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N G Beratis, … , G U LaBadie, K Hirschhorn

J Clin Invest. 1978;62(6):1264-1274. https://doi.org/10.1172/JCI109247.

Research Article

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Characterization of the Molecular Defect in Infantile and Adult Acid α-Glucosidase Deficiency Fibroblasts

NICHOLAS G. BERATIS, GUNDULA U. LABADIE, and KURT HIRSCHHORN, *Division of Medical Genetics, Department of Pediatrics, Mount Sinai School of Medicine of the City University of New York, New York 10029, and Department of Human Genetics, New York State Institute for Basic Research in Mental Retardation, Staten Island, New York 10314*

ABSTRACT Different clinical expressions of acid α-glucosidase deficiency have been described. The present study was undertaken to investigate the basic metabolic defect in the infantile and adult forms of the disease. Acid α-glucosidase (EC 3.2.1.20) was purified from normal and from adult acid α-glucosidase deficiency fibroblasts. The pH optimum; Michaelis constant; electrophoretic mobility in starch; thermal denaturation at pH 4.0 and 7.0; and inhibition by turanose, α-methylglucoside, and trehalose were the same in purified enzyme from normal and mutant cells. Placental acid α-glucosidase was purified to, or near, homogeneity. Monospecific antibodies raised against the enzyme in each of three enzyme peaks obtained from the last purification step were found to cross-react with the enzyme of all three peaks, and with purified, normal fibroblast enzyme. Cross-reacting material (CRM) also was identified in fibroblast lysates from normal subjects and from both forms of acid α-glucosidase deficiency. The amount of CRM in the adult form appeared to be significantly less than in normal cells or cells from the infantile form. Enzyme activity was demonstrated in the immune complexes of the normal and adult acid α-glucosidase deficiency fibroblasts, but not of the infantile form. Competition for antibody binding sites was observed between normal and both types of mutant enzyme. The findings indicate that this case of infantile acid α-glucosidase deficiency is the result of a structural gene mutation which causes the synthesis of a catalytically inactive (CRM-positive) enzyme protein. It appears that in the adult form, the mutation causes a reduction in the amount of the enzyme protein present in the cells.

Part of this paper was presented at the Annual Meeting of the American Society of Human Genetics, 19–22 October 1977, San Diego, Calif., and in abstract form: 1977. *Am. J. Hum. Genet.* **24**: 22A.

Received for publication 15 February 1978 and in revised form 23 August 1978.

INTRODUCTION

Deficiency of the lysosomal enzyme acid α-glucosidase (EC 3.2.1.20) is the basic metabolic defect in glycogen storage disease type II (1). At least three different clinical entities have been described in patients with acid α-glucosidase deficiency. In the infantile form (Pompe's disease), massive cardiomegaly, severe hypotonia, hepatomegaly, and muscular weakness may be present at birth, or develop during the first weeks of life; most infants die by the age of 1 yr as a result of cardiorespiratory failure (2). The late infantile or juvenile form starts with slowly progressive muscular weakness during the first years of life, and patients die during the first or the second decade (3, 4). Cardiomegaly has been found in some of these patients. The clinical manifestations of the adult form are limited to skeletal muscle. Generalized weakness and wasting of the muscles appear in the second to the fourth decade of life and death usually occurs in the forties (5, 6).

Absence of both acid α-1,4-glucosidase and acid α-1,6-glucosidase activities have been found in the infantile form of acid α-glucosidase deficiency (7). However, with maltose as substrate, a residual α-1,4-glucosidase activity has been observed in leukocytes (6, 8), muscle (5, 6), urine (9), and cultured skin fibroblasts (10) of the adult form of the disease. By use of antibodies made against purified human placental acid α-glucosidase, lack (11–13) or severe reduction (14) of cross-reacting material has been observed in the liver and the fibroblasts of patients with glycogenosis type II. Considerable α-glucosidase activity at pH 4.0 has been found in the kidney of patients with the infantile form of Pompe's disease (15). No inhibition of this apparent acid α-glucosidase activity, however, was observed by antiserum against purified liver acid α-glucosidase (16).

The present study describes the purification of acid α-glucosidase from human placenta, from normal cultured skin fibroblasts, and from skin fibroblasts de-

 J. Clin. Invest. © *The American Society for Clinical Investigation, Inc.*, *0021-9738/78/1201-1264* $1.00
Volume 62 December 1978 1264–1274

rived from a patient with the adult form of acid α-glucosidase deficiency. The kinetics of the purified enzymes and the immunologic properties of the enzyme protein from patients with both the infantile and the adult forms of acid α-glucosidase deficiency are reported.

METHODS

Skin fibroblasts

Skin biopsies were obtained from a patient with the infantile form of acid α-glucosidase deficiency and a patient with the adult form of the disease. Cutured fibroblasts derived from normal subjects were used as controls. The biopsies were obtained with informed consent from adults, or, in the case of children, with the informed consent of their parents. The patient with the infantile acid α-glucosidase deficiency developed severe hypotonia, which started at the age of 2 mo. At the age of 4 mo he developed congestive heart failure with cardiomegaly. He died at the age of 5 mo. The patient with the adult form was studied when she was 46 yr old. She demonstrated progressive muscle weakness and muscle wasting since the age of 30 yr. She died at the age of 47 yr because of respiratory failure. Skin fibroblasts were cultured and harvested at early confluence as described (17). Cells were washed three times with 0.9% saline, and stored at −80°C until used. Fibroblasts were lysed in distilled water by seven cycles of rapid freezing and thawing. The lysates were centrifuged at 1,800 *g* for 5 min and the supernatant fractions were used for further studies.

Enzyme assays

Acid α-glucosidase activity was measured at pH 4.0. The reaction mixture for the assay with maltose as substrate contained 5–20 μg of cellular protein, and 40 μl 20 mM maltose in 0.05 M (in reference to phosphate) citrate-phosphate buffer, pH 4.0, in a total volume of 50 μl. After incubation for 60 min at 37°C, the reaction was stopped with 0.1 M Tris-HCl buffer, pH 7.0. Glucose released from maltose was measured enzymatically with Glucostat Special (Worthington Biochemical Corp., Freehold, N. J.) which was added to the Tris-HCl buffer used to terminate the reaction. Neutral α-glucosidase was measured at pH 6.3 with the same amount of fibroblast protein and the same substrate concentration as for the acid isozyme.

