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Studies of Lysosomal α-Glucosidase. I. Purification and Properties of the Rat Liver Enzyme*

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ABSTRACT: An α-glucosidase has been purified 1300-fold from the lysosomal fraction of rat liver. The preparation appears to be homogeneous when subjected to equilibrium ultracentrifugation. The degree of purification of the enzyme at each step in its preparation was essentially the same whether the assay substrate was maltose, glycogen, or isomaltose. Thus, the enzyme appears to have α-1,6-glucosidase as well as α-1,4-glucosidase activity. Both of these activities sedimented at the same rate when the purified enzyme was centrifuged in a linear sucrose gradient. The apparent molecular weight of the protein is about 114,000. The enzyme has maximal activity toward maltose at pH 3.7, toward isomaltose at pH 4.2, and toward glycogen at pH 4.4. Similar pH optima were found in acetate and citrate buffers. Glucose formation from glycogen can be stimulated fivefold at pH 4.0 by 0.2 M KCl. This stimulation was found for several monovalent and divalent cations. The influence of cation concentration on the rate of maltose and isomaltose hydrolysis is much less than on that of glycogen. The enzyme can act also as a transglucosylase at pH 4.0 when it is incubated with oligosaccharides such as maltose. Maltotriose and maltotetraose are formed together with a branched trisaccharide of unknown structure which has been shown to be distinct from panose and from 4,6-di-*O*-(α-D-glucopyranosyl)-D-glucose. The enzyme also catalyzes transglucosylation from maltose to glycogen. The structure of the polysaccharide product has been studied. Since the specific activity of the enzyme as a transglucosylase parallels its specific activity as a hydrolytic enzyme during purification, it is concluded that the lysosomal glucosidase has intrinsic transglucosylase activity.

The lysosomes which are present in liver are known to contain a large number of hydrolases with specificities directed toward a variety of intracellular and extracellular macromolecular substances as well as toward compounds of simpler structure which occur within the cell. Presumably, the metabolic role of these enzymes, whose activities are most pronounced under acidic conditions, is to convert their substrates into monomeric units so that these may again become available to the cell for biosynthetic and for energy-yielding reactions. This concept has been discussed in detail by de Duve and his coworkers (de Duve, 1965; de Duve and Wattiaux, 1966; Coffey and de Duve, 1968; Aronson and de Duve, 1968). The action on glycogen of enzymes within the lysosome is of special interest since Lejeune *et al.* (1963) showed that a lysosomal α-1,4-glucosidase, active at pH 4, is found in rat liver. Hers (1963) found that a similar activity is present in human liver, presumably within lysosomes as well. The further discovery was made by Hers (1963) that in children who have a fatal form of glycogen storage disease

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known as type II glycogenosis (Pompe's disease), this α-1,4-glucosidase activity is not present in the liver, heart, and skeletal muscles. Baudhuin *et al.* (1964a) found that electron micrographs of liver tissue from such patients show that a large amount of glycogen is sequestered within intracellular bodies which have a single membrane and which may be lysosomes. These observations have been confirmed by others and have been extended by studies on heart, kidney, and skeletal muscle (Zellweger *et al.*, 1965; Cardiff, 1966; Hernandez *et al.*, 1966; Hug *et al.*, 1966; Witzleben, 1969). Although the lysosomal α-1,4-glucosidase is often referred to as a maltase, the enzyme is known to have a broader specificity in that it acts also on the outer chains of glycogen (Lejeune *et al.*, 1963). Auricchio *et al.* (1968) have described the purification from rat liver of an α-glucosidase which acts on maltose and glycogen optimally at pH 4.5. The possibility that the enzyme might act on other than α-1,4-glucosidic bonds was not studied. Recently Bruni *et al.* (1969) have described the purification of a homogeneous protein from bovine liver which acts at pH 4.5 to catalyze the hydrolysis of α-1,4-glucosidic bonds in maltose and glycogen. These latter authors state that the enzyme can also form glucose from isomaltose but details of this observation have not been given. The preparation from bovine spleen of an enzyme with a similar pH optimum for activity toward maltose and glycogen has also been described (Fujimori *et al.*, 1968). The lysosomal origin of these purified rat liver, bovine liver, and bovine spleen enzymes has not been shown but is suggested by their pH optima for activity. Hers (1964) and Hers and van Hoof (1968) have stated that lysosomes contain an "acid isomaltase," but the properties of such an enzyme have not been described. Torres and Olavarria (1964) found that a protein fraction prepared from dog liver had only a weak activity as a glucosidase for isomaltose at acid pH. The present study describes the purification from rat liver lysosomes of an enzyme capable of catalyzing the total hydrolysis of glycogen to glucose at acid pH. In this paper some properties of the enzyme are also reported. The following paper (Jeffrey *et al.*, 1970) describes the kinetic behavior of the enzyme toward several oligosaccharide and polysaccharide substrates. As a result of all of these studies, it is concluded that the purified enzyme acts both as an α-1,4- and an α-1,6-glucosidase. An accompanying paper (Brown *et al.*, 1970) contains data on the corresponding enzyme from human tissues, and in this paper it is shown that both types of glucosidase activity are simultaneously missing in the liver and muscle of children with type II glycogenosis.

