SUMMARY

Rabbit pups were maintained from birth either on a high fat, low protein diet or on a low fat, high protein diet for as long as 10 days. Rates of palmityl coenzyme A oxidation, glutamate + malate-supported respiration, and cytochrome oxidase activity in heart and liver homogenates were the same in both groups and similar in all respects to rates observed in mother-fed animals. In view of these findings, the hypothesis that the amount of lipid in the postnatal diet influences the ability to oxidize long chain fatty acids must be re-evaluated. It seems likely that other perinatal stimuli may be more important than diet in regulating oxidative energy metabolism to ensure survival during this critical transition period.

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Chemotaxis
lysosomal hydrolases
mannosidosis
α-mannosidosis
neutrophil
Zn-dependent enzymes

Mannosidosis: Clinical, Morphologic, Immunologic, and Biochemical Studies

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Extract

The primary metabolic defect in mannosidosis is the deficiency of the acidic α-mannosidase A and B activities which results in the lysosomal accumulation of mannose-rich substrates. Our studies demonstrate that the enzymatic diagnosis of suspect homozygotes can be made reliably using plasma, isolated leukocytes, or cultured skin fibroblasts assayed carefully at the appropriate acidic pH.

Immunologic studies of a mannosidosis homozygote revealed significant abnormalities of neutrophil function; these included a depressed chemotactic responsiveness and impaired phagocytosis of bacteria. Lymphocyte transformation studies showed a 20% of normal response to purified phytohemagglutinin and a 25% of normal response to concanavalin A.

Three major components of α-mannosidase activity in normal human liver were resolved by ion exchange chromatography on DEAE-cellulose and electrophoresis on cellulose acetate gels. Electrophoresis of the liver extract from *homozygote 1* with mannosidosis revealed only one band of activity which coelectrophoresed with the α-mannosidase C isozyme partially purified from normal liver. However, ion exchange chromatography revealed the presence of residual hepatic acidic activities; the residual A

isozyme was eluted in a position corresponding to that of normal α-mannosidase A whereas the residual B activity was eluted at a slightly more electronegative position than that of normal B isozyme.

The apparent K_m values for α-mannosidase activity as determined from Lineweaver-Burk plots were 1.1 mM for normal liver and 0.9 mM for normal leukocytes. In contrast, the residual activity in these sources from *homozygote 1* could not be saturated within the solubility range of the substrate; the apparent K_m value was estimated at 15.4 mM in liver extracts.

Zinc significantly lowered the apparent K_m value of the acidic activity in normal liver (from 1.2 to 0.24 mM), whereas this metallic ion had little effect on the values for mannosidosis hepatic activity (from 15.4 to 12.3 mM). Unlike zinc, cobalt had its major effect on the acidic activity in the mannosidosis liver extract, lowering the apparent K_m from 15.4 to 3.9 mM, whereas the apparent K_m for the normal activity was increased from 1.2 to 1.9 mM. The residual acidic activities were markedly stimulated by zinc in both leukocytes (~300%) and plasma (~400%) from the homozygotes and to a lesser extent in those sources from normal individuals. In contrast, cobalt enhanced the residual acidic activities in leukocytes (~500%) and plasma (~200%) from the homozygotes while inhibiting these acidic activities (78.9% and 47.7%, respectively) in normal individuals.

Speculation

The susceptibility of homozygotes with mannosidosis to severe recurrent infections may be the direct consequence of impaired leukocyte membrane recognition processes resulting from the defective catabolism of substrates with α-D-mannose residues. The metabolic defect in mannosidosis presumably results from a structural gene mutation which alters the kinetic and metal binding properties of the lysosomal α-mannosidase activities. Appropriate metal cation supplementation may stimulate the residual activity of the mutant isozymes and provide an effective therapeutic approach to patients with this inborn error of metabolism.

Mannosidosis, a systemic lysosomal storage disease first described by Öckerman (33), is characterized by psychomotor retardation, a facial dysmorphia resembling that of the Hurler syndrome, dysostosis multiplex, hepatosplenomegaly, hearing loss, recurrent infections, and autosomal recessive inheritance. The primary metabolic defect responsible for these manifestations is the deficiency of the acidic α-mannosidase A and B activities (α-D-mannoside mannohydrolase, EC. 3.2.1.24) (9, 29, 35), which results in the lysosomal accumulation of mannose-rich oligosaccharides in neural (34) and visceral tissues (1, 33) and in the urine (1, 31, 32, 46) of affected homozygous patients. Presumably this enzymatic defect also leads to the accumulation of other pathogenic glycoconjugate substrates with terminal α-mannosyl residues as evidenced by the recent finding of abnormal glycopeptides, rich in mannose, in cultured skin fibroblasts from a homozygote with mannosidosis (47).

Since Öckerman's original report in 1967 (33), only 18 patients with this lysosomal storage disease have been described (1, 2, 6, 14, 24, 26, 27, 32, 46). We have seen five patients in two unrelated families at the University of Minnesota Hospitals during the past 4 years. The major clinical and laboratory findings of our cases and those in the literature have been summarized in Table 1. In this report, we describe the clinical course of a previously unreported patient (*homozygote 1*), who expired at age 3½ years, and emphasize the morphologic and laboratory features of this disorder. In addition, we report the results of our investigations to further characterize the molecular pathology of this disorder including correlative studies of the ultrastructural, enzymatic, and immunologic defects in mannosidosis.

CASE REPORT

Homozygote 1, a 2⁷/₁₂ year-old Caucasian female (*ML; UMH 1194722*), was initially referred to the University of Minnesota Hospitals for evaluation of recurrent infections (Fig. 1). She was the firstborn (June 6, 1971) of unrelated parents following an uncomplicated pregnancy, labor, and delivery. Birth weight was 3.2 kg and the neonatal and early infancy periods were unremarkable. At 6 months of age she was noted to have a greater occipital-frontal circumference than chest circumference and was hospitalized for the evaluation of suspected hydrocephalus. At that time, the diagnosis of arrested hydrocephalus was made. During the next 19 months she had chronic otitis media and eight episodes of upper respiratory tract infections, four of which required hospitalization. In addition, a mild developmental delay was noted; she rolled over at 4 months, sat at 8 months, crawled at 12 months, and walked with and without assistance at 18 and 23 months, respectively.

On admission, physical examination revealed a well developed 2⁷/₁₂-year-old female with a prominent forehead and a mild facial dysmorphia resembling that of the Hurler syndrome. Her height was 91.5 cm (+1 SD), weight 14.9 kg (+1.7 SD), and occipital-frontal circumference 54.0 cm (+3 SD). Pertinent physical findings included frontal bossing, dull gray tympanic membranes, and hepatosplenomegaly. The liver edge was firm, smooth, and palpable 5 cm below the right costal margin and the splenic tip was palpable 2 cm below the left costal margin. In addition, there was a small reducible umbilical hernia, unusually thick-feeling skin without lesions, and prominent lymphadenopathy. Cardiac examination and EKG were normal. Neurologically, she had normal motor strength, tone, and sensory response. Cranial nerves were grossly intact except for a 70-dB hearing loss. Cerebellar function was intact, although her gait was broad-based for age. Electroencephalographic studies were normal. Denver Developmental Screening indicated gross motor, fine motor, language, and personal-social skills at 13, 21, 6, and 14 months of age, respectively. The delay in language development presumably was related to the severe hearing loss.

Radiologic examination revealed chronic pulmonary infiltrates consistent with repeated infections. Extensive bony abnormalities compatible with dysostosis multiplex were present, including a "J-shaped" sella, a hypoplastic L2 lumbar vertebra with anterior beaking, tapered proximal metacarpals, mild flaring of the iliac wings, and extensive sclerosis of the cranial vault and particularly of the skull base. No corneal or lenticular opacities were seen by slit lamp microscopy; normal discs, retinae, and maculae were noted on funduscopic examination.

Clinical laboratory studies included normal blood-urea-nitrogen, creatinine, sodium, potassium, chloride, bicarbonate, calcium, phosphorus, bilirubin, serum glutamic oxalacetic transferase, alkaline phosphatase, serum protein electrophoresis, cholesterol, triglycerides, and total phospholipids. Sweat chlorides were normal and the quantitative immunoglobulins were IgA, 157 mg/100 ml (normal range for age, 34–109 mg/100 ml); IgG, 940 mg/100 ml (557–1,100 mg/100 ml); and IgM, 127 mg/100 ml (40–119 mg/100 ml). Coagulation studies were within normal limits. The hemoglobin was 11.5 g/100 ml and the leukocyte count was 9,200/mm³ with a normal differential. On examination of a peripheral smear, approximately 90% of the lymphocytes were vacuolated. The urinary metabolic screen was normal, including a Berry spot test.

