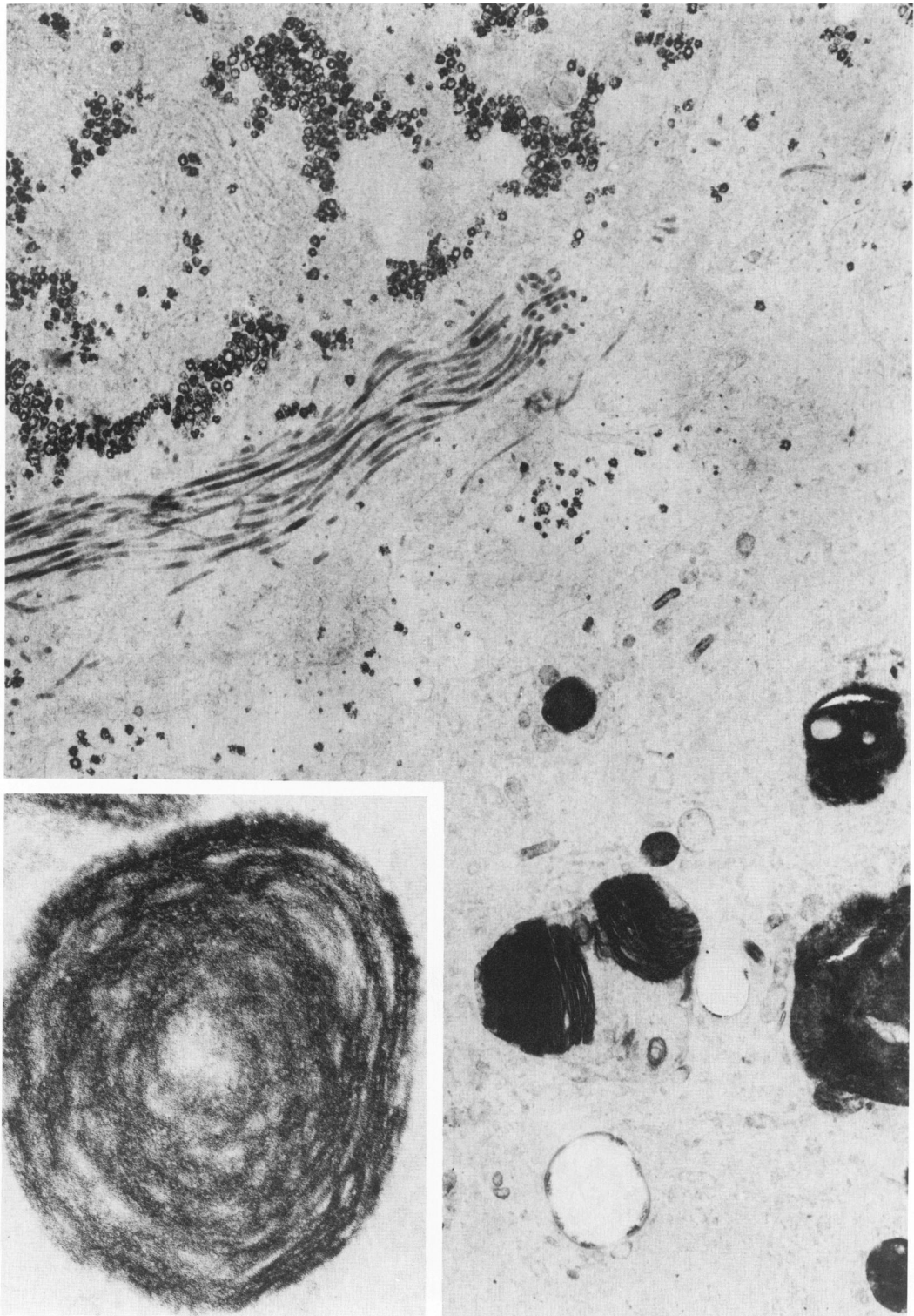


Figure 15—Same procedure as in Figure 14. Note the selectivity of the stain for carbohydrates. Only glycogen, collagen, and Fabry inclusions are stained. The treatment with hydrogen peroxide has the effect of etching the epoxy resin and also removes the interference of the osmium electron density. ($\times 19,800$) **Inset**—Higher magnification of a storage vacuole. Note the lamellar pattern. ($\times 110,000$)