Materials and Methods

Materials. Isomaltose was obtained from Mann Research Laboratories and from Pierce Chemical Co. The commercial samples contained a number of unidentified contaminating oligosaccharides. Purification of isomaltose was by descending chromatography on Whatman No. 1 paper, using 1-butanol–pyridine–water (3:2:1.5, v/v) as the developing solvent. A benzidine–trichloroacetic acid spray (Bacon and Edelman, 1951) was used for detection of all sugars with reducing properties. The identity of the purified isomaltose was established by comparison in the 1-butanol–pyridine–water system with chromatographic standards kindly provided by Dr. M. L. Wolfrom and by Dr. J. H. Pazur. Glucose was determined enzymatically by the spectrophotometric measurement of NADPH in the presence of added $NADP^+$, Mg^{2+}, ATP, hexokinase, and glucose 6-phosphate dehydrogenase. These two enzymes were obtained from the Boehringer Corporation and they were free of detectable invertase. This fact permitted their use for the measurement of glucose in the presence of sucrose, as was necessary in assays done in the early steps of the enzyme purification. Uniformly labeled [^{14}C]maltose (6.7 mCi/mmole) was obtained from Nuclear-Chicago and was purified by paper chromatography using the 1-butanol–pyridine–water solvent system.

Glycogen isolated from rabbit liver was obtained from Mann Research Laboratories and was purified by ethanol precipitation following exhaustive dialysis. The per cent of nonreducing end groups in glycogen (branch point content) was determined enzymatically by a modification of the method of Illingworth *et al.* (1952) using a pure preparation of glycogen phosphorylase *a* to which was added oligo-α-1,4→1,4-glucantransferase–amylo-1,6-glucosidase. The latter enzyme was prepared by the method of Brown and Brown (1966). The modification involved the direct assay of glucose in the total digest of glycogen by the coupled enzymatic system described above. Glucose 1-phosphate was determined by an assay which was similar except that crystalline phosphoglucomutase (Boehringer) was used instead of hexokinase and ATP.

Maltose and *p*-nitrophenyl β-*N*-acetylglucosaminide were obtained from Sigma Chemical Co. D-(+)-Cellobiose, stachyose, D-(+)-melibiose, D-(+)-raffinose, D-(+)-melezitose, and D-(+)-turanose were commercial products from Mann Research Laboratories. A sample of 4,6-di-*O*-(α-D-glucopyranosyl)-D-glucose was generously given by Dr. I. J. Goldstein. Panose was a gift from Dr. J. H. Pazur. DEAE-cellulose was obtained from the Brown Co.; before use it was treated by the method of Peterson and Sober (1962). Sephadex G-100 (40–120 mesh) was obtained from Sigma and before use it was treated in 6 M urea and then exhaustively washed with water according to the procedure of Auricchio and Sica (1967).

Assay for α-1,4-Glucosidase. The reaction mixture contained 0.05 M maltose–0.05 M potassium acetate buffer (pH 4.0), and enzyme in a final total volume of 0.4 ml. After incubation for 30 min at 37°, the reaction was stopped either by heating in a boiling-water bath for 1 min (in the cases where crude subcellular fractions were assayed), or by adding 1 ml of 0.2 M Tris buffer (pH 8.0) in the case of assays of more purified enzyme fractions. The reaction mixture was centrifuged, if necessary, to remove coagulated protein and the glucose content of the clear supernatant fluid was determined enzymatically as described above. The formation of glucose by α-1,4-glucosidase action on glycogen was determined in a similar way, except that the enzyme fraction was incubated for 20 min at 37° with 2% glycogen (*ca.* 8 mM in glucose end groups) in 0.10 M potassium acetate buffer, pH 4.2, containing 0.2 M KCl. One unit is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmole of maltose/hr or the formation of 1 μmole of glucose/hr from glycogen.