Special diagnostic studies included quantitative urinary amino acids and mucopolysaccharides which were within normal limits. Bone marrow biopsy revealed foamy appearing macrophages similar to those seen in Niemann-Pick disease as well as vacuolated lymphocytes. No inclusion bodies were observed by phase microscopy in cultured skin fibroblasts. Ultrastructural examination of hepatic tissue obtained by percutaneous biopsy showed numerous, enlarged lysosomes containing amorphous mucopolysaccharide-or glycoprotein-like material. Assays of various lysosomal hydrolase activities were performed; the demonstration of deficient acidic α-mannosidase activity in plasma, isolated peripheral leukocytes, and cultured skin fibroblasts established the diagnosis of mannosidosis.

At 3½ years of age, the patient was transported to the

Table 1. *Major clinical and laboratory features of enzymatically confirmed homozygotes with mannosidosis*

Author and reference	Case no.	Sex	Facial dysmorphology	Dysostosis multiplex	Mental retardation	Hearing loss	Corneal/ lenticular opacities	Hepatosplenomegaly	Vacuolated lymphocytes	Decreased serum IgG	Recurrent infections	Mucopolysaccharuria
Ockerman (33, 34)	1	M	+	+	+		+	+	+	+	+	−
Autio *et al.* (1)	2	M	+	+	+	+	−	−	+	+	+	−
	3	M	+	+	+	−	+	−	+	+	+	−
	4	M	+	+	+	−	+	−	+	+	+	−
Norden *et al.* (32)	5	F	+	+	+		+	+	+			±
	6	F	+	+	+		+	+	+		+	±
Tsay *et al.* (46)	7	M	+	+	−		−	+	−			−
Farriaux *et al.* (14)	8	M	+	+	−	+	−		+		+	−
	9	M	+	+	+		−		+			−
	10	M	+	+	+		−		+			−
	11	F	+	+			−		+			−
	12	M	+	+			−		+			−
Loeb *et al.* (26, 27)	13	F	+	−	+	+	−	−	+		+	−
	14	M	+	±	+	+	−	−	+		+	+
Booth *et al.* (6)	15	F	+	±	+	+	−	−			−	−
	16	F	+	±	+	+	−	−			−	−
	17	M	+	±	+	+	−	−			−	−
Aylsworth *et al.* (2)	18	M	+	+	+	−	+	+	+	−	+	−
Present cases												
Homozygote 1	19	F	+	+	+	+	−	+	+	+	+	−
Homozygote 2	20	M	+	+	+	+	+	−	+	−	+	−
Homozygote 3	21	M	+	+	+	+	+	−	+	−	+	−
Homozygote 4	22	F	+	+	+	+	+	−	+	−	+	−
Homozygote 5	23	F	+	+	+	+	+	−	+	−	−	−
Total	23	14M/ 9F	23/23	22/23	19/21	12/15	10/23	6/18	19/20	5/10	13/17	3/23

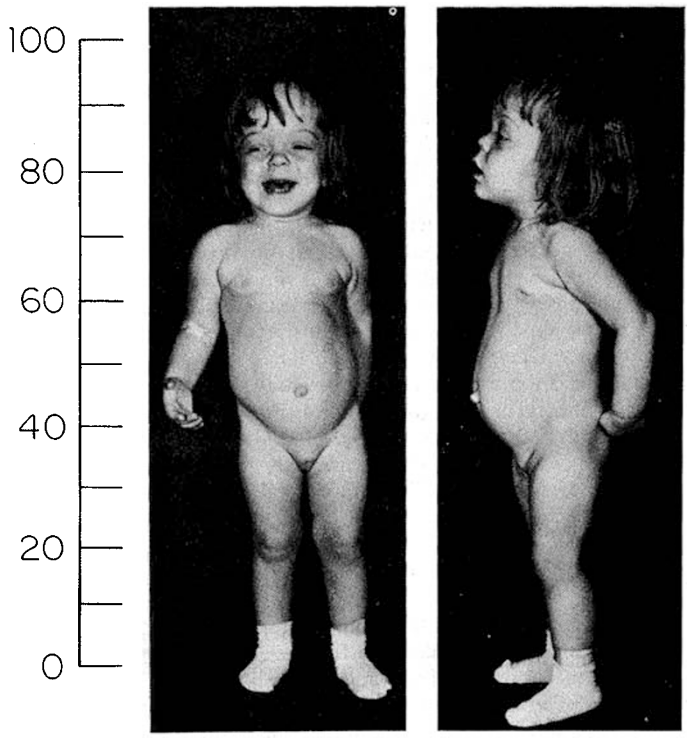

Fig. 1. *Homozygote 1* at the age of 2½ years old.

University of Minnesota Hospitals after 4 months of almost continual hospitalization elsewhere for recurrent pneumonitis, otitis, and upper respiratory tract infections. Quantitative immunoglobulins on admission were IgA, 105 mg/100 ml (normal range for age, 47–129 mg /100 ml); IgG, 576 mg/100 ml (680–1,270 mg/100 ml); IgM, 66 mg/100 ml (69–168 mg/100 ml); IgE, 18 ng/ml (21–198 ng/ml). She was anergic on skin testing to PPD, streptokinase/streptodornase, Candida, and mumps antigens. During this hospitalization she developed severe respiratory distress with nasal flaring, intercostal retraction, diffuse rhonchi, and a temperature of 105° F. Chest x-ray revealed marked bilateral basilar infiltrates. The respiratory status deteriorated rapidly over a week period despite intensive chemotherapy and respiratory support. She expired from respiratory failure and disseminated intravascular coagulation. Adenovirus type 7 was cultured from urine, stool, and throat cultures obtained before death and from lung tissue obtained at autopsy.

MATERIALS AND METHODS

MORPHOLOGIC STUDIES

Routine peripheral blood and bone marrow smears were stained with Wright's-Giemsa. Smears were also subjected to periodic acid-Schiff, Sudan black B, Toluidine blue O, and Oil Red O reactions. Particles of bone marrow were processed for electron microscopy in a manner previously described (8).

Percutaneous liver biopsy was obtained with a 1.6-mm Menghini needle by previously described procedures (41). Neuropathologic studies were carried out on brain tissues obtained at autopsy within 30 min of death. Liver and brain were fixed for electron microscopy in Millong's buffer containing 1.25% glutaraldehyde and 1.25% osmic acid. After 1 hr, the tissues were dehydrated in graded alcohols, initially with 70% ethanol, and propylene oxide and embedded in Epon. Thin sections stained with uranyl acetate and lead citrate were examined in a Phillips 200 electron microscope.

IMMUNOLOGIC STUDIES

Neutrophil chemotactic assays were performed by the method of Mowat and Baum (30) as modified by Hill *et al.* (19). Leukocytes

were separated by gravity sedimentation from heparinized blood, washed, counted, and diluted with balanced salt solution to approximately 2.5×10^6 neutrophils/ml. Aliquots (0.4 ml) of this suspension were centrifuged onto 5-μm Millipore filters in a cytocentrifuge. The filters were placed in a modified Boyden chamber with *Escherichia coli* bacterial factor as a chemotactant. After a 3-hr incubation, the filters were removed and stained with hematoxylin. The number of neutrophils that migrated completely through each filter in 10 random high power fields (using a 5- by 5-mm reticule) were counted. A chemotactic index was derived by dividing this number by the number of cells ($\times 10^6$) delivered to the starting side of the filter. Simultaneous control neutrophil chemotaxis assays were performed on the same day using neutrophils from adult laboratory personnel. All assays were done in triplicate and compared with control values. Normal values for this technique were 35–99; the reproducibility among triplicate assays of a given neutrophil sample was 13% of the mean (19).

For these experiments, a bacterial chemotactic factor produced by the overnight growth of *E. coli* grown in balanced salt solution at 37° was used. The culture broth was passed through a 0.22-μm Millipore filter and this filtrate was then stored at −70° in 1-ml ampules. On each test day, the ampules were thawed, diluted 1:20 with balanced salt solution, and used as the chemotactic attractant. Random migration was determined by counting the number of neutrophils which migrated completely through the Millipore filter when balanced salt solution was substituted for the chemotactic factor.