blockade, which suppresses all osmium-induced Schiff positivity, is periodic-acid-resistant,²⁶ we believe that when performed before the PAS sequence, it can improve the specificity of the PAS reaction. On the other hand, every fixative interferes with the PAS specificity: even formalin reacts with lipids and can introduce unspecific Schiff positivity.^{27,28} In a similar way, we tried to improve the selectivity of oil red O stain in osmicated tissues. Hydrogen peroxide bleaching of the sections completely removed the brown background color of the granules, probably by reoxidizing and solubilizing the osmium. No structures other than the granules stained with this technique. Although we cannot prove the specificity of the method, since, as mentioned before, lipids are no longer extractable, the selectivity appeared to be excellent. However, hydrogen peroxide bleaching did not restore the eosinophilia of tissues lost through the osmium-induced deamination.²³

In summary, although it carries several theoretical shortcomings, osmium postfixation is useful in the study of the morphology of the glycolipid granules. The specificity of some of the histochemical reactions can be partly restored by appropriate blocking and bleaching treatments. The substitution of paraffin with methacrylate as embedding medium also proved to reduce to a significant degree the loss of lipids, even in nonosmicated tissues.

These findings are not surprising. Although methacrylate has been shown to extract some phospholipids more than ethanol,²⁹ it has been used to preserve lipids in a case of Farber granulomatosis,³⁰ and it has been reported that all the conventional lipid histochemistry tests were positive with methacrylate embedding.³¹ Methacrylate embedding also offers the advantage of allowing the use of thin sections with excellent resolution of details and minimal distortion.

The histochemistry of Fabry's disease has been described in several reports, some of which are quite detailed.^{1,32-33} Our results on frozen, as well as methacrylate-embedded, tissues confirm the previous data and are consistent with the glycolipid nature of the storage material.

It is of great interest that preservation of glycolipids with methacrylate embedding affected various cells in a different manner, suggesting different solubility and/or composition of the lipid moiety in different cells. Also consistent with the variable composition of the storage granules was the finding of a performic acid-Schiff positivity in the most easily extractable (arterial wall) granules. Altogether, however, the performic acid-Schiff reaction was faint or absent in this case, even on frozen sections. This is in keeping with the biochemical results of Bagdade et al,³⁴ which re-

vealed that 70% of the lipids extracted from the lung of a Fabry's case were saturated.

The findings of more than one kind of lipid in this disease is not surprising. Storage of nonspecific lipids in lipidoses may be due to physiochemical binding of other lipids by the primary deposits. Cholesterol, for instance, has been found in Tay-Sachs disease.⁸ It is also known that a primary enzymatic defect can greatly influence the activity of other enzymatic complexes, particularly in lysosomes.³⁵ We also confirmed the positivity of the PAS reaction as modified by Adams.^{1,36} In spite of the doubts expressed about the necessity of this time-consuming procedure,³⁷ we consider it the most specific test for glycolipids presently available.

In order to characterize more specifically the carbohydrate moiety of the glycolipids, we used two peroxidase-labeled lectins, known to react specifically with D-galactose. As expected, the two procedures showed strong reactivity of the granules. In fact, Fabry's substrate contains two terminal molecules of α - and β -linked galactose. Lectin from *Ricinus* (RCA 120) is known to display a selective affinity for D-galactose,³⁸ and lectin from *Bandeiraea simplicifolia* is highly reactive with α -linked D-galactose.³⁹ In a detailed study at the light-microscopic level, using a technique similar to the one used by us, Yamada and Shimizu¹³ showed that peroxidase-labeled lectin from *Ricinus* specifically reacts with galactose in histologic sections. A similar technique had been used by Bretton and Bariéty⁴⁰ to stain glomeruli of the normal rat at the ultrastructural level.

So far no report has been published on the use of peroxidase-labeled lectin from *Bandeiraea* in human tissue. However, Peters and Goldstein⁴¹ described the use of a fluorescein conjugate to stain a galactopyranoside group. The fact that chloroform-methanol extraction completely abolishes all reactivity of the granules in our case supports the view that they are galactolipids. It is interesting to note that besides the storage granules, we found staining of red blood cells and the brush border of the proximal kidney tubules. These structures possibly represent the substrate of α -galactosidase.