The reaction mixture for measurement of acid α-glucosidase activity with glycogen as substrate contained 5–25 μg of fibroblast protein, and 40 μl 0.05 M (in reference to phosphate) citrate-phosphate buffer, pH 4.0, to which was added 50 mg glycogen/ml (2 mg/assay). The reaction was allowed to continue for 2 h at 37°C and the glucose released was measured as above.

Fluorometric determination of α-glucosidase activity was performed with 4-methylumbelliferyl-α-D-glucopyranoside (Research Products International Corp., Elk Grove Village, Ill.) as substrate. Protein was measured according to Lowry et al. (18). All determinations were performed in duplicate. One enzyme unit was defined as the activity which liberated 1 nmol of glucose/h at 37°C.

Acid α-glucosidase purification

Placental enzyme. The placental tissue was homogenized in a Virtis Homogenizer (Virtis Co., Inc., Gardiner, N. Y.) with a double amount (wt/vol) of water. The homogenate was centrifuged at 36,000 *g* for 20 min and the pH of the supernate was adjusted to 5.0 with 0.1 N HCl. Subsequently CM-Sephadex C-50 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) was added (4 g/100 g of placenta), and the mixture was filtered in a Buchler funnel (Buchler Instruments, Div. Searle Diagnostics Inc., Fort Lee, N. J.). The pH of the filtrate was adjusted to 4.0 with 1 N HCl and centrifuged at 650 *g* for 20 min. Acid α-glucosidase was precipitated with 50% ammonium sulfate and stored overnight at 4°C. The precipitate was collected by centrifugation at 650 *g* for 20 min, the pellet was resuspended in a solution of 25 mM NaCl that contained 1 mM EDTA, pH 5.0, and dialyzed against the same solution. The dialysate was centrifuged at 2,000 *g*, and the supernate was concentrated on an Amicon PM-10 filter (Amicon Corp., Lexington, Mass.). Acid α-glucosidase was purified further on a Sephadex G-100 (Pharmacia Fine Chemicals) column essentially as described by Auricchio et al. (19). A 4.5 × 45-cm column was used to increase the flow rate. The pH of the NaCl-EDTA solution used for the elution of the column was adjusted to 5.0. Human acid α-glucosidase binds to Sephadex (11) and it was released from the Sephadex with 0.25% maltose added to the eluant. 10-ml fractions were collected at a flow rate of 30 ml/h. Fractions of the enzyme peak eluted with maltose were pooled and concentrated by ultrafiltration (Amicon PM-10). The eluting buffer was removed from the solution that contained the enzyme by washing the eluate three times with 5 mM Tris-2 mM EDTA-5 mm 2-mercaptoethanol, pH 7.0, on the same Amicon filter. The concentrated enzyme preparation was applied to a 1.5 × 30-cm DEAE-cellulose (Pharmacia Fine Chemicals) column equilibrated with the buffer used to wash the pooled fractions. The enzyme was eluted from the column with a 700-ml linear NaCl gradient from 0 to 1 M. Fractions of the eluate that contained acid α-glucosidase activity were washed with 0.9% NaCl, concentrated by ultrafiltration, and stored at −85°C. No additional protein was necessary to stabilize the enzyme. All purification steps were carried out at 4°C.

Cultured skin fibroblast enzyme. Fibroblasts derived from the patient with the adult form of acid α-glucosidase deficiency and a normal subject were cultivated in 840-cm^2 roller bottles and were used for purification of acid α-glucosidase. Cells obtained from 40 bottles were lysed in distilled water, the lysate was centrifuged at 12,000 *g* for 10 min, and the supernatant fraction was applied to the same Sephadex G-100 column used for the purification of the placental enzyme. A total of 165 and 145 mg of protein from the normal and mutant fibroblasts, respectively, was applied to the column. Elution of the enzyme, washing and concentration of the eluate were performed as for the placental enzyme. Because the protein concentration in the concentrated preparation which contained the purified enzyme was very low, the fibroblast enzyme was stabilized by the addition of bovine albumin in a final concentration of 0.8 mg/ml.

Starch and polyacrylamide gel electrophoresis

Horizontal starch gel electrophoresis was performed at 5 V/cm with a 0.1-M Na_2HPO_4/NaH_2PO_4 gel buffer and a 0.2-M Na_2HPO_4/NaH_2PO_4 bridge buffer, both at pH 6.5, as described by Swallow et al. (20). The isozymes of α-glucosidase were visualized with 2 mM 4-methylumbelliferyl-α-D-glucopyranoside in 0.1 M (in reference to phosphate) citrate-phosphate buffer, at pH 4.0 and 6.3.

Polyacrylamide disc-gel electrophoresis at pH 4.3 was carried out according to the procedure of Reisfeld et al. (21). Electrophoresis was for 6 h at 5 mA per tube, in 0.5 × 7.0-cm 7% acrylamide gels. Acid α-glucosidase was located after

electrophoresis with the fluorogenic substrate at pH 4.0, as on the starch gels. The gels were stained for protein with 0.2% Coomassie Blue stain in 10% acetic acid (vol/vol) and 50% methanol (vol/vol).

Antibody production, immunodiffusion, immunotitration

Acid α-glucosidase present in the three enzyme peaks obtained from the DEAE-cellulose ion-exchange chromatography (see Results) was homogenized in Freund's complete adjuvant (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.). 60, 735, and 130 μg enzyme protein from the first, second, and third peak, respectively, were injected intradermally in several dorsal sites of three New Zealand albino rabbits. 2 and 5 wk later, the rabbits were injected i.m. with 60, 360, and 60 μg of protein from the first, second, and third peak, respectively. The first booster dose was mixed with incomplete Freund's adjuvant, whereas the last dose was given without adjuvant. 10 days after the last booster dose, the rabbits were bled, and serum from clotted blood was collected and stored at −20°C. Immunoglobulin G was partially purified as described (22).

Double immunodiffusion was performed on agarose plates which contained 1% agarose (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.), 4% polyethylene glycol 6,000 (BBL Division, Becton, Dickinson & Co.), and 0.02% sodium azide in phosphate-buffered saline, pH 7.0. Preparations to be tested were allowed to diffuse against the rabbit anti-human acid α-glucosidase antibodies for 24–48 h at room temperature in a moist chamber. Immune precipitin bands were tested for enzymatic activity with 4-methylumbelliferyl-α-D-glucopyranoside; and, subsequently, the plates were stained for protein with Coomassie Blue. When crude fibroblast lysates were used, the fibroblast extract was lyophylized, and the material obtained was reconstituted in a small volume of water (40–100 mg protein/ml).