Neutrophil phagocytosis and bacterial killing studies were carried out by the Maaloe method as modified by Quie *et al.* (39). Neutrophil nitroblue tetrazolium (NBT) reduction was performed by the method of Park *et al.* (35).

BIOCHEMICAL STUDIES

Plasma was obtained from heparinized blood by centrifugation at 2,000 × *g* for 10 min at 4°. Leukocytes were isolated as previously described (7, 12). Liver from the patient and from age-matched control subjects was obtained within 15 min after death and frozen immediately at −70° for subsequent analyses.

The acidic (pH 4.4) and neutral (pH 6.0) α-mannosidase activities in various sources were determined by the following methods. Plasma, leukocyte, and cultured skin fibroblast extracts were diluted 10-fold in McIlvaine's citrate-phosphate buffer (17), pH 4.4 and 6.0. Hepatic tissue was homogenized in distilled water (1:2, w/v), centrifuged at 30,000 × *g* for 25 min, and the supernatant assayed for enzymatic activity. The standard reaction mixture contained 300 μl 1 mM 4-methylumbelliferyl-α-D-mannopyranoside (49) in citrate-phosphate buffer, pH 4.4 or pH 6.0, and 100 μl of enzyme source. Leukocytes, cultured skin fibroblast and hepatic supernatants, and column fractions (100 μl of the 2.0-ml fractions) were incubated for 30 min, and plasma for 1 hr at 37°. The reactions were terminated by the addition of 4.6 ml 0.1 M ethylenediamine, pH 11.4. Fluorescence was measured in a Turner model 111 fluorometer with an excitation wave length of 365 nm and an emission wave length of 450 nm and compared with 4-methylumbelliferone as a standard. Under these assay conditions, the rates of the enzyme reaction were linear with respect to time and protein concentration for each enzyme source.

For studies with metal ions, the reaction mixture was modified to contain 50 μl of enzyme source, 50 μl of the appropriate metal ion ($CoSO_4$, $ZnSO_4$ or $MnCl_2$) in distilled water, and 300 μl substrate in the appropriate buffer and then assayed as described above. Zinc and copper concentrations were determined in liver extracts from normal and mannosidosis tissues which were prepared as described for enzyme assay, according to standard techniques (42, 43).

The activities of other lysosomal hydrolases were determined in various sources with the appropriate artificial substrate according to the following methods: total α- and β-galactosidases (12), β-glucuronidase (48), total β-hexosaminidase (13), α-L-iduronidase (18), arylsulfatase A (4), and α-L-fucosidase (25). Protein concentrations were determined according to the method of Lowry (28).

The α-mannosidase isozymes in normal human liver and mannosidosis liver obtained at autopsy were resolved by ion exchange chromatography. Liver (2.0 g) was homogenized in distilled water (1:4, w/v), centrifuged at 30,000 × *g* for 25 min, and the supernatant (5 ml) was chromatographed on DEAE-cellulose (Whatman DE 52) (50), essentially by the method of Ikonne *et al.* (23).

Electrophoresis of partially purified α-mannosidases A, B, and C and tissue extracts was performed on cellulose acetate gels (Cellogel, 350 μm, 16 by 17 cm) (51) according to general procedures (15) with the following modifications for these isozymes. Electrophoresis was carried out in 0.04 M potassium phosphate buffer, pH 7.3, at 4° with the electrophoresis tank on ice. Gels were prerun at 0.88 ma/cm, constant current, for 20 min; then samples (5–10 μl containing 3.5–6.5 nmol/hr) were applied with capillary micropipets and allowed to equilibrate for 10 min. Constant current (0.88 ma/cm) was then applied; the duration of the electrophoretic run was 5 hr. The gel was removed from the tank and incubated for 1 min in 1.5 mM 4-methylumbelliferyl-α-D-mannopyranoside in 0.1 M citrate-phosphate buffer, pH 4.5. The gel was removed, gently blotted with filter paper, and placed in a moist chamber consisting of two glass plates separated by a border of felt strips saturated with water. The chamber was incubated at 37° for 40 min. Bands of fluorescence were developed by placing the gel in 0.17 M glycine-carbonate buffer, pH 10.7, for 2 min. The gel was blotted with filter paper and again placed in the moist chamber; the bands of enzymatic activity were viewed under long wave length ultraviolet light and photographed immediately.

RESULTS

DIAGNOSTIC ENZYME DETERMINATIONS

Table 2 shows the levels of acidic and neutral α-mannosidase activities in plasma, isolated peripheral leukocytes, and cultured skin fibroblasts from five homozygotes with mannosidosis, their obligate heterozygous parents, and normal individuals. In each enzyme source from the homozygotes, there was a marked deficiency of α-mannosidase activity at pH 4.4, whereas the activity at pH 6.0 was normal, with the exception of leukocytes, which demonstrated reduced neutral activities (29). The levels of acidic and neutral enzymatic activities obtained for the obligate heterozygotes were within the normal range; their ratios of acidic α-mannosidase activity to total β-hexosaminidase activity at pH 4.4 in plasma and leukocytes from the parents of *homozygotes 1–5* were within the respective ranges calculated for normal individuals and did not discriminate the heterozygous state (29). The arylsulfatase A, α-L-fucosidase, α- and β-galactosidase, β-glucuronidase, β-hexosaminidase A and B, and α-L-iduronidase activities in leukocytes and cultured skin fibroblasts from the homozygotes with mannosidosis were all within normal control ranges.

MORPHOLOGIC STUDIES

Approximately 80% of the lymphocytes in the peripheral blood smear contained 15–20 sharply defined, clear cytoplasmic vacuoles as shown in Figure 2. The vacuoles were not arranged in any particular array and essentially occurred wherever the cytoplasm was most abundant. The bone marrow smears contained numerous foamy macrophages, measuring approximately 40–50 μm in diameter, which occurred both singly and in small groups as shown in Figure 3*A*. Generally, the nucleus was centrally or slightly eccentrically located. The appearance of the cytoplasm varied slightly in different macrophages; in some cells, the contents appeared as closely packed, sharply defined, clear vacuoles similar to those found in the lymphocytes. In other macrophages there

Table. 2. *Acidic and neutral α-mannosidase activities in various sources from homozygotes and heterozygotes for mannosidosis and normal individuals*[1]

Source	Plasma, nmol/hr/ml		Leukocytes, nmol/hr/mg protein		Cultured skin fibroblasts, nmol/hr/mg protein	
	pH 4.4	pH 6.0	pH 4.4	pH 6.0	pH 4.4	pH 6.0
Family 1						
Homozygote 1						
ML	0.15	36.3	0.18	1.9	<0.01	11.9
Heterozygotes[2]						
JL	3.75	38.5	75.0	30.0		
RL	7.35	37.3	125	39.2		
Family 2						
Homozygotes 2–5						
GG	0.30	35.5	0.80	6.2		
JG	0.18	46.5	0.84	5.5		
MaG	0.02	49.0	0.91	4.5		
MeG	0.15	34.5	0.52	3.5		
Heterozygotes[2]						
HG	5.50	33.2	45.9	11.4		
EG	6.50	43.5	35.4	5.23		
Normal mean	12.3	30.9	83.2	24.4	166	58.4
(Range)	(3.5–35.6)	(13.5–127)	(22.8–153)	(9.2–536)	(36.3–483)	(13.7–184)
n	40	40	14	14	21	21

[1] Enzymatic activities determined using 4-methylumbelliferyl-α-D-mannopyranoside as substrate as described in *Materials and Methods*.
[2] The heterozygous individuals were the parents of the respective homozygotes.