In conclusion, although techniques using these two lectins are still in an experimental phase, we believe that they represent the most specific histochemical test to demonstrate the presence of carbohydrates in complex lipid molecules. Moreover, this approach allows the specific determination of monosugars, and it could be therefore a very useful tool in the further characterization of other glycolipid storage diseases on histologic level, with obvious practical diagnostic advantages.

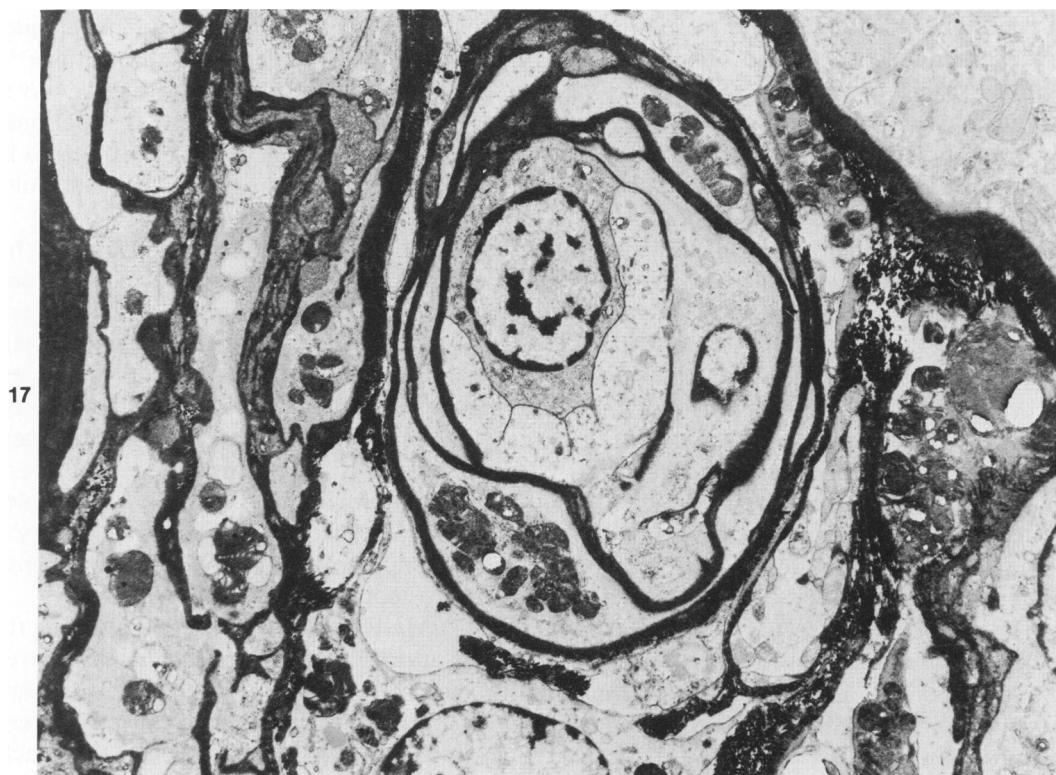
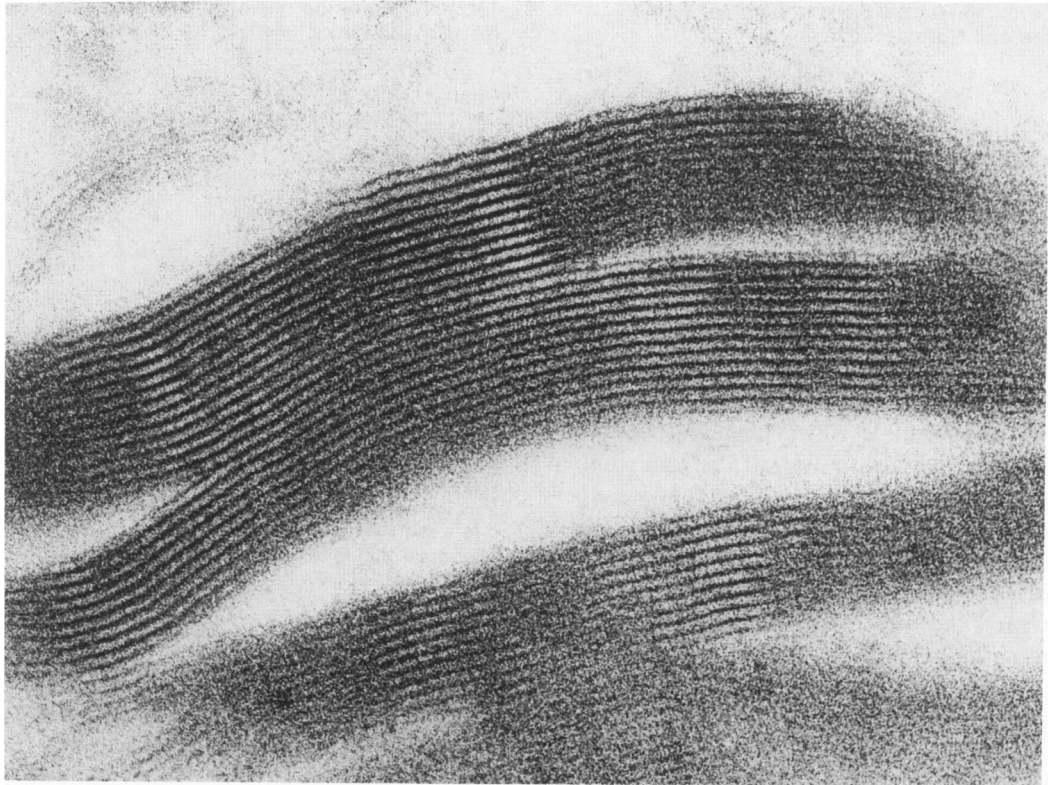
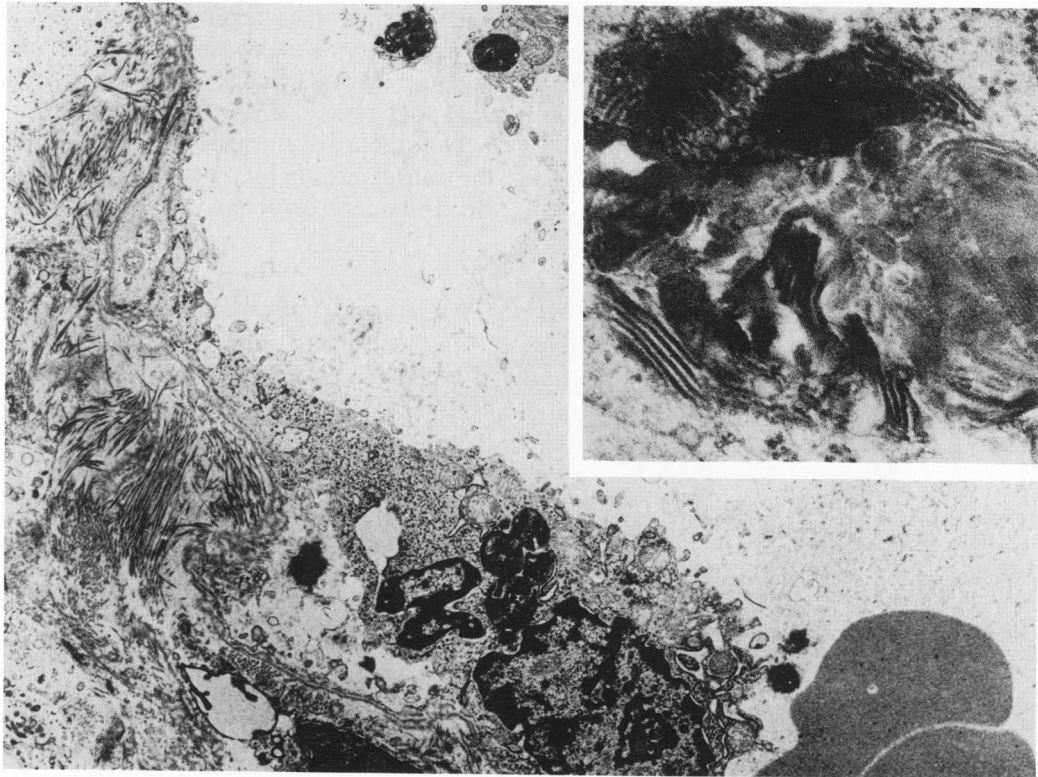