Assay for α-1,6-Glucosidase. The incubation mixture contained 0.022 M isomaltose–0.05 M potassium acetate buffer (pH 4.0), and enzyme in a final total volume of 0.2 ml. After incubation for 30 min at 37°, the quantity of glucose formed was determined as in the assay for α-1,4-glucosidase. The use in this assay of an even higher initial concentration of isomaltose is desirable if enough of the pure compound is available, be-

cause of the high K_m of the enzyme for this substrate (Jeffrey *et al.*, 1970). One unit is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmole of isomaltose/hr.

Assay of Glucosyltransferase. The action of the glucosidase in transferring glucose units was investigated in two ways. In one case, 0.1 ml of the enzyme preparation was incubated with uniformly labeled 0.05 M [^{14}C]maltose (6.1×10^5 cpm) in 0.05 M potassium acetate buffer, pH 4.0 (final volume, 0.2 ml). After 1 hr at 37°, the reaction was stopped by heating for 1 min in a boiling-water bath. Aliquots of the solution were chromatographed as described below, without prior deionization. The radioactivity incorporated into each of the various oligosaccharide products was determined with a strip scanner. The second kind of transferase assay involved the transfer of glucose units from maltose to glycogen. The enzyme preparation (0.1 ml) was incubated with 10 mg of glycogen and 0.04 M [^{14}C]maltose (uniformly labeled, 6.1×10^5 cpm) in 0.04 M potassium acetate buffer (pH 4.2) containing 0.1 M KCl (final volume 0.25 ml). After 4 hr at 37°, the reaction was stopped by heating for 1 min in a boiling-water bath. Glycogen (1 ml, 1%) was added followed by 1.5 volumes of ethanol to precipitate the polysaccharide. The glycogen was dissolved in 20% KOH and heated at 100° for 30 min. After precipitation by ethanol, solution of the precipitate in water, and neutralization of the solution, the glycogen was precipitated again. This process was repeated three times. The ^{14}C content of the glycogen was determined by plating it and counting in a low-background counter (Nuclear-Chicago). The glycogen content was found by measuring the glucose present in a hydrolysate of the polysaccharide after heating it for 3 hr in 1 M HCl. The coupled hexokinase and glucose 6-phosphate dehydrogenase assay for glucose was used as described above. One unit of activity in the assay in which only [^{14}C]maltose was added is defined as the amount of enzyme required to form 1 μmole of each transfer product/hr. When [^{14}C]maltose and glycogen were used together as substrates, one unit of enzyme is defined as the amount required to incorporate 1 nmole of glucose into 1 μmole of glycogen end group/hr.

Assay for Glucose 6-Phosphatase (EC 3.1.3.9). The reaction mixture contained 0.04 M glucose-6-P–0.001 M EDTA–0.02 M histidine–HCl buffer (pH 6.6), and enzyme in 0.5-ml final volume. Incubation was for 30 min at 37°. After addition of 0.5 ml of 10% trichloroacetic acid, P_i was determined in the protein-free supernatant by the method of Fiske and Subbarow (1925).

Other Enzyme Assays. Acid phosphatase (EC 3.1.3.2) was assayed by the method of Appelmans *et al.* (1955), except that incubation was for 30 min. Glutamate dehydrogenase (EC 1.4.1.3) was assayed in the direction of NAD^+ reduction according to a modification of the method of Beaufay *et al.* (1959), consisting of the use of 60 mM glycylglycine as the buffer at pH 7.7. EDTA (1 mM) was used instead of cysteine. β-*N*-Acetylglucosaminidase (EC 3.2.1.30) was determined by a slight modification of the method described by Weissmann *et al.* (1967) in which *p*-nitrophenyl β-*N*-acetylglucosaminide is the substrate. In the present work 25 mM potassium citrate, pH 4.2, was the buffer, and the reaction mixture was deproteinized by 20% trichloroacetic acid following incubation. The formation of *p*-nitrophenol was measured spectrophotometrically in the deproteinized filtrates in the presence of added potassium borate (pH 10.4). All protein fractions which might have contained any subcellular particles were pretreated with 0.1% Triton X-100 (Rohm and Haas Co.) before they were assayed. In view of this, no additional Triton was added to any assay system. Protein was determined by the method of Lowry *et al.* (1951) adapted to a microscale. Crystallized bovine plasma albumin (Armour) was used as the protein standard.