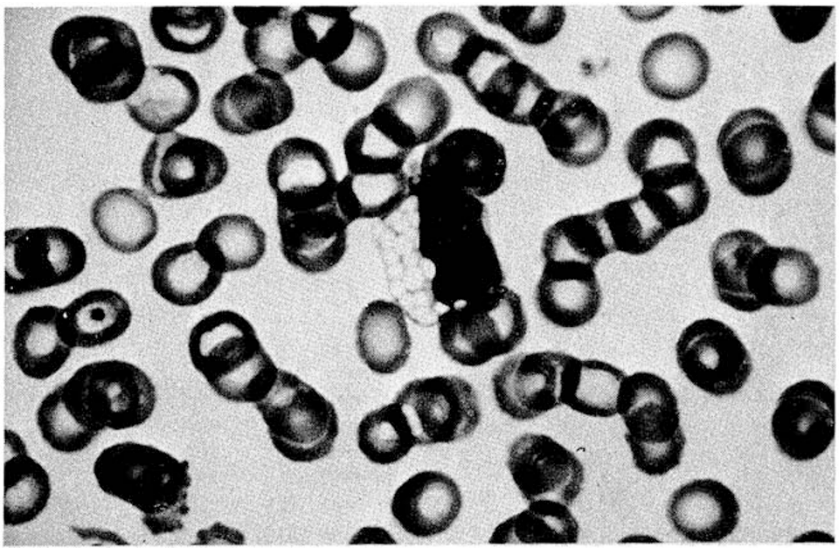

Fig. 2. Peripheral blood lymphocyte containing numerous clear, sharply defined cytoplasmic vacuoles (Wright's-Giemsa stain, × 1000).

appeared to be a fusion of the vacuoles and a loss of their clear outlines. The macrophages in which several vacuoles appeared fused usually had a rim of sharply defined vacuoles at the periphery of the cytoplasm.

All histochemical stains were negative except for periodic acid-Schiff which reacted positively only at the junction of the fused vacuoles in bone marrow macrophages. On ultrastructural examination, all marrow macrophages exhibited vacuolar structures, most containing amorphous, osmophilic inclusion materials as shown in Figure 3, *B* and *C*.

Examination of hepatic tissue obtained by percutaneous biopsy revealed enlarged hepatocytes with multiple clear, periodic acid-Schiff negative vacuoles in their cytoplasm. Ultrastructural examination revealed numerous single membrane-bound vacuolar structures, presumably lysosomes, in the cytoplasm of almost all hepatic cells; these structures contained an abnormally accumulated, amorphous matrix-material with occasional membranous and/or filamentous structures.

A complete neuropathologic examination was performed within 1 hr after death. The brain and spinal cord were not remarkable on gross examination. Histologic examination of the central nervous system, however, revealed striking neuronal storage in almost all neurons. The affected neurons were ballooned in varying degrees with either watery or finely granular cytoplasm as shown in Figure 4. The dorsal spinal, trigeminal, and paravertebral sympathetic ganglion cells were similarly affected. On ultrastructural examination, the cytoplasm of the affected neurons was often packed with storage vacuoles which were limited by a single membrane (Fig. 5). The storage vacuoles were remarkably electron-lucent or watery, and contained sparsely dispersed reticulogranular material, a few fine vacuoles, and varying amounts of fine fibrils in stacks. The size and contents of the vacuoles varied considerably in different

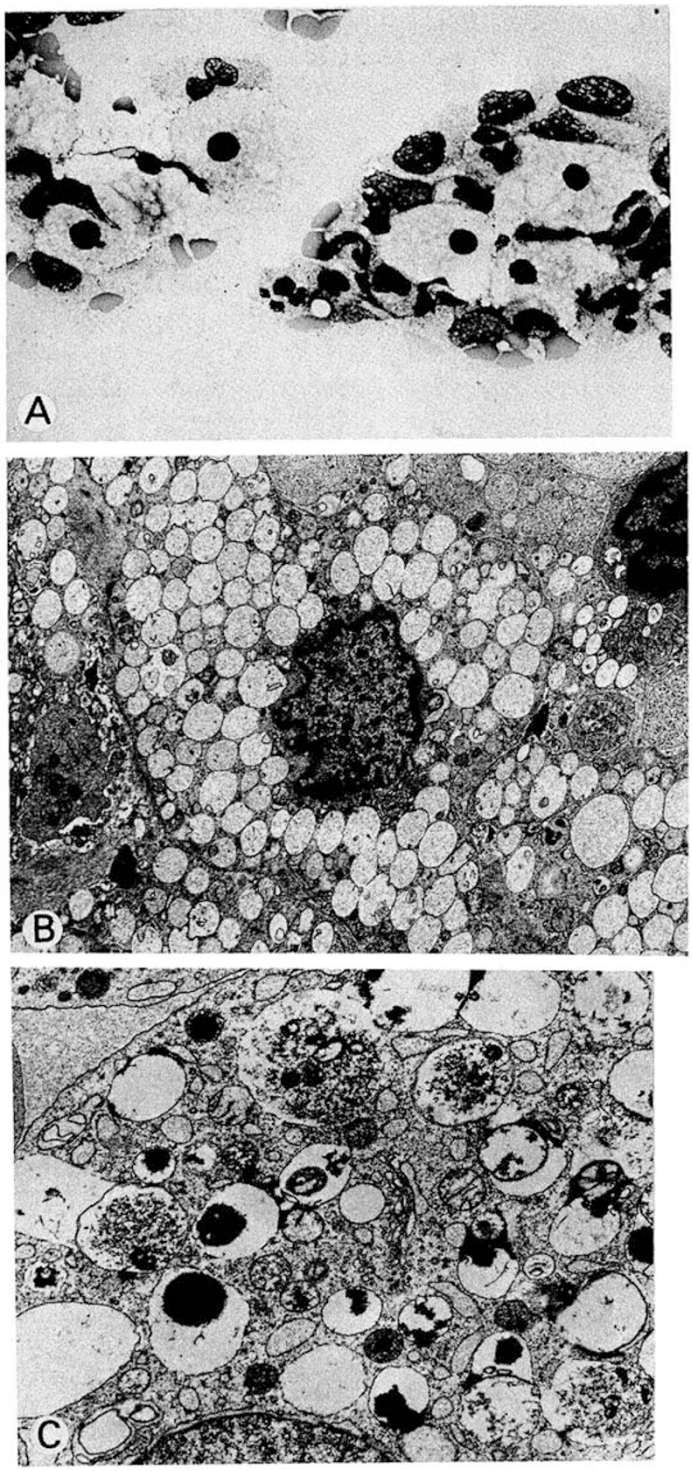

Fig. 3. *A*: bone marrow smear showing numerous foamy macrophages (Wright's-Giemsa stain, × 400). *B*: electron photomicrograph of a bone marrow macrophage showing numerous cytoplasmic vacuoles, a few containing some amorphous material (uranyl acetate and lead citrate, × 5,600). *C*: electron photomicrograph showing abundant amorphous debris in cytoplasmic vacuolar structures in a bone marrow macrophage (× 13,800).

groups of neurons and the vacuoles were often fused or confluent. Detailed description of the pathologic findings will be reported elsewhere (44).

IMMUNOLOGIC STUDIES

The results of the neutrophil function evaluations of *homozygote 1*, including chemotaxis, phagocytosis, and bacterial killing and NBT reduction studies are summarized in Table 3. A profound defect in leukocyte response to chemotactic attraction was observed, whereas the mechanism for random migration was intact. Phagocytosis, as measured by bacterial killing studies, revealed slower phagocytosis by the patient's neutrophils than by control neutrophils. The NBT response in unstimulated neutrophils was normal and there was a normal increase in the NBT reduction on exposure to endotoxin (Table 3). Lymphocyte studies revealed 58% B cells and 42% T cells. Lymphocyte transformation studies showed a 20% of normal response to purified phytohemagglutinin and a 25% of normal response to concanavalin A. In addition, the levels of IgG and IgE were slightly lower than the lower limit of the respective normal ranges for age.

BIOCHEMICAL STUDIES

Effect of pH and Substrate Concentration. The effect of pH on total α-mannosidase activities in leukocytes and liver extracts from normal individuals revealed an optimum of pH 4.4 in both sources, in agreement with previously reported values (3, 20, 29, 37). In contrast, a marked deficiency of activity at pH 3.5–5.0 was observed in these sources from *homozygote 1* with mannosidosis.