Figure 16—Pituitary gland. Paraformaldehyde fixation; osmium postfixation; Epon embedding; periodic acid–thiocarbohydrazide; silver proteinate. Note the strong reactivity of the inclusion material as well as the selectivity of the procedure. However, after osmium postfixation, this, like any other procedure involving periodate oxidation, loses some of its specificity. ($\times 24,000$) **Figure 17**—Kidney. Paraformaldehyde fixation; osmium postfixation; Epon embedding; periodic acid–silver methenamine. Note the reactivity of the vacuolar inclusions in the vascular wall. ($\times 5300$)



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Figure 18—Pituitary gland. Paraformaldehyde fixation; osmium postfixation; lead stain. Note the typical, although not specific, periodicity (60 Å) of the storage material. ($\times 350,000$) **Figure 19**—Transplanted kidney. Paraformaldehyde fixation; osmium postfixation; Epon embedding; uranyl and lead stain. Ultrastructural investigation proved the only reliable method of detecting the recurrence of the disease in the transplanted kidney. Note the small vacuoles in the endothelial cell of an intertubular capillary. ($\times 5900$) **Inset**—Higher magnification of another vacuole. ($\times 67,000$)

Table 2—Levels of Neutral Glycosphingolipids in Transplanted and Normal Kidneys

Source	Lactosyl- ceramide	Gala- biosyl- ceramide	Globo- glycosyl- ceramide	Tetra- hexosyl- ceramide
Allograft (this case)	0.12	0.13	0.39	0.52
Normal kidney				
Mean	0.16	0.10	0.35	0.42
Range (n = 3)	0.09–0.25	0.03–0.15	0.25–0.40	0.21–0.55

Our ultrastructural studies confirmed the result already reported by others and reviewed by Desnick et al.⁴ The histochemical tests reported here, periodic acid–thiocarbohydrazide–silver proteinate and periodic acid–silver methenamine, suffer from some of the limitations already discussed, since they were performed on osmium-fixed tissues. However, the Thiéry method showed considerable selectivity for the storage granules. The phosphotungstic acid stain (PTA) was apparently the most selective for carbohydrate-containing structures. The pretreatment with hydrogen peroxide both etched the Epon, allowing a more intense staining with phosphotungstic acid, and bleached the osmium, reducing the interference caused by its electron density. Although PTA positivity has been assumed to indicate the presence of mucopolysaccharides in Fabry's disease,⁴² this is not supported by other available literature. The use of this method, that of hydrogen peroxide–phosphotungstic acid, could turn out to be the easiest and one of the more reliable ways of studying glycolipids at the ultrastructural level in Fabry's disease.

Table 3—Inhibition of Normal α -Galactosidase A by Normal and Fabry Hemizygote Plasma*

Source	Case	% Inhibition
Normal:	CK	20
	DB	12
	GP	15
Fabry hemizygotes:	JC	18
	BC	13
	FS	20
Fabry hemizygotes (transplanted):	RG	19
	DG	20
	This case	43
	This case†	43

* Plasma samples were adjusted to pH 7.2 with HCl following the addition of 1/10 volume of 0.25 M sodium HEPES, pH 7.2. The buffered plasma samples (22 μ l) plus purified human splenic α -galactosidase A (3 μ l, 4 nmoles of artificial substrate cleaved per hour) were mixed together and incubated with 150 μ l of artificial substrate solution (5 mM 4-methylumbelliferyl- α -D-galactopyranoside in 0.15 M citrate-phosphate buffer, pH 4.6). The 100% control solution contained instead of plasma, 22 μ l of 1 mg/ml human serum albumin in 25 mM sodium HEPES, pH 7.2.

† Dialyzed.

The reoccurrence of Fabry's disease in a renal allograft has not been previously reported.^{43–50} Actually, kidney transplantation has been regarded by some as a possible way of correcting the enzymatic deficiency through enzyme replacement by means of an implant of a normal organ which continually produces the endogenous activity of α -galactosidase A.^{43,44}

The amount of storage material found at autopsy in the transplanted kidney was very limited. In fact, only electron-microscopic investigation proved successful