Immunotitration of cultured skin fibroblast acid α-glucosidase with rabbit anti-acid α-glucosidase antibodies was performed in a final vol of 20 μl, which contained 7.5 or 15 μg of fibroblast protein and increasing concentrations of immune IgG. The mixture was kept at 37°C for 30 min, and at 4°C for 60 h. Because centrifugation at this stage did not affect enzyme activity, the samples were not centrifuged, but were assayed directly for acid α-glucosidase activity with maltose as substrate.

RESULTS

The amount of glucose released by the acid α-glucosidase in fibroblast lysates was linear from 5 to 20 and from 5 to 25 μg protein per assay, when maltose or glycogen was used as substrate, respectively. The reaction was linear up to 2 h with both substrates. Maximal activity was obtained at a maltose concentration in the reaction mixture of ≅30 mM. However, enzyme assays were performed at a substrate concentration of 15.5 mM because greater substrate concentrations gave high blank readings. At this maltose concentration, 80% of the maximal activity was obtained. 2 mg glycogen per assay gave maximal activity. Normal skin fibroblasts showed the highest activity at pH 4.0–4.3, whereas two minor peaks of activity were present at pH 6.3 and 7.0. Skin fibroblasts from the patient with the adult acid α-glucosidase deficiency showed an activity of 13–16% of normal values at pH 4.3. Mixing experiments between lysates of adult acid α-glucosidase deficiency fibroblasts and normal fibroblasts showed the expected enzyme activities.

The partially purified preparation of placental α-glucosidase, which was chromatographed on the Sephadex G-100 column, did not contain any neutral α-glucosidase activity as tested by starch gel electrophoresis. The bulk of the acid α-glucosidase that was applied to the column was eluted after addition of maltose to the eluant. A minor peak of activity was eluted with the void volume before the application of maltose (Fig. 1). Although hydrolysis of the substrate was observed at pH 6.3 in fractions of the enzyme peak eluted with maltose, no neutral isozymes of α-glucosidase were identified by starch gel electrophoresis.

Three peaks or two peaks and a shoulder of enzymatic activity were obtained from pooled fractions of the enzyme eluted from the Sephadex G-100 column with maltose, when chromatographed on the DEAE-cellulose ion-exchange column (Fig. 2). The second peak always showed the greatest total and specific activity, whereas the third peak or shoulder showed the lowest. A protein peak, which corresponded to each of the enzyme activity peaks, was present. In addition, one protein peak was eluted before the appearance of any measurable α-glucosidase activity. The purification steps of placental acid α-glucosidase are listed in Table I.

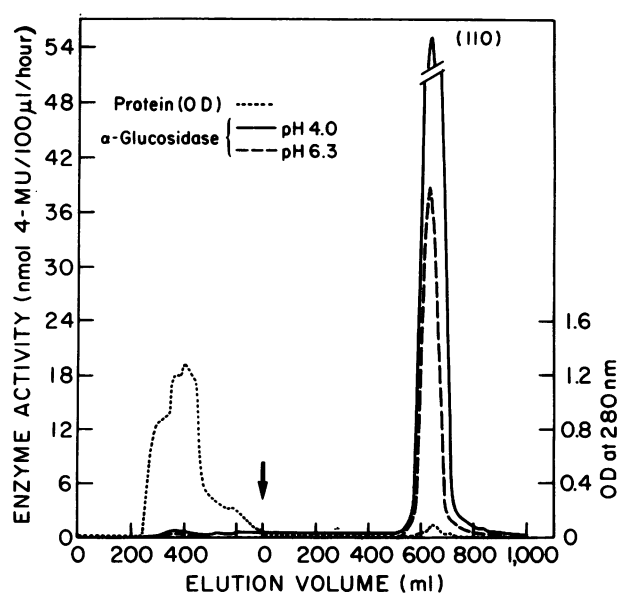

FIGURE 1 Elution of α-glucosidase and proteins of a partially purified placental preparation from a Sephadex G-100 column. 10-ml fractions were collected. The arrow shows addition of maltose to the eluant. 4-Methylumbelliferyl-α-D-glucopyranoside was used as substrate. 4-MU = 4-methylumbelliferone.

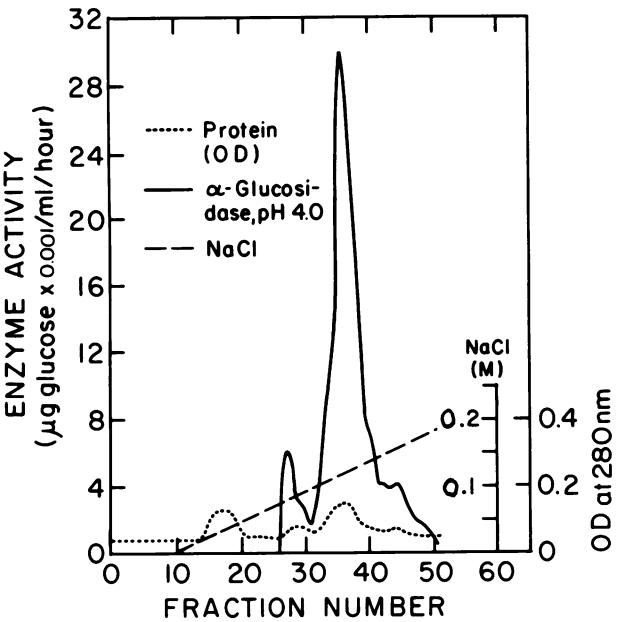

FIGURE 2 DEAE-cellulose ion-exchange chromatography of pooled fractions of placental acid α-glucosidase which was eluted with maltose from a Sephadex G-100 column. 2-ml fractions were collected. Maltose was used as a substrate.

Polyacrylamide disc-gel electrophoresis at pH 4.3 revealed a broad- and a weak-enzyme activity band in the Sephadex G-100 peak eluted with maltose, and one band in each of the three DEAE-cellulose peaks (Fig. 3A). Protein bands, which corresponded to the enzyme activity bands, were observed when the gels were stained for protein (Fig. 3B).

α-Glucosidase from normal cultured skin fibroblasts was eluted from the Sephadex G-100 column into three peaks of activity. The first two peaks had a relatively small amount of activity, and appeared before the application of maltose to the column. The major enzyme peak was eluted with maltose (Fig. 4A). In the first peak, α-glucosidase activity was higher at pH 6.3 than at 4.0. Although maximal activity in the major peak was at pH 4.0, hydrolysis of the substrate was also observed at pH 6.3. Inhibition of the acid α-glucosidase activity by anti-acid α-glucosidase antibodies also inhibited the activity at pH 6.3. Starch gel electrophoresis revealed that the major peak of activity consisted only of the acid isozyme. 21% of the acid α-glucosidase activity that was applied to the column was recovered in the major enzyme peak.