Figure 6*A* shows the relationship between substrate concentration and α-mannosidase activities, assayed at pH 4.4, in leukocyte and liver extracts from a normal individual and *homozygote 1* with mannosidosis. Using the synthetic substrate, 4-methylumbelliferyl-α-D-mannopyranoside, the acidic α-mannosidase activity was saturated at a substrate concentration of about 2.5 mM in both sources from the normal individual. A striking finding was the presence of detectable levels of acidic α-mannosidase activity in these sources from the homozygote with mannosidosis which could not be saturated within the solubility range of the substrate. Apparent K_m values for α-mannosidase activity as determined from Lineweaver-Burk plots were 1.1 mM for normal liver (Fig. 6*B*) and 0.9 mM for normal leukocytes (Fig. 6*C*). In contrast, the apparent K_m values in the homozygote with mannosidosis were extremely difficult to calculate; the apparent K_m was estimated at 15.4 mM in liver extracts.

Effect of Inhibitors. To rule out the possible existence of endogenous inhibitors of acidic α-mannosidase activity in *homozygote 1* with mannosidosis, the acidic α-mannosidase activity in 1:1 mixtures of both liver and leukocyte extracts from normal

Table 3. *Polymorphonuclear function studies*

	Homozygote 1	Control
Chemotaxis (chemotactic index[1])		
Random migration	9	9
Response to chemotactic attractant	15	102
Phagocytosis and bacterial killing[2] (no. of colonies)		
Staphylococcus aureus		
0 min	650	640
30 min	710	170
60 min	290	54
120 min	120	18
Escherichia coli		
0 min	1,000	960
30 min	180	38
60 min	80	56
120 min	52	2
Nitroblue tetrazolium reduction (% cells with blue formazan)		
Unstimulated	10	9
Stimulated with endotoxin	27	24

[1] The chemotactic index was calculated as described in *Materials and Methods*.

[2] The bacteria-neutrophil ratio was 1:1.

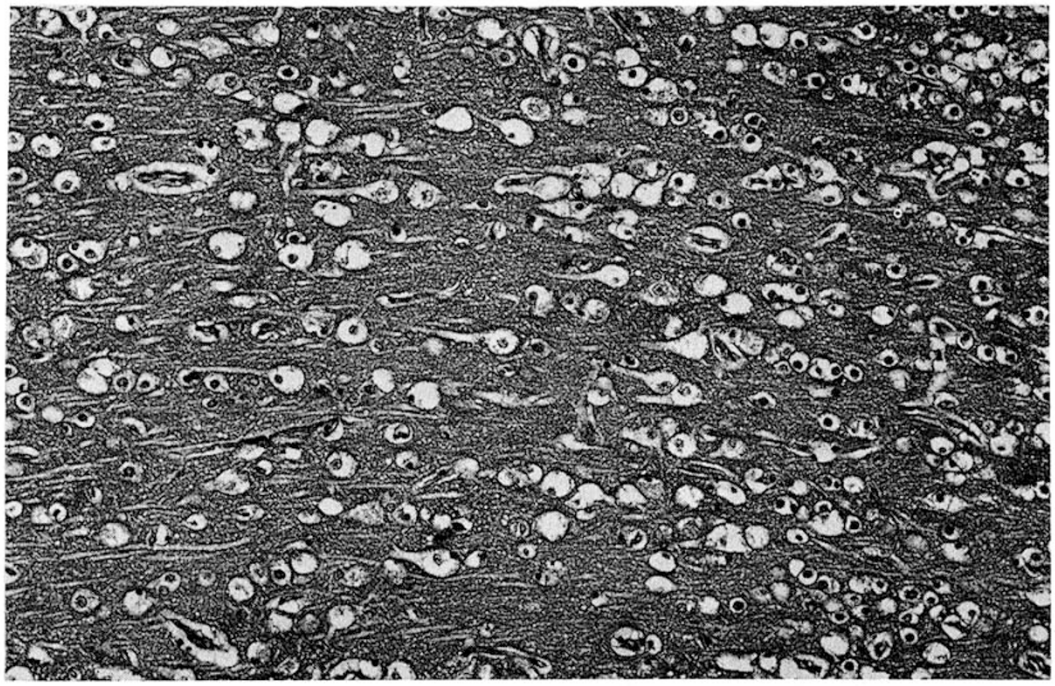

Fig. 4. Cerebral cortex: all of the neurons are ballooned and their cytoplasm is watery or clear (azocarmine stain, × 160).

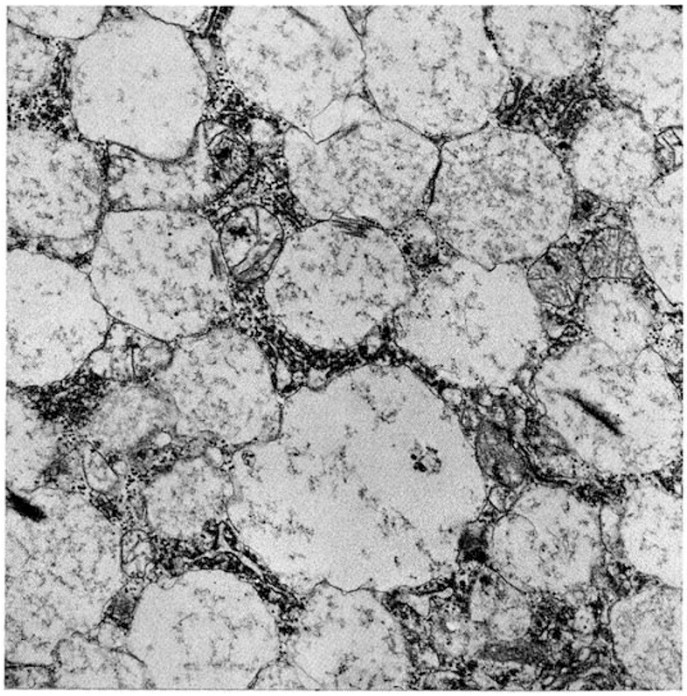

Fig. 5. Ventral horn cell of the spinal cord. The cytoplasm is packed with electron-lucent, single membrane-bound vacuoles which contain fine reticulogranular material and occasional stacks of fine fibrils (uranyl acetate and lead citrate, × 17,000).

individuals and the homozygote was determined. The observed activity in each mixture approximated the expected average of the activities in the normal and enzyme-deficient sources.

Effect of $ZnSO_4$, $CoSO_4$, and $MnCl_2$. Figure 7*A* shows the differential effects of increasing concentrations of zinc and cobalt ions on the acidic α-mannosidase activity in liver extracts from *homozygote 1* and an age-matched normal individual. Zinc stimulated the acidic activity in normal liver to about 150–190% of initial activity at $ZnSO_4$ concentrations between 0.01 and 5.0 mM with maximal stimulation at 2.5 mM. In contrast, the acidic α-mannosidase activity from the mannosidosis homozygote was only slightly stimulated to approximately 115% of initial activity at all concentrations from 0.01–10.0 mM $ZnSO_4$. Whereas cobalt inhibited acidic α-mannosidase activity in normal liver at all concentrations studied, a marked stimulation of the residual acidic activity in the homozygote was observed at final concentrations from 0.01 mM to 8.0 mM, with maximal stimulation, 280% of initial activity, at 1 mM $CoSO_4$. $MnCl_2$ was found to inhibit the acidic activities in normal and mannosidosis liver extracts; at 5.0 mM, both activities were inhibited to approximately 30% of initial activity.

Figure 7, *B* and *C*, shows the effect of 2.5 mM $ZnSO_4$ and 1.0 mM $CoSO_4$ on the V_{max} and apparent K_m values of the acidic activity in the normal and mannosidosis liver extracts as determined by Lineweaver-Burk plots. Zinc significantly lowered the apparent K_m value of the acidic activity in normal liver (from 1.2 to 0.24 mM), whereas this metallic ion had little effect on the values for mannosidosis hepatic activity (from 15.4 to 12.3 mM). Unlike zinc, cobalt had its major effect on the acidic activity in the mannosidosis liver extract, lowering the apparent K_m from 15.4 to 3.9 mM, whereas the apparent K_m for the normal activity was increased from 1.2 to 1.9 mM.

As an internal control, the endogenous concentrations of zinc and copper in the liver extracts from the homozygote with mannosidosis and normal individuals were determined by atomic absorption spectroscopy. Compared with the concentration in a normal age-matched extract, the levels of zinc were higher in the homozygote, 4.8 and 28.6 μg/g wet weight, respectively. When expressed as the ratio of zinc to copper, the values for the mannosidosis and normal liver extracts were 13.0 and 7.5, respectively.