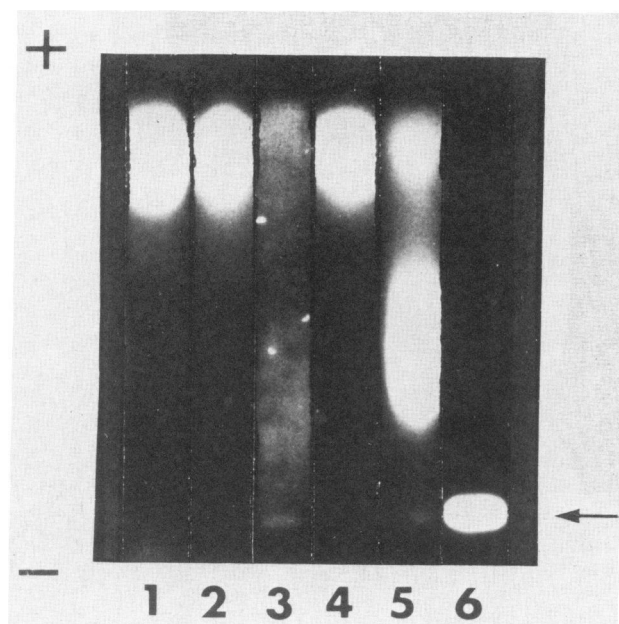


Figure 20—Cellulose acetate electrophoresis of plasma α -galactosidase A in the presence of normal and Fabry plasma and anti- α -galactosidase A. The electrophoretic method and fluorescent activity stain were as described previously. In each lane, 1 μ l of solution was applied and electrophoresed which contained 1 unit of human plasma α -galactosidase A activity mixed with 1) 1 mg/ml human serum albumin in 25 mM HEPES, pH 7.2; 2) normal human plasma containing 25 mM HEPES and adjusted to pH 7.2; 3) Fabry plasma, case FR, buffered as in 2; 4) Fabry plasma, case BC (nontransplant patient); 5) 0.3 g rabbit antihuman splenic α -galactosidase A; and 6) 3.0 g rabbit antihuman splenic α -galactosidase A.

in detecting the typical myelinlike structures. This is in keeping with the fact that the increase in Fabry's substrate in this organ was below the levels of biochemical detection. It is interesting that the cells most involved at this early stage were the endothelial cells of the intertubular capillaries, which are most likely to come in contact with the highest levels of Fabry's substrate present in the circulating blood. Although this finding remains unexplained, there are several possible explanations. The cells degradative capacity may have been overwhelmed by the large amount of substrate. The endothelial cells in which the storage material was observed may have populated the vascular wall from endogenous reticuloendothelial stem cells. Thus, they would be deficient in α -galactosidase activity and store the substrates. Alternatively, the substrate may have accumulated due to the presence of an inhibitor to normal α -galactosidase A. Support for this latter hypothesis is based on the finding of a circulating inhibitor in the recipient's plasma (Table 3), which behaved on electrophoresis in a way similar to that of anti- α -galactosidase antibodies. This hypothesis is not inconsistent with the fact that the recipient experienced a rejection episode 2 months after transplantation, which may have resulted in the formation of anti- α -galactosidase antibodies. Further studies to confirm the immunoglobulin nature of the circulating inhibitor are in progress.