The Sephadex G-100 elution pattern of α-glucosidase from the adult acid α-glucosidase deficiency fibroblasts was different from that obtained from the normal skin fibroblasts. Two peaks of activity were seen, the first

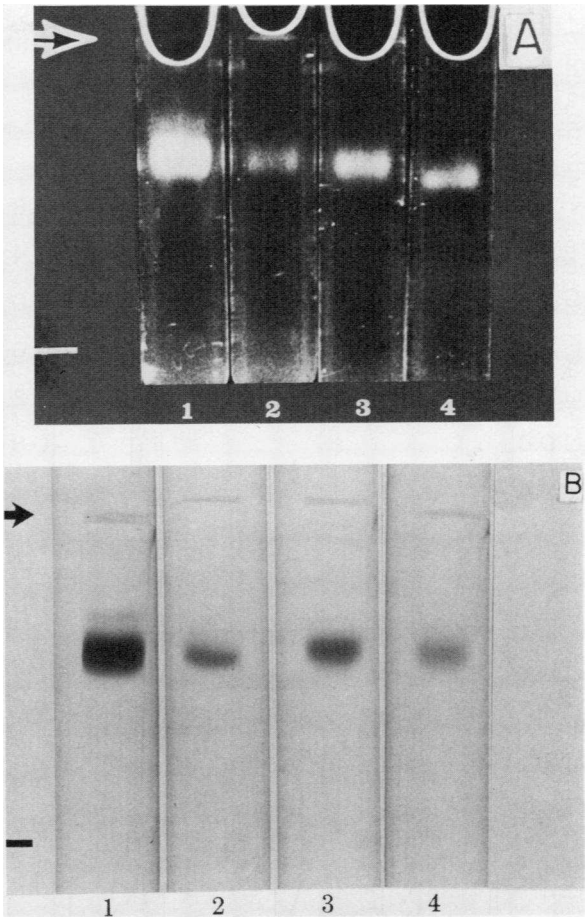

FIGURE 3 Polyacrylamide disc-gel electrophoresis at pH 4.3 of purified preparations of placental acid α-glucosidase. (1) second Sephadex G-100 peak eluted with maltose; (2) first DEAE-cellulose chromatography peak; (3) second DEAE-cellulose chromatography peak; (4) third DEAE-cellulose chromatography peak. Arrow indicates the origin. Migration is towards the cathode. (A) Gels were stained for enzyme activity with the fluorogenic substrate 4-methylumbelliferyl-α-D-glucopyranoside. (B) Gels were stained for protein with Coomassie Blue. Each sample electrophoresed contained 10 μg protein.

TABLE I
Purification of Acid α-Glucosidase from Human Placenta

Step	Specific activity	Purification	Recovery
	μg glucose/ mg protein/h	*-fold*	%
Homogenate	1,511	1.0	100
CM-Sephadex	4,744	3.1	70.4
$(NH_4)_2SO_4$, dialysis	5,268	3.4	44.3
Sephadex G-100	534,363	354	28.6
DEAE-cellulose			
Peak I	620,726	411	1.6
Peak II	703,245	465	19.9
Peak III	263,600	174	0.9
Total recovery			22.4

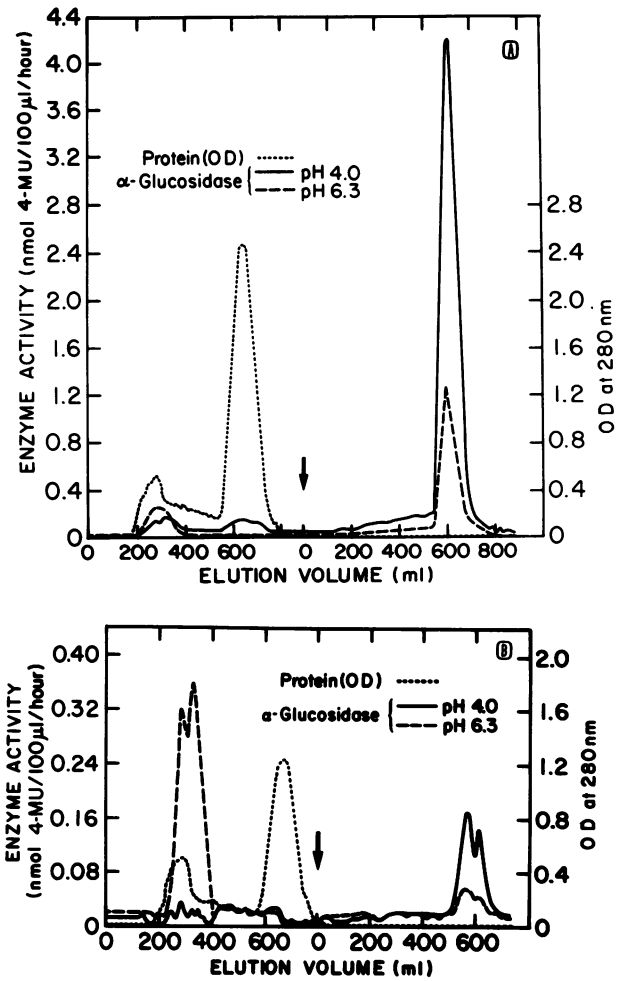

FIGURE 4 Chromatography on a Sephadex G-100 column of crude lysate supernatant fluids from cultured skin fibroblasts. Fractions of 10 ml were collected. The arrow indicates addition of maltose to the eluant. 4-Methylumbelliferyl-α-D-glucopyranoside was used as substrate. 4-MU = 4-methylumbelliferone. (A) Normal fibroblasts. (B) Adult acid α-glucosidase deficiency fibroblasts.

being greater than the second (Fig. 4B). The first peak was eluted with the void volume, whereas the second one was eluted after the addition of maltose to the eluant. The former peak consisted of an α-glucosidase with maximal activity at pH 6.3, whereas the enzyme in the latter peak was more active at pH 4.0. Recovery of the acid α-glucosidase activity applied to the column was 26%.

Acid α-glucosidase from normal and adult acid α-glucosidase deficiency fibroblasts had the same electrophoretic mobility in starch gel (Fig. 5). There was no difference in substrate saturation kinetics between the purified enzymes from normal and deficient skin fibroblasts, and from placenta. With Lineweaver-Burk plots, no difference in the apparent Michaelis constant was observed between the enzymes obtained from these sources. Apparently the V_{max} of the acid α-glucosidase from the adult acid α-glucosidase deficiency fibroblasts was only 6.5% of the normal enzyme (Table II).