In leukocytes from all the homozygotes, zinc and cobalt stimulated both the residual acidic and neutral activities as shown in Table 4. In leukocytes from normal individuals the mean acidic activity was stimulated by zinc whereas cobalt was inhibitory; the mean neutral activity was stimulated both by zinc and cobalt ions.

Differential effects of these ions also were observed on the plasma acidic and neutral activities from the homozygotes compared with those from normal individuals. As shown in Table 4, the residual acidic activities from the homozygotes were stimulated by both zinc and cobalt; in contrast, zinc stimulated and cobalt inhibited the mean acidic activity from normal individuals. The

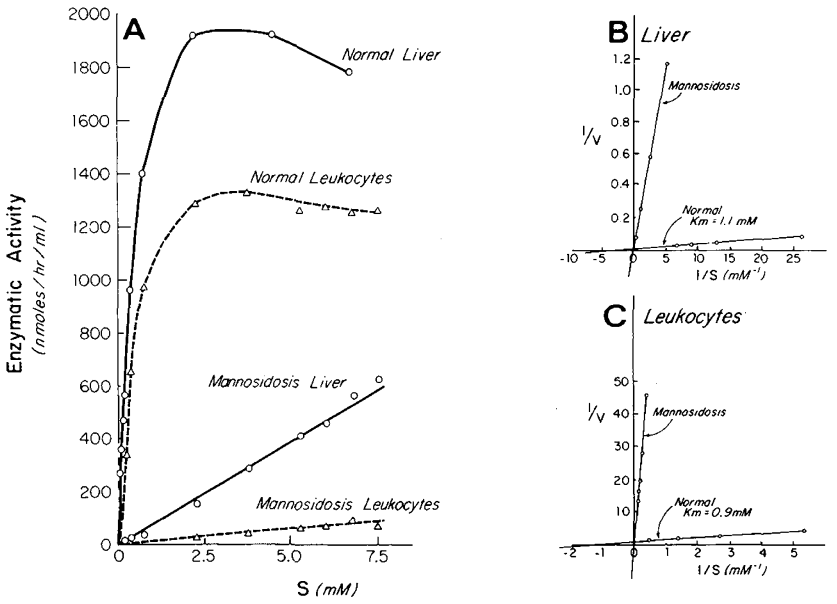

Fig. 6. *A:* effect of substrate concentration on acidic α-mannosidase activities in liver and leukocyte extracts from a homozygote with mannosidosis and a normal individual. Lineweaver-Burk plots of acidic α-mannosidase activities in liver (*B*) and leukocyte extracts (*C*) from indicated sources.

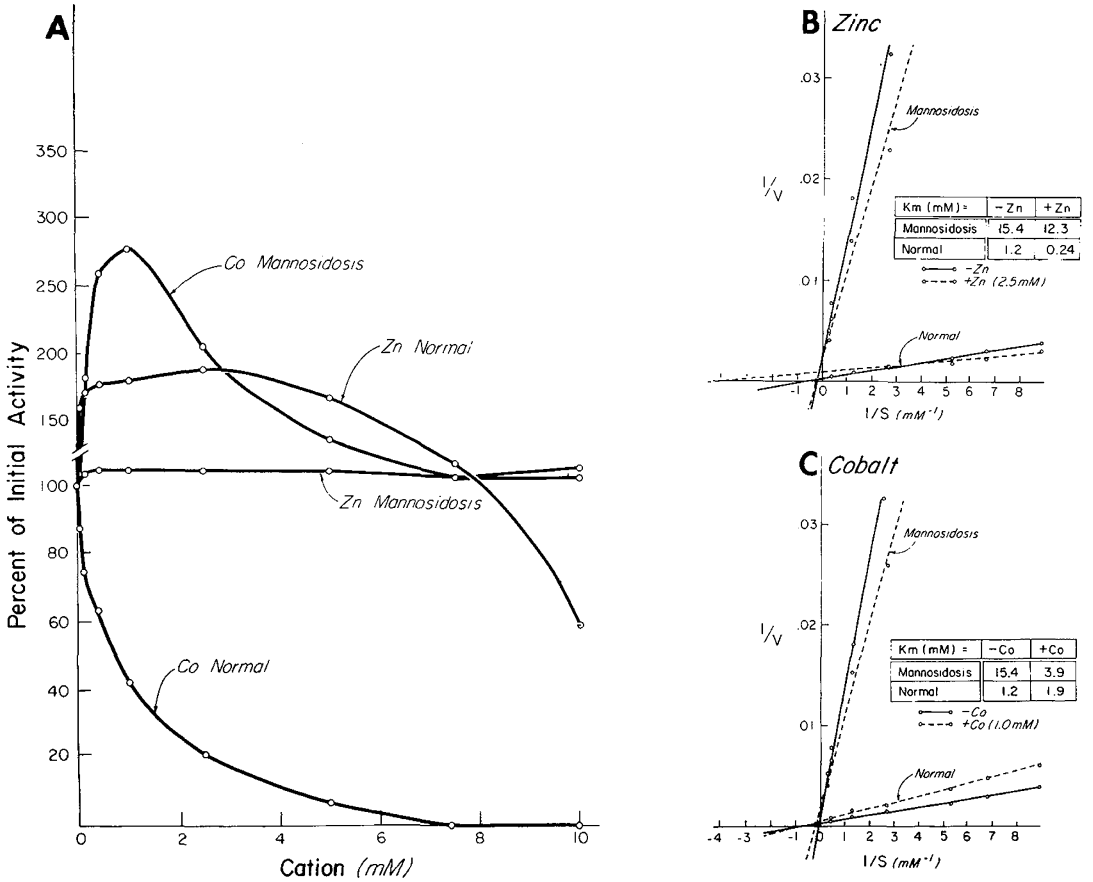

Fig. 7. *A:* effect of $ZnSO_4$ and $CoSO_4$ on acidic α-mannosidase activities from indicated hepatic sources. Lineweaver-Burk plots of acidic α-mannosidase activities in liver extracts from indicated sources in the presence and absence of (*B*) 2.5 mM $ZnSO_4$ and (*C*) 1.0 mM $CoSO_4$.

neutral activities from the homozygotes and normal individuals were essentially unaffected by either cation.

Resolution and Purification of α-Mannosidase Isozymes. Three peaks of α-mannosidase activity, A, B, and C, were observed after chromatography of normal liver extract on DEAE-cellulose as shown in Figure 8. The A and B isozymes were maximally detected at pH 4.4, whereas the C isozyme was most active at pH 6.5; low levels of A and B activity were also detected at pH 6.5. The A isozyme was eluted with the initial buffer wash and the B and C isozymes at 0.06 M and 0.12 M concentrations, respectively, on the linear KCl gradient. In contrast, when a liver extract from *homozygote 1* was subjected to anion exchange chromatography, the activities of the A and B isozymes were detected, but at markedly low levels, whereas the C isozyme was eluted at the

same salt concentration and with activity comparable to that of the normal C isozyme (Fig. 8). However, the residual B activity was eluted as a broad peak in a position between the normal liver B and C isozymes. These findings were consistent with our observations of residual acidic α-mannosidase activities in crude leukocyte and liver extracts which could not be saturated with substrate (Fig. 6).

Further purification of the α-mannosidase isozymes was achieved by differential ammonium sulfate precipitation followed by combined conventional and "oligo-affinity" (concanavalin A-Sepharose) chromatography (11, 22). By these procedures, normal hepatic α-mannosidase A and B isozymes were purified about 150- and 550-fold with yields of 2% and 13%, respectively, based on their initial pH 4.4 activities. The neutral α-mannosidase C was purified about 60-fold with a 31% recovery based on the pH 6.5 activity in the crude extract. Using these methods, the residual α-mannosidase A and B activities from hepatic tissues from *homozygote 1* with mannosidosis were also purified. The partially purified residual activities had apparent K_m values which were more than 50 times greater than those observed for the partially purified normal A and B isozymes; these residual activities were stimulated by cobalt and manganese cations which inhibited the activities of the normal A and B isozymes. The C isozymes purified from both sources had identical kinetic properties and similar responses to metal ions. The characterization of the physical and kinetic properties of the partially purified normal and residual activities will be reported elsewhere (22).