References

- Lehner T, Adams CW: Lipid histochemistry of Fabry's disease. *J Pathol Bacteriol* 1968, 95:411-415
- Sweeley CC, Klionsky B: Fabry's disease: Classification as a sphingolipidosis and partial characterization of a novel glycolipid. *J Biol Chem* 1963, 238:3148-3150
- Brady RO, Gal AE, Bradley RM, Martensson E, Warsaw AL, Laster L: Enzymatic defect in Fabry's disease: Ceramide trihexosidase deficiency. *N Engl J Med* 1967, 276:1163-1167
- Desnick RJ, Klionsky B, Sweeley CC: Fabry's disease: Defective α -galactosidase activity, The Metabolic Basis of Inherited Diseases. Edited by JB Stanbury, JB Wyngaarden, DS Fredrickson, New York, McGraw-Hill, 1978, pp 810-840
- Roth J, Schulze E, Raabe G, Waldmann G: Analytische Studie des Morbus Fabry. *Virchows Arch [Pathol Anat]* 1974, 363:287-301
- Burkholder PM, Updike SJ, Ware RA, Reese OG: Clinicopathologic, enzymatic and genetic features in a case of Fabry's disease. *Arch Pathol Lab Med* 1980, 104:17-25
- Gubler MC, Lenoir G, Grünfeld J-P, Ulmann A, Droz D, Habib R: Early renal changes in hemizygous and heterozygous patients with Fabry's disease. *Kidney Int* 1978, 13:223-235
- Adams CWM: Lipid histochemistry of lipids, Neurochemistry. Edited by CWM Adams. Amsterdam, Elsevier, 1965, pp 67-108
- McNamara TE, Goodloe S, Butkus DE: Myelin bodies in patients without clinical Fabry's disease. *Arch Pathol Lab Med* 1980, 104:14-16
- Desnick RJ, Allen KY, Desnick SJ, Raman MK, Bernlohr RW, Krivit W: Fabry's disease: Enzymatic diagnosis of hemizygotes and heterozygotes: α -galactosidase activities in plasma, serum, urine, and leukocytes. *J Lab Clin Med* 1973, 81:157-171
- Barsotti P, D'Agostino M, Marinozzi V: GMA embedding of osmium fixed tissues. *J Submicr Cytol* 1980, 12:233-240
- Pearse AGE: Histochemistry, Theoretical and Applied. Vol 1, 3rd edition. London, Churchill, 1968
- Yamada K, Shimizu S: The histochemistry of galactose residues of complex carbohydrates as studied by peroxidase-labeled *Ricinus communis* agglutinin. *Histochemistry* 1977, 53:143-156
- Leduc EH, Bernhard W: Recent modifications of the glycol methacrylate embedding procedure. *J Ultrastruct Res* 1967, 19:196-199
- Marinozzi V: Silver impregnation of ultrathin sections for electron microscopy. *J Biophys Biochem Cytol* 1961, 9:121-133
- Thiery J-P: Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. *J Microsc* 1967, 6:987-1018
- Faraggiana T, Marinozzi V: Phosphotungstic acid staining of polysaccharides containing structures on epoxy embedded tissues. *J Submicrosc Cytol* 1979, 11:263-265
- Desnick RJ, Sweeley CC, Krivit W: A method for the quantitative determination of neutral glycosphingolipids in urine sediment. *J Lipid Res* 1970, 11:31-37
- Desnick RJ, Dawson G, Desnick SJ, Sweeley CC, Krivit W: Diagnosis of glycosphingolipidoses by urinary-sediment analysis. *N Engl J Med* 1971, 284:739-744
- Bishop DF, Dean KJ, Sweeley CC, Desnick RJ: Enzyme therapy XVII: Purification and characterization of human α -galactosidase isozymes: Comparison of tissue and plasma forms and evaluation of purification methods, Enzyme Therapy in Genetic Diseases. Vol 2. Edited by RJ Desnick. New York, Alan R. Liss Publishing Co., 1980, pp 17-32
- Desnick RJ, Dean KJ, Grabowski G, Bishop DF, Sweeley CC: Enzyme therapy in Fabry's disease: Differential *in vivo* plasma clearance and metabolic effectiveness of plasma and splenic α -galactosidase A isozymes. *Proc Natl Acad Sci USA* 1979, 76:5326-5330
- Frost P, Tanaka Y, Spaeth GL: Fabry's disease - glycolipid lipidosis. *Am J Med* 1966, 40:618-627
- Wolman M: The reaction of osmium tetroxide with tissue components. *Exp Cell Res* 1957, 12:231-240
- Wolman M: The lipids stained by the periodic acid-Schiff technic. *Stain Technol* 1956, 31:241-245
- Holzinger L: Über die unspezifische Sudanophilie. *Acta Histochem* 1965, 20:374-380
- Bayliss OB, Adams CWM: Resistance of aldehyde blockades to periodic acid. *Histochem J* 1976, 8:71-75
- Wolman M, Greco J: The effect of formaldehyde on tissue lipids and on histochemical reactions for carbonyl groups. *Stain Technol* 1952, 27:317-324
- Jones D: Reactions of aldehydes with unsaturated fatty acids during histological fixation. *Histochem J* 1972, 4:421-465
- Cope GH, Williams MA: Quantitative studies on the preservation of choline and ethanolamine phosphatides during tissue preparation for electron microscopy: II. Other preparative measures. *J Microscopy* 1969, 90:47-60
- Moser V, Prenskey AL, Wolff HJ, Rosman NP: Farber granulomatosis. *Am J Med* 1969, 47:869-890
- Halbhuber K-J: Einbettungen für die lichtmikroskopische Lipidhistochemie. *Acta Histochem* 1977, Suppl 19:223-227
- Ferrans VJ, Hibbs RG, Burda CD: The heart in Fabry's disease: A histochemical and electron microscopic study. *Am J Cardiol* 1969, 24:95-110