Crude lysates from both normal and adult acid α-glucosidase deficiency fibroblasts, when heated at 53°C at a fibroblast protein concentration of 1.5 mg/ml, showed an initial increase of the specific enzyme activity. This increase of specific activity was not present when the protein concentration was reduced to 0.75 mg/ml. No significant difference in the acid α-glucosidase inactivtion rate was observed between normal and mutant enzyme (Fig. 6A). The pH of the heat-inactivated samples was adjusted to 4.0 and the ionic strength was kept the same. Thermal denaturation at 58°C of purified acid α-glucosidase from normal and mutant cultured skin fibroblasts proceeded more rapidly at pH 7.0 than at pH 4.0. The heat inactivation rate, however, was the same between the wild type and the mutant enzyme (Fig. 6B). Also, no difference of the heat inactivation rate was observed between purified cultured skin fibroblast and placental enzyme. Heat inactivation of the purified enzymes was performed in the presence of 0.8 mg/ml of bovine serum albumin, which was added to stabilize the enzyme.

Purified acid α-glucosidase from placenta, and from normal and deficient cultured skin fibroblasts was inhibited with turanose, α-methylglucoside, and trehalose to a greater extent when glycogen, rather than maltose, was the substrate. No significant difference, however, was observed in the inhibition rate in relation to the source of the enzyme (Table III).

On double immunodiffusion, an immunoprecipitin band was formed between the antibodies against the acid α-glucosidase from the three enzyme peaks of the DEAE-cellulose column and crude placental extract, acid α-glucosidase eluted with maltose from the Sephadex G-100 column, the three enzyme peaks from the ion-exchange column, and purified enzyme from normal fibroblasts (Fig. 7A, B, and C). Antigenic identity was observed between skin fibroblast and placental enzyme. Also, one continuous immunoprecipitin band was obtained, when crude placental extract was allowed to diffuse against antibodies to the first, second, and third DEAE-cellulose enzyme peaks (Fig. 8A). Fluorescent bands of acid α-glucosidase were visualized after staining of enzyme-active immunoprecipitates with the fluorogenic substrate (Fig. 8B). Fibroblast lysates from normal subjects, from the patients with the infantile and the adult form of acid α-glucosidase deficiency, and purified acid α-glucosidase from normal skin fibroblasts formed a single immunoprecipitin band when diffused against antibodies to placental enzyme. The precipitin bands from the lysates of both types of deficient fibroblasts shared antigenic identity with the enzyme from the normal

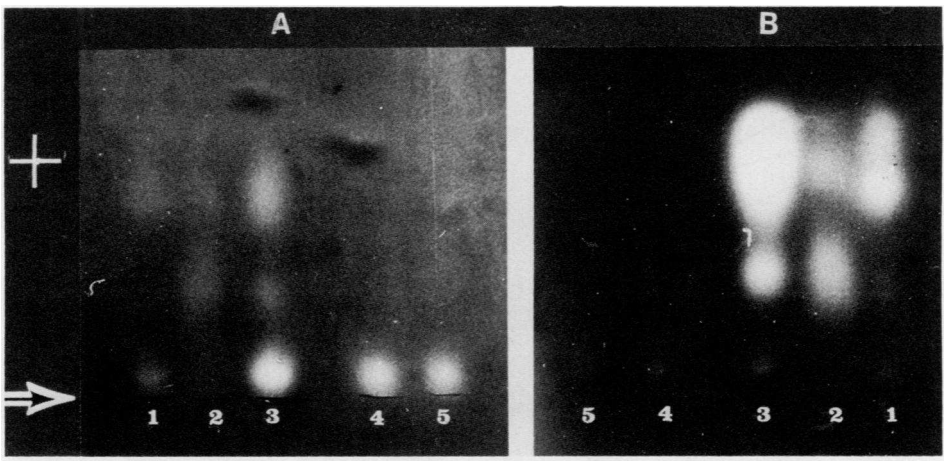

FIGURE 5 Horizontal starch gel electrophoresis of α-glucosidase. (A) Gel stained with the fluorogenic substrate 4-methylumbelliferyl-α-D-glucopyranoside at pH 4.0. (B) Gel stained at pH 6.3. (1) 135 μg protein (30 U) acid α-glucosidase from a crude fibroblast lysate from a patient with adult acid α-glucosidase deficiency; (2) 135 μg protein (2.5 U) from a crude fibroblast lysate from a patient with infantile acid α-glucosidase deficiency; (3) 67 μg protein (180 U) from a crude fibroblast lysate from normal skin fibroblasts; (4) 143 U purified acid α-glucosidase from adult acid α-glucosidase deficiency fibroblasts; (5) 143 U purified acid α-glucosidase from normal cultured skin fibroblasts. Acid α-glucosidase has the same mobility in all samples, except in the sample from the infantile form of the disease, where no activity was visualized. The rapidly migrating bands of the crude lysates are the neutral isozymes of α-glucosidase.

cells. The immunoprecipitin band from the infantile acid α-glucosidase deficiency cells had approximately the same intensity as the band from the normal cells, whereas the band from the adult acid α-glucosidase deficiency cells was faint, and a higher amount of fibroblast protein was needed to make it clearly visible (Fig. 9A). When the immune complexes were treated with the fluorogenic substrate for α-glucosidase, enzyme activity was demonstrated in the bands of the lysates from normal and adult acid α-glucosidase deficiency fibroblasts and the purified wild type fibroblast enzyme, but not from the infantile acid α-glucosidase deficiency cells. In samples that contained the same amount of cellular protein, the fluorescent band of the adult acid α-glucosidase deficiency fibroblasts was fainter than that of normal fibroblasts (Fig. 9B).

TABLE II

Specific Activity of Acid α-Glucosidase in Crude Fibroblast Lysates, and Specific Activity, V_{max}, and Michaelis Constant of Purified Enzyme from Fibroblasts of a Normal Subject and a Patient with the Adult Form of Acid α-Glucosidase Deficiency

Preparation	Specific activity	V_{max}	K_m
	μg glucose/mg protein/h		*mM*
Crude FB lysate*			
Normal	475	—	—
Mutant	75	—	—
Purified enzyme			
Normal FB	525,000	986,000	22.2
Mutant FB	53,000‡	64,000‡	18.5

Maltose was used as substrate.
* FB, cultured skin fibroblasts.
‡ Because small amounts of nonenzyme protein present may interfere with accurate estimation of specific activity and V_{max}, the observed differences may not be genuine.