Electrophoresis of α-Mannosidase Isozymes. Figure 9 shows that electrophoresis on cellulose acetate gel at pH 7.3 distinctly separated the α-mannosidase A, B, and C isozymes which were

Table 4. *Effect of zinc and cobalt on acidic and neutral α-mannosidase activities in leukocytes and plasma from homozygotes with mannosidosis and normal individuals*

Source	Leukocyte initial activity, nmol/hr/mg protein	Leukocytes, % of initial activity			Plasma, initial activity, nmol/hr/ml	Plasma, % of initial activity		
		Zn	Co	Zn + Co		Zn	Co	Zn + Co
Effect on residual acidic α-mannosidase activities[1]								
Homozygote 1	0.27	389	785	389	0.10	332	200	119
Homozygote 2	0.45	251	280	256	0.30	443	167	192
Homozygote 3	0.14	476	676	186	0.18	639	264	264
Homozygote 4	0.26	273	393	195	0.20	390	225	212
Homozygote 5	0.15	450	927	433	0.15	300	200	167
Normal mean	59.2	165	78.9	131	12.3	157	47.7	119
(Range), $n = 3$	(26.7-121)	(149-190)	(52.0-108)	(121-150)	(9.5-17.0)	(138-168)	(38.5-55.0)	(97.1-147)
Effect on neutral α-mannosidase activity[2]								
Homozygote 1	1.92	183	1,083	146	36.3	106	95.0	74.4
Homozygote 2	1.41	326	1,960	390	35.5	104	107	76.1
Homozygote 3	1.82	299	2,000	242	35.5	118	122	80.3
Homozygote 4	2.47	239	1,220	251	33.0	103	108	80.3
Homozygote 5	1.57	344	2,340	484	34.5	108	108	84.1
Normal mean	12.9	203	483	173	31.0	115	117	75.8
(Range), $n = 3$	(6.9-23.5)	(182-241)	(239-672)	(139-214)	(28.5-38.5)	(110-123)	(98.2-139)	(65.0-83.3)

[1] Enzymatic activity assayed at pH 4.4 in the presence of no metal ions, 2.5 mM $ZnSO_4$, 1.0 mM $CoSO_4$, and 2.5 mM $ZnSO_4$ + 1.0 mM $CoSO_4$.
[2] Enzymatic activity assayed at pH 6.0 in the presence of no metal ions, 2.5 mM $ZnSO_4$, 1.0 mM $CoSO_4$, and 2.5 mM $ZnSO_4$ + 1.0 mM $CoSO_4$.

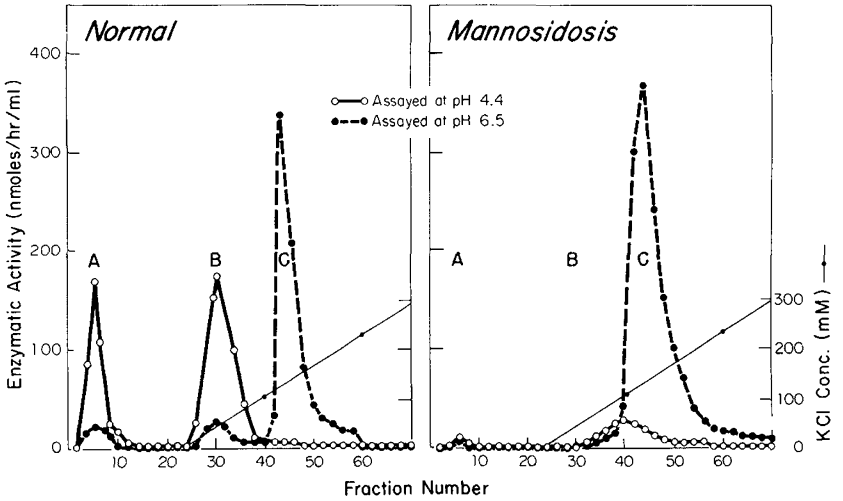

Fig. 8. Elution profile from the DEAE-cellulose chromatography of α-mannosidase A, B, and C isozymes in normal and mannosidosis liver. For details, see *Materials and Methods*.

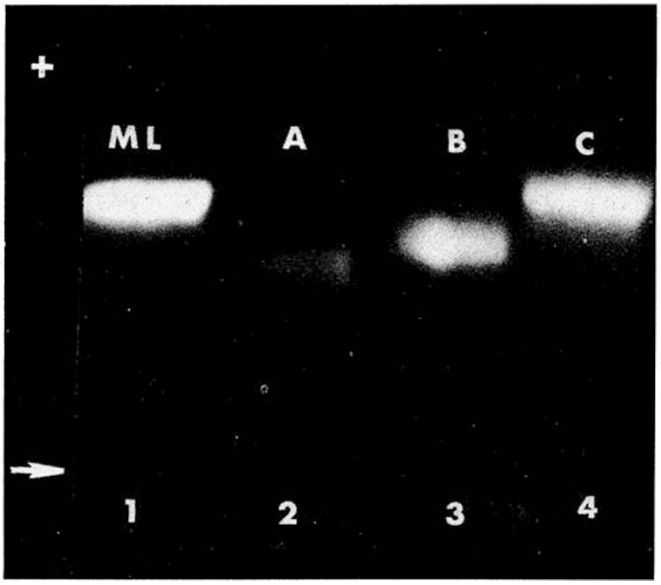

Fig. 9. Fluorescent bands of enzymatic activity after electrophoresis on cellulose acetate gel and staining with 4-methylumbelliferyl-α-D-mannopyranoside. *Lane 1:* liver from the homozygote with mannosidosis (1:3 w/v extract); *lanes 2, 3,* and *4:* partially purified normal human liver α-mannosidase A, B, and C isozymes, respectively. *Arrow:* point of application.

partially purified from normal human liver. The B isozyme migrated between the A isozyme and the more electronegative C isozyme. In a liver extract from *homozygote 1* with mannosidosis, a single band of activity was visible which was comparable in mobility to partially purified, normal hepatic α-mannosidase C; the levels of residual A and B activities were not sufficient for detection in this system.

DISCUSSION

Since the original clinical and biochemical delineation of mannosidosis by Öckerman (33) in 1967, only 18 enzymatically diagnosed homozygotes have been reported in the world literature. Presumably, many patients have been misdiagnosed since the phenotypic features, as well as hematologic and radiologic findings, resemble those of the mucopolysaccharidoses. Thus, suspect patients should be confirmed biochemically. Our studies demonstrate that the enzymatic diagnosis of suspect homozygotes can be made reliably using plasma, isolated leukocytes, or cultured skin fibroblasts assayed carefully at the appropriate acidic pH (Table 2). The recent demonstration that total urinary mannose may be unreliable because of the wide variability in glycoprotein excretion in normal individuals (46) underscores the necessity for measurement of acidic α-mannosidase activities rather than the levels of urinary mannose (32, 33).

Unfortunately, the enzymatic identification of heterozygotes for mannosidosis has been difficult (1, 14), as evidenced by our results (Table 2). Even when the ratio of α-mannosidase to total β-hexosaminidase activities at pH 4.4 is calculated, the values do not always discriminate heterozygotes as previously suggested (29). Thus, multiple determinations of acidic α-mannosidase activity in several different sources may be required for heterozygote identification. The difficulty in heterozygote detection is unusual among the lysosomal storage diseases and suggests a unique interaction of the mutant and active gene products or associated cofactors *in vivo*.

The immunologic studies of the mannosidosis homozygote revealed significant abnormalities of neutrophil function; these included a depressed chemotactic responsiveness and impaired phagocytosis of bacteria. However, endotoxin-stimulated neutrophils showed normal NBT reduction, suggesting normal oxidative responsiveness in these cells. In addition, there were depressed lymphocyte responses to T cell mitogens and phytohemagglutinin and concanavalin A. These findings suggest defective function of leukocyte plasma membrane-mediated processes.

It is tempting to speculate that the leukocyte abnormalities result from abnormal mannose catabolism. Partially degraded oligosaccharides, glycopeptides, and glycoproteins with terminal α-D-mannose residues may be bound to the leukocyte plasma membranes as well as accumulated in their lysosomes. In addition, circulating mannose-rich substrates may interfere with leukocyte function. These abnormal metabolites may lead to impaired neutrophil responses to chemotactic attractants, impaired neutrophil bactericidal mechanisms, and decreased lymphocyte binding of phytomitogens by altering leukocyte membrane surface structure or charge (16). The chemical alteration of the receptors for these membrane-recognition processes may account for the observed abnormalities of leukocyte function and the increased susceptibility to recurrent bacterial and viral infections which characterize the clinical course of this disease.