33. Van Mullem PJ, Ruiter M: Histochemische Untersuchungen anlässlich der Lipoidspeicherung bei der sogenannten Fabry'schen Krankheit (Angiokeratoma corporis diffusum). *Arch Klin Exp Dermatol* 1968, 232:148-167
34. Bagdade JD, Parker F, Ways PO, Morgan TE, Lagunoff D, Eidelman S: Fabry's disease: A correlative clinical, morphologic, and biochemical study. *Lab Invest* 1968, 18:681-688
35. Holtzman: Lysosomes, A Survey. New York, Springer-Verlag, 1976
36. Adams CW, Bayliss OB: Histochemical observations on the localisation and origin of sphingomyelin, cerebroside and cholesterol in the normal and atherosclerotic human artery. *J Pathol Bacteriol* 1963, 85: 113-119
37. Elleder M: Lipidhistochemistry. A critical survey. *Acta Histochem* 1977, Suppl 19:239-265
38. Nicolson GL, Blaustein J: The interaction of *Ricinus communis* agglutinin with normal and tumor cell surfaces. *Biochim Biophys Acta* 1972, 266:543-547
39. Goldstein IJ, Hayes CE: The lectins; carbohydrate-binding proteins of plants and animals. *Adv Carbohydr Chem Biochem* 1978, 35:127-340
40. Bretton R, Bariéty J: A comparative ultrastructural localization of concanavalin A, wheat germ and *Ricinus communis* on glomeruli of normal rat kidney. *J Histochem Cytochem* 1976, 24:1093-1100
41. Peters BP, Goldstein IJ: The use of fluorescein-conjugated *Bandeiraea Simplicifolia* B4-isolectin as a histochemical reagent for the detection of α -D-galactopyranosyl groups: Their occurrence in basement membranes. *Exp Cell Res* 1979, 120:321-334
42. Tondeur M, Resibois A: Fabry's disease in children: An electron microscopic study. *Virchows Arch Cell Pathol* 1969, 2:239-254
43. Philippart M, Franklin SS, Gordon A: Reversal of an in-born sphingolipidosis (Fabry's disease) by kidney transplantation. *Ann Int Med* 1972, 77:195-200
44. Desnick RJ, Simmons RL, Allen KY, Woods JE, Anderson CF, Najarian JS, Krivit W: Correction of enzymatic deficiencies by renal transplantation: Fabry's disease. *Surgery* 1972, 72:203-211
45. Jacky E: Fabry'sche Erkrankung (Angiokeratoma corporis diffusum universale): günstiger Verlauf nach Nierentransplantation. *Schweiz Med Wochenschr* 1976, 106:703-709
46. Buhler FR, Thiel G, Dubach VC, Enderlin F, Gloor F, Tholen H: Kidney transplantation in Fabry's disease. *Br Med J* 1973, 3:28-29
47. Clarke JTR, Guttman RD, Wolfe LS, Beaudoin JG, Morehouse DD: Enzyme replacement therapy by renal allotransplantation in Fabry's disease. *N Engl J Med* 1972, 287:1215-1218
48. Spence MW, Mackinnon KE, Burgess JK, D'Entremont DM, Belitsky P, Lannon SG, MacDonald AS: Failure to correct the metabolic defect by renal allotransplantation in Fabry's disease. *Ann Intern Med* 1976, 84:13-16
49. Grünfeld JP, LePorrier M, Droz D, Bensaude I, Hinglais N, Crosnier J: La transplantation rénale chez les sujets atteints de maladie de Fabry: Transplantation du rein d'un sujet hétérozygote à un sujet sain. *Nouv Presse Med* 1975, 4:2081-2085
50. Van den Bergh FAJTM, Rietra PJGM, Kolk-Vegter AJ, Bosch E, Tager JM: Therapeutic implications of renal transplantation in a patient with Fabry's disease. *Acta Med Scand* 1976, 200:249-256

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