Paper Chromatographic Procedures. When prior deionization of solutions was necessary, short columns of the mixed-bed resin, Amberlite MB-3, were used, and these were washed thoroughly in order to minimize the nonspecific adsorption of small quantities of neutral sugars. The solutions were then concentrated by evaporation at reduced pressure and aliquots were chromatographed on Whatman No. 1 paper using as descending solvent, 1-butanol–pyridine–water (3:2:1.5). Standard reference substances were located by the use of a benzidine–trichloroacetic acid spray. When ^{14}C-labeled substrates were used, the chromatographic strips were scanned in a Vanguard 880 Autoscanner equipped with an automatic data system. In all experiments in which the specific activity of a sugar was determined, the substance was eluted from the strip with distilled water and its ^{14}C-content was determined using a low-background, thin window counter (Nuclear-Chicago). Analysis of the quantity of the substance was by enzymatic assay of glucose following hydrolysis of the oligosaccharide for 3 hr in 1 M HCl at 100°. In kinetic experiments in which [^{14}C]-glucose formation was measured, it was found that potassium acetate did not interfere with the chromatographic separation of the sugars; hence, prior deionization by Amberlite was not required.

Polyacrylamide Disc Gel Electrophoresis. The procedure which Reisfeld *et al.* (1962) described for the separation of proteins at low pH was modified by using a gel containing 5.5% acrylamide and 0.15% *N,N'*-methylenebisacrylamide, but buffered at pH 4.5 as described by these authors. The sample (up to 50 μl) was transferred to the top of the gel column, and 50 μl of 40% sucrose was added and carefully mixed with the sample. The advantage of using a nonelectrolyte such as sucrose rather than polymerizing the sample into a large-pore gel matrix has been pointed out by Hjertén *et al.* (1965) and by Dietz and Lubrano (1967). In the present work a short column of 7.5% acrylamide gel (pH 4.5) was polymerized above the sucrose-containing sample to stabilize the system. The reservoir buffer (pH 4.2) was that described by Reisfeld *et al.* (1962). Addition of the sample to the gel columns and all subsequent operations including the electrophoresis itself were done in a cold room at 3°. The lower reservoir contained the cathode, and a constant current of 2.5 mA/tube was passed through the apparatus (Canalco) for 1 hr. In some cases, the gel columns were immediately sliced transversely into disks 1 mm thick, using an apparatus obtained from Canalco. Each disk was extracted by cutting it up after dropping it into 0.2 ml of ice-cold 0.1 M potassium acetate buffer (pH 4.0). Enzyme assays then were done on aliquots of the extract. Other gel columns were stained for 1 hr in 0.5% aniline blue black (Matheson Coleman and Bell) in 7.5% acetic acid. Destaining was accomplished electrophoretically in an apparatus obtained from Canalco. Visual comparison was made between the stained protein zones and the location of various enzyme activities extracted from the gel disks.

Isolation and Fractionation of Subcellular Components. Sprague–Dawley strain rats weighing about 250 g were fasted

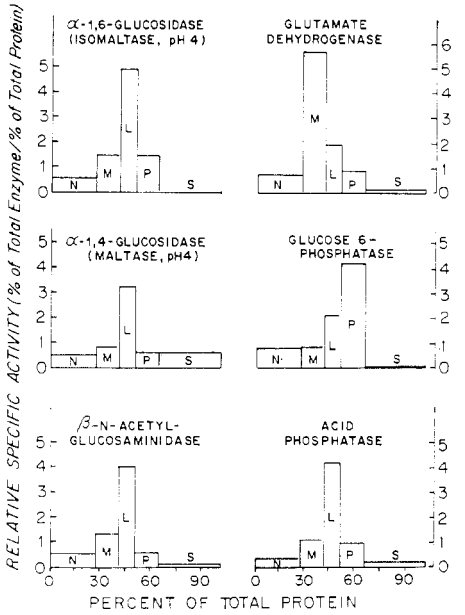

FIGURE 1: Intracellular distribution of enzymatic activities. On the abscissa, fractions are represented by their relative protein content, in the order in which they are isolated, *i.e.*, from left to right: nuclear (N), mitochondrial (M), lysosomal (L), microsomal (P), and supernatant (S).