Immunotitrations of acid α-glucosidase in lysates from normal and adult acid α-glucosidase deficiency fibroblasts with increasing concentrations of rabbit anti-acid α-glucosidase antibodies progressively removed the acid α-glucosidase activity. The enzyme inhibition curves, however, were different between the normal and the adult acid α-glucosidase deficiency fibroblast lysates as well as in mixtures of normal and both forms of acid α-glucosidase deficiency fibroblast lysates (Fig. 10). At an antibody dilution of 1:30, 50 and 95% of the acid α-glucosidase activity in the samples with 15 μg protein of normal and adult acid α-glucosidase deficiency fibroblasts was inhibited, respectively. At the same antibody dilution, 84% of the enzyme activity in the sample with 7.5 μg of normal protein was inhibited. The enzyme inhibition in the mixture of 7.5 μg protein each of normal and infantile acid α-glucosidase deficiency fibroblast lysate was only 66%. Similarly, in the mixture of 7.5 μg normal and 7.5 μg adult acid α-glucosidase deficiency extract, the inhibition was 72%. These results indicate competition

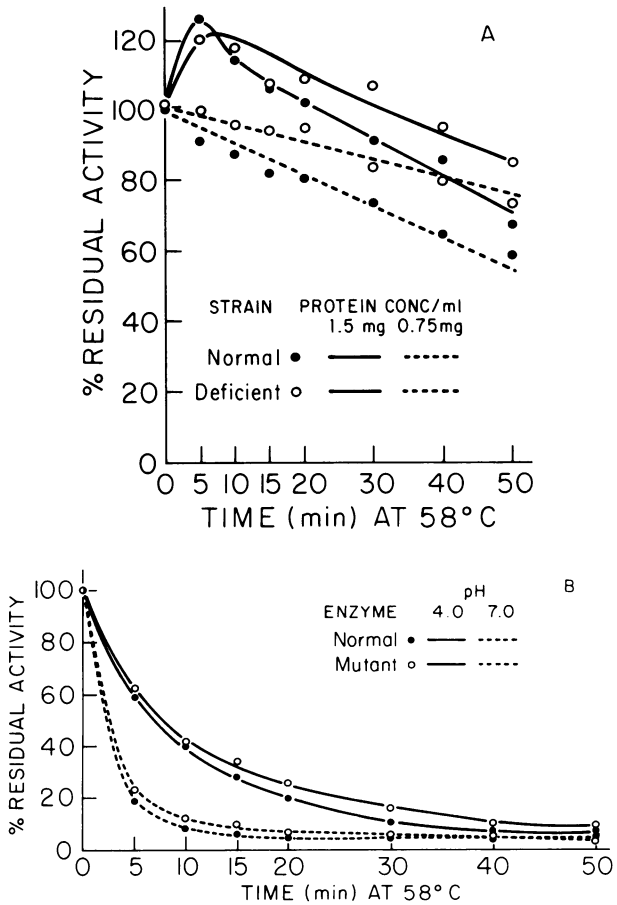

FIGURE 6 Heat denaturation curves. All samples were assayed for enzyme activity at pH 4.0. Maltose was used as substrate. (A) Acid α-glucosidase in crude cultured skin fibroblast lysates from normal and adult acid α-glucosidase deficiency fibroblasts. The activity at zero time (100% activity) was 223 and 74 μg glucose released per milligram protein per hour for the normal and the deficient cells, respectively. The normal fibroblasts were harvested at early confluency, whereas the mutual cells were harvested at late confluency to increase the specific activity. Cells were lysed and heated in 0.1 M (in reference to phosphate) citrate-phosphate buffer, pH 4.0. (B) Purified acid α-glucosidase from normal and adult acid α-glucosidase deficiency fibroblasts heated at pH 4.0 and 7.0 in 0.1 M (in reference to phosphate) citrate-phosphate buffer. The enzyme preparations from the normal and deficient cells contained 74 and 40 U acid α-glucosidase, respectively. Both preparations contained 0.8 mg bovine albumin per milliliter added to stabilize the enzyme. Conc, concentration.

for binding sites between the normal and both forms of mutant enzymes. Immunotitrations of lysates from normal and from adult acid α-glucosidase deficiency fibroblasts, with approximately the same total enzyme activity, but different amounts of fibroblast protein, showed similar inhibition curves (Fig. 11). No enzyme inhibition was observed when nonimmune immunoglobulin G, prepared from the same rabbit before immunization, or saline was used instead of antibodies to the enzyme.

TABLE III

Inhibition of Purified Acid α-Glucosidase from Placenta, and from Normal and Deficient Cultured Skin Fibroblasts by Turanose, α-Methylglucoside, and Trehalose

Preparation	Inhibitor	Inhibitor: Maltose	Inhibitor: Glycogen
	mM	%	
Placenta	Turanose (10)	60.0	72.7
	α-Methylglucoside (50)	19.3	67.4
	Trehalose (100)	11.7	84.2
Normal FB*	Turanose (10)	58.9	72.2
	α-Methylglucoside (50)	16.0	56.7
	Trehalose (100)	5.8	82.2
Deficient FB	Turanose (10)	57.6	71.0
	α-Methylglucoside (50)	13.5	51.2
	Trehalose (100)	10.5	81.0

Both maltose and glycogen were used as substrate.
* FB, cultured skin fibroblasts.

α-Glucosidase present in the fetal calf serum used for culturing did not form an immunoprecipitin band when allowed to diffuse against antibodies to human placental acid α-glucosidase and its activity was inhibited by only 15% when incubated in solution with the antibody. Also, the calf enzyme was significantly more heat-labile than the fibroblast enzyme ($t_{1/2}$, 53°C:7 min).

DISCUSSION

α-Glucosidase eluted from the Sephadex G-100 column with maltose had maximal activity at pH 4.0–4.3, but hydrolysis of the substrate was also observed at pH 6.3. This enzyme preparation, however, contained only acid α-glucosidase as demonstrated by starch gel electrophoresis and the inhibition of the activity at pH 6.3 by anti-acid α-glucosidase antibodies. Neutral α-glucosidase does not bind to Sephadex and, therefore, it was eluted in the void volume. This isozyme is more labile than the acid isozyme (23), and lost most of its activity during the purification process.