A most intriguing aspect of our kinetic studies was the demonstration of residual acidic α-mannosidase activity in crude liver and leukocyte extracts from *homozygote 1* with mannosidosis. Using the synthetic substrate, 4-methylumbelliferyl-α-D-mannopyranoside, this acidic activity could not be saturated in either enzyme source. The apparent K_m values for the residual hepatic and leukocytic activities, estimated from Lineweaver-Burk plots (Fig. 6), were more than 15 and 20 times greater than those calculated for the respective normal sources. These results are supported by our preliminary observations that the K_m values for the partially purified residual A and B isozymes were more than 50 times greater than those calculated for the partially purified normal hepatic isozymes (11, 22). In agreement with these findings, a residual acidic α-mannosidase activity with altered kinetics and increased heat lability has been recently described in crude extracts of cultured skin fibroblasts from four unrelated homozygotes with mannosidosis (5).

The effects of zinc (stimulation) and cobalt (inhibition) on acidic α-mannosidase activity in normal human liver extracts (10, 37) and mannosidosis extracts (21) have been reported recently. In addition, the specific activity of α-mannosidase at pH 5.0 has been correlated directly with the zinc content of various rat tissues (43) and α-mannosidase purified to homogeneity from the jack-bean has been shown to be a zinc metalloenzyme (42), indicating the importance of zinc to this enzymatic activity. Our studies also demonstrated a stimulatory effect of zinc and an inhibitory effect of cobalt ions on acid α-mannosidase activity in normal liver extracts. In the presence of 2.5 mM $ZnSO_4$ and 1.0 mM $CoSO_4$, the V_{max} and apparent K_m values estimated for the acidic activity in normal liver extracts from Lineweaver-Burk plots were altered; the apparent K_m values were decreased in the presence of zinc and slightly increased when cobalt was present in the crude extract. However, a differential effect was observed when these metal ions were incubated in the liver extracts from the homozygote with mannosidosis. The apparent K_m values for the residual acidic activity were decreased slightly with zinc and markedly with cobalt. In contrast to the significant effect of zinc on the kinetics of normal acidic activity, the minimal effect of zinc on the residual acidic activity may be related to the physical properties of the mutant isozymes (*e.g.*, metal binding capacity) or possibly to the presence of higher endogeneous concentrations of zinc in the extract as well as in other sources from the mannosidosis homozygote.

Zinc and cobalt markedly stimulated the residual acidic activities in the plasma and both the residual acidic and neutral activities in the leukocytes from the homozygotes (Tables 2 and 4). These *in vitro* studies demonstrated that 1.0 mM cobalt enhanced the residual acidic and neutral activities in leukocytes from homozygotes more than the 2.5 mM zinc concentration. The cobalt

enhancement of this residual acidic activity in the homozygote (compared with the inhibiting effect of cobalt on the acidic activity in leukocytes from normal individuals) may be an assay artifact, presumably due to partial activity detected at pH 4.4 of the neutral component, which was markedly stimulated (10-fold) by cobalt. However, this effect of cobalt was not observed on the neutral activity in plasma from homozygotes and normal individuals (Table 4). It is intriguing to note that the molecular defect in mannosidosis altered both the acidic and neutral activities in leukocytes, but only the acidic activities in plasma and hepatic tissues from homozygotes, whereas the obligate heterozygotes had completely normal levels of neutral leukocytic activity (Table 2). It is possible that the residual acidic activity in patients with mannosidosis might be increased following the administration of appropriate trace metals based on the *in vitro* characterization of the specific response of a particular family's mutation to metal ion supplementation. The effectiveness of this therapeutic approach should be evaluated in Angus calves with mannosidosis, an animal model of the human enzymatic deficiency disease (36) and in cultured skin fibroblasts obtained from the patient before *in vivo* trials.

Three major components of α-mannosidase activity in normal human liver were resolved by ion exchange chromatography on DEAE-cellulose (Fig. 8) and electrophoresis on cellulose acetate gels (Fig. 9) in agreement with previously reported findings (9, 10, 29, 37, 38, 40). Two isozymes, α-mannosidases A and B, had optimal activities at pH 4.4 and the third isozyme, α-mannosidase C, had an optimum at pH 6.5. Differential ammonium sulfate precipitation followed by conventional and affinity chromatographic (40) procedures has already resulted in significant purification of these isozymes from normal human liver for subsequent physical and kinetic characterization (11, 22).

Electrophoresis of the liver extract from *homozygote 1* with mannosidosis revealed only one band of activity which coelectrophoresed with the α-mannosidase C isozyme partially purified from normal liver; similar results have been previously reported in tissues (38) and cultured skin fibroblasts (6, 45) from homozygotes with mannosidosis. Although no residual acidic activities were detected electrophoretically in the mannosidosis liver extract, ion exchange chromatography revealed the presence of residual acidic activities; the residual A isozyme was eluted in a position corresponding to that of normal α-mannosidase A whereas the residual B activity was eluted at a slightly more electronegative position than that of normal B isozyme.

Our studies of the acidic α-mannosidase isozymes in the crude tissue extracts and in the partially purified preparations have provided intriguing data concerning the residual acidic activities in tissues from the homozygote with mannosidosis. These findings suggest that the enzymatic defect in *patient 1* may have resulted from a missense mutation in the structural gene coding for the acidic α-mannosidase activities. Apparently, this mutation resulted in a gene product which rendered the enzyme protein for the B isozyme more electronegative and altered the kinetic and metal binding properties of both acidic isozymes. Although this hypothesis is appealing, clearly, the implications of these studies, particularly with crude extracts, require further documentation.

From a clinical perspective, mannosidosis appears to present in at least two major forms: type I homozygotes (*e.g., homozygote 1*) have severe disease with hepatosplenomegaly, severe recurrent infections, and early demise whereas type II homozygotes (*e.g., homozygotes 2–5* and *cases 15–17*, Table 1) have a milder course characterized primarily by hearing loss, mental retardation, milder dysostosis multiplex, and survival into adulthood. Indeed, careful characterization of the residual activities purified from various sources from type I and type II homozygotes may provide insight into the marked clinical differences among these patients (Table 1) which suggest heterogeneity of the genetic defects in different families with mannosidosis. Further investigation of the kinetic, physical, and immunologic properties of highly purified α-mannosidase isozymes with natural as well as synthetic substrates will be required to elucidate the molecular and genetic interrelationships among the normal isozymes and the molecular pathology of the residual activities in mannosidosis.

SUMMARY

The clinical and laboratory features of a 3½-year-old patient with mannosidosis are described and compared with those of all the cases reported in the world's literature. The clinical diagnosis of our patient was established by the observation of vacuolated peripheral lymphocytes, foamy bone marrow macrophages, lysosomal inclusions in hepatic cells obtained by percutaneous biopsy, and the deficient activities of α-D-mannosidases A and B in the plasma, isolated peripheral leukocytes, and cultured skin fibroblasts. Immunologic investigations revealed leukocyte functional defects which presumably account for the susceptibility to intercurrent infections which characterize the clinical course of this disorder. An intriguing finding was the presence of residual hepatic α-D-mannosidase A and B activities which were less than 5% of normal mean hepatic activities; the residual activities were stimulated more than 2½-fold when assayed in the presence of 1.0 mM $CoSO_4$ *in vitro*, suggesting that cobalt and/or zinc supplementation might be of therapeutic value in patients with this enzymatic defect.

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Airway resistance
specific airway resistance
thoracic gas volume

A Simplified Approach to the Measurement of Specific Airway Resistance

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Extract

A simple algebraic manipulation of known formulas leads to a direct expression for the specific airway resistance (SR_{aw}) which precludes separate measurements of the airway resistance (R_{aw}) and the thoracic gas volume (TGV). The equation is: $SR_{aw} = tg\ \beta\ (P_{Bar} - P_{H_2O})\ e_2$, in which tg β stands for the relation between the plethysmographic box volume and pneumotachograph flow fluctuations; $P_{Bar} - P_{H_2O}$ is the barometric pressure corrected for water vapor pressure at body temperature, and e_2 is a calibration for graphical units. Thanks to this new formula SR_{aw} can now be easily measured with great precision and quickly calculated, even in the case of those children (Table 1) with whom this was not previously possible. No further cooperation is needed than breathing at a