for 24 hr before use. The animals (usually 5) were sacrificed by intraperitoneal injection of Nembutal, and their livers were removed quickly and put into ice-cold 0.25 M sucrose–0.002 M EDTA, pH 7.2. The intact livers were washed by transferring them to fresh portions of sucrose–EDTA until their exterior surfaces were essentially free of blood. Usually three such transfers sufficed. The liver tissue (about 30 g) was cut with scissors into small pieces and immediately homogenized in 200 ml of the ice-cold sucrose–EDTA, using a Sorvall Omni-mixer operated at 50 V for 30 sec. The homogenate (1:8) was fractionated centrifugally at 3° according to a procedure based on that described by Appelmans *et al.* (1955) and by de Duve *et al.* (1955) In the present work the nuclear fraction (N), the mitochondrial fraction (M), and the lysosomal fraction (L) were removed successively in the SS-34 rotor of a Sorvall RC2-B centrifuge. This rotor has a radius of 10.8 cm at the tube bottom and 5.7 cm at the fluid meniscus when tubes contain 40 ml. The microsomal fraction (P) was removed using a Spinco Model L2-65 centrifuge and a Ti 50 rotor. The integrated forces used to separate the first three particulate fractions were: N, 4,900g_{av} × min; M, 18,560g_{av} × min; and L, 160,000g_{av} × min. These values were calculated from the measured time of acceleration to the plateau value of the maximum speed used (2,250 rpm for N; 9,500 rpm for M; 19,500 rpm for L), and from the time of centrifugation at maximum speed. They do not include any contribution to the integrated force which the time of deceleration provided. Thus, they are comparable in meaning with data published from the laboratory of de Duve. It should be emphasized that the present procedure for the preparation of a lysosome-rich fraction was designed especially to minimize the loss of lysosomes into the heavy mitochondrial fraction, since a high yield of lysosomal protein was of importance in view of the several subsequent steps in the fractionation procedure which are described below. Similarly, consideration of the total amount of the lysosomal fraction which could be prepared conveniently at one time led to the use of the RC2-B centrifuge rather than the Spinco Model L centrifuge. In the present centrifugation procedure each supernatant fluid was separated from its underlying precipitate by decantation essentially as described by de Duve *et al.* (1955). The fact that these separations are difficult to reproduce and critical to the success of the fractionation has been discussed by de Duve *et al.* (1955) and Baudhuin *et al.* (1964b).

Results

Evaluation of the effectiveness of the procedure described for the fractionation of the liver homogenate into various subcellular components was made by assays for four marker enzymes. The results are shown in Figure 1 in which all graphs pertain to a single, representative preparation. The area of each block is proportional to the percentage of enzyme activity recovered in the corresponding fraction, and its height is proportional to the degree of purification achieved over the whole homogenate. The lysosomal fraction was characterized by the concentration in it of β-*N*-acetylglucosaminidase and of acid phosphatase. The relative specific activity of each of these enzymes is that which would be expected from the studies of de Duve and coworkers, when consideration is given to the fact that about 9% of the total protein of the homogenate was included in the L fraction isolated here. The recent studies of Coffey and de Duve (1968) and of Aronson and de Duve (1968) show that rat liver lysosomal enzymes can be purified as much as 50-fold over the homogenate and in a 10% yield by the use of more refined methods of centrifugal fractionation of Triton-filled lysosomes obtained by pretreatment of the animal with Triton WR-1339 (Trouet, 1964). In the present work much of the protein of the isolated L fraction was of mitochondrial and microsomal origin (Figure 1). Glutamate dehydrogenase activity was used as a marker for the presence of mitochondria and glucose 6-phosphatase for microsomes. These two enzymes, and apparently other protein contaminants as well, were removed from the glucosidase preparation in subsequent steps of the procedure. It was of special interest that both the α-1,6-glucosidase activity and the α-1,4-glucosidase activity were concentrated in the L fraction. That some maltase active at pH 4 remains soluble has been a constant finding in various preparations and accounts for the lower relative specific activity of the L fraction when it is assayed with maltose as a substrate as compared with its activity as an α-1,6-glucosidase using isomaltose as a substrate. The latter activity was not found in the soluble fraction. For this reason, in the purification procedure described below, the increase in specific activity toward each substrate is calculated on the basis of the activity present in the L fraction rather than on that found in the whole homogenate. All steps of the purification procedure were carried out in a cold room at 3°.

Solubilization of the Lysosomal Fraction. The lysosomal fraction (L) was washed twice with a volume of 0.25 M sucrose–2 mM EDTA (pH 7.2) which was equal to two times the weight of liver tissue which had been homogenized. After each washing, centrifugation was for 160,000g_{av} × min as in the first separation of the fraction. The washed pellet was sus-

pended in 80 ml of 5 mM Tris–2 mM EDTA–5 mM 2-mercaptoethanol (pH 7.0), and the suspension was frozen rapidly in a Dry Ice–2-propanol bath and then thawed under cold running water. This treatment was repeated four times. The particulate fraction which remained was separated from the