The three acid α-glucosidase peaks obtained from the ion-exchange column appear to contain enzyme purified to homogeneity or near homogeneity. The acid α-glucosidase purified by deBarsy et al. (11) was a mixture of the enzyme present in these peaks. In addition, it contained at least one more protein without enzyme activity, which was eluted from the ion-exchange column before the three enzyme peaks (Fig. 2). This protein was not identified as a separate band by polyacrylamide gel electrophoresis (Fig. 3), possibly be-

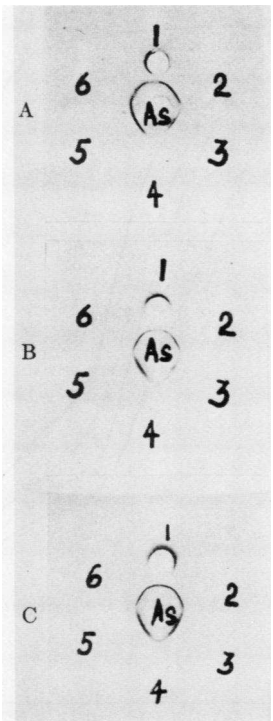

FIGURE 7 Double immunodiffusion plates of rabbit anti-acid α-glucosidase serum (As: center well) against crude placental extract and purified acid α-glucosidase. (1) crude placental extract; (2) purified placental acid α-glucosidase from the first DEAE-cellulose peak; (3) purified placental acid α-glucosidase from the second DEAE-cellulose peak; (4) purified placental acid α-glucosidase from the third DEAE-cellulose peak; (5) placental acid α-glucosidase eluted with maltose from the Sephadex G-100 column; (6) purified acid α-glucosidase from normal fibroblasts. Of the six outer wells, only well 1 is visualized in the figure. In A, B, and C the antiserum used was raised against acid α-glucosidase from the first, second, and third DEAE-cellulose peaks, respectively. Plates were stained with Coomassie Blue.

cause it did not migrate toward the cathode, or because it co-migrated with the enzyme protein. It is not completely resolved why the intensity of the bands in the polyacrylamide gels is different between the enzyme preparation eluted with maltose from the Sephadex G-100 column and the three enzyme preparations obtained by the ion-exchange chromatography. If the contaminating protein(s) co-migrated with the enzyme, bands of different intensity could be obtained as different proteins take up the stain differentially. The weaker fluorescent bands of activity observed in the samples obtained from the DEAE-cellulose column is probably the result of the rapid enzyme inactivation observed in these highly purified preparations.

The specific activity and the V_{max} of the purified acid α-glucosidase from the adult acid α-glucosidase deficiency fibroblasts were 10 and 6.5% of the purified normal enzyme, respectively, but the Michaelis constant values were similar. It is not certain whether or not the low specific activity and V_{max} of the enzyme from the mutant cells represent genuine catalytic differences from the normal enzyme caused by a mutation that affects the active site of the enzyme molecule. It is possible that a minor contamination of the purified enzyme preparations with other proteins has affected these measurements. The thermal denaturation curves of acid α-glucosidase in both crude cell lysates and the purified enzyme preparations indicate that the enzyme deficiency in the adult acid α-glucosidase deficiency fibroblasts did not result from the synthesis of a heat-labile enzyme.

The initial increase of the specific activity of acid α-glucosidase in heated crude fibroblast lysates which had a high protein concentration, may result from the presence in these lysates of enzyme aggregates, which release free enzyme molecules during heating. This would cause previously covered active sites of the enzyme to become accessible to the substrate, in a similar manner as reported for placental alkaline phosphatase (24, 25). Although no significant difference in the

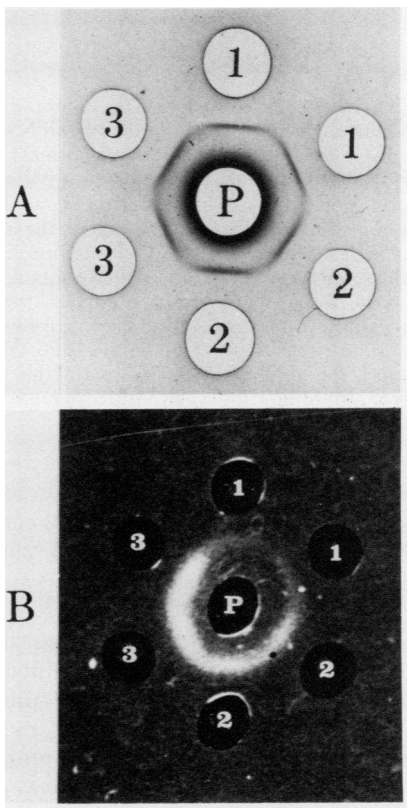

FIGURE 8 Double immunodiffusion plate of 130 μg protein of crude placental extract (center well) against antiserum to the first (1), the second (2), and the third (3) DEAE-cellulose enzyme peaks. (A) Plate was stained with Coomassie Blue. (B) Same plate stained for acid α-glucosidase activity with 4-methylumbelliferyl-α-D-glucopyranoside.

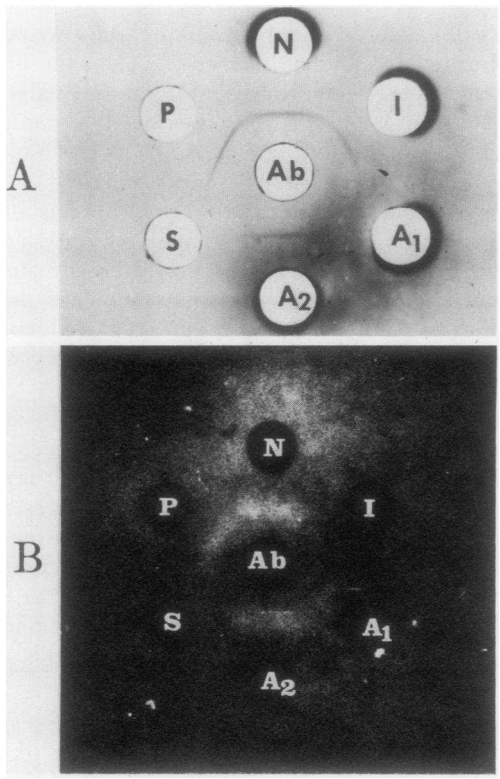

FIGURE 9 Agar double diffusion. The center well contains rabbit anti-acid α-glucosidase antibodies; well N contains 600 μg cultured skin fibroblast protein from a normal subject; well I contains 600 μg fibroblast protein from a patient with the infantile form of acid α-glucosidase deficiency; well A_1 contains 600 μg fibroblast protein from a patient with the adult form of acid α-glucosidase deficiency; well A_2 contains 1,500 μg protein from the same fibroblast strain; well S contains saline; and well P contains 0.6 μg protein of the purified preparation of acid α-glucosidase from normal cultured skin fibroblasts. (A) Plate stained for protein with Coomassie Blue. (B) The same plate stained for enzyme activity with the fluorogenic substrate 4-methylumbelliferyl-α-D-glucopyranoside.

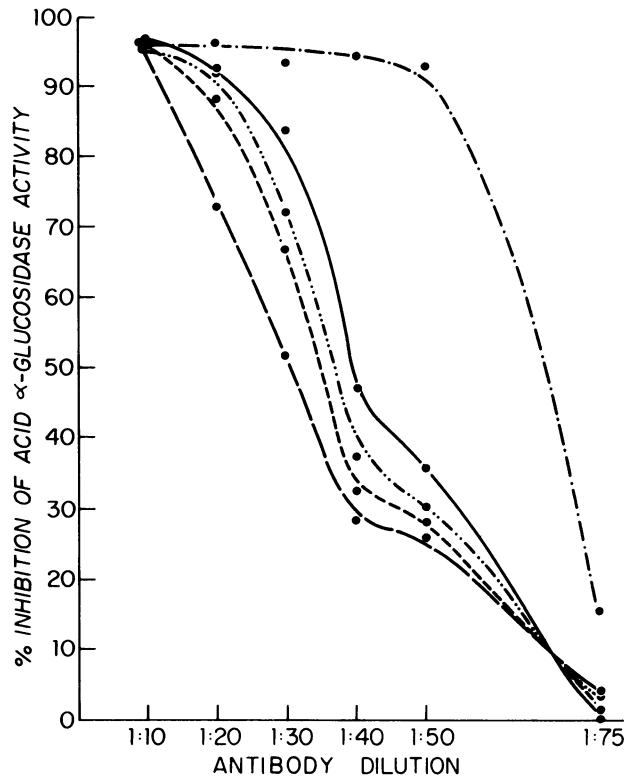

FIGURE 10 Immunotitrations of acid α-glucosidase from various fibroblast strains with partially purified rabbit anti-acid α-glucosidase antibodies. The titrations depicted are with 15 μg normal skin fibroblast protein (— —), with a mixture of 7.5 μg protein each of normal and infantile acid α-glucosidase deficiency fibroblasts (- - -), with a mixture of 7.5 μg protein each of normal and adult acid α-glucosidase deficiency fibroblasts (— · · —), with 7.5 μg protein from normal fibroblasts (——), and 15 μg protein from adult acid α-glucosidase deficiency fibroblasts (— · —). Antibody at a dilution of 1:10 contained 0.24 μg protein. No enzyme inhibition was observed when nonimmune immunoglobulin or saline was substituted for antibodies to acid α-glucosidase.

heat inactivation rate was observed between the enzyme from normal and from the adult form of the deficiency, an increased resistance to heat has been reported in crude lysates from two such patients (26). In addition, the mutant enzyme showed an increased mobility by cellulose acetate electrophoresis (26). The findings of the mixing experiment suggest that the enzyme deficiency in the adult acid α-glucosidase deficiency fibroblasts is not caused by the presence of a soluble inhibitor or the absence of an activator.

The presence of a continuous precipitin line in double immunodiffusion experiments when anti-acid α-glucosidase antibodies were diffused against either crude placental or fibroblast extracts, or purified acid α-glucosidase indicates that there is antigenic identity between the placental and the fibroblast enzyme. The staining of the immunodiffusion plates for acid α-glucosidase activity directly showed that enzyme ac-

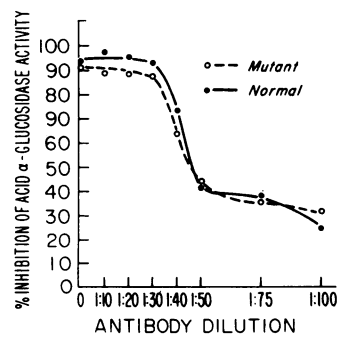

FIGURE 11 Immunotitrations of acid α-glucosidase from normal and adult acid α-glucosidase deficiency fibroblasts with partially purified rabbit anti-acid α-glucosidase antibodies. Both lysates contained approximately the same amount of enzyme activity (normal fibroblast lysate: 25 U; deficient fibroblast lysate: 27 U). Antibody dilution of 1:10 contained 0.24 μg protein. This antibody preparation was different from that used in Fig. 10.

tivity was present in the enzyme-antibody precipitate from normal crude placental and normal skin fibroblast extracts, and from purified enzyme from normal tissues. The presence, however, of cross-reacting, enzymatically inactive acid α-glucosidase protein in fibroblast lysates from the patient with the infantile acid α-glucosidase deficiency indicates that in this patient the disease was the result of a structural gene mutation at the acid α-glucosidase locus.

The faint immune precipitin band observed in adult acid α-glucosidase deficiency fibroblast lysates suggests that either the cells produce an enzyme protein which is altered in such a fashion that it is only partially recognized by the antibodies to normal acid α-glucosidase or that the amount of the enzyme protein present in the cells is reduced. However, the observation that the enzyme activity in lysates from the adult acid α-glucosidase deficiency fibroblasts showed maximal inhibition at an antibody dilution of 1:50, whereas the enzyme activity in the same amount of cellular protein from normal skin fibroblasts was gradually inhibited up to an antibody dilution of 1:10 indicates that the mutant cells have a reduced amount of enzyme protein. This was confirmed by the identical inhibition curves obtained when the same amount of activity from both normal and adult acid α-glucosidase deficiency fibroblasts was inhibited by increasing concentrations of antibodies. A reduction of the number of the enzyme molecules present in the cells of the adult form of the disease was also recently reported by Reuser et al. (14). In addition, the findings of the immunotitration confirm the presence of catalytically inactive enzyme protein in fibroblasts from the infantile form of the disease, as this mutant enzyme protein competes for antibody binding sites with the normal enzyme. A similar competition for antibody binding sites also was shown between normal and adult acid α-glucosidase deficiency fibroblast enzymes. The apparently contradictory findings of this study with the earlier reported absence or severe reduction of immunologically reacting enzyme protein in tissues of patients with glycogenosis type II (11–14, 27) could be interpreted as the result of genetic heterogeneity.

ACKNOWLEDGMENTS

We are grateful to Dr. Rochelle Hirschhorn and Frank Martiniuk for helpful suggestions regarding the enzyme purification.

This study was supported in part by grants from the Muscular Dystrophy Association, the National Foundation—March of Dimes (I-535), and the National Institutes of Health (GM 19433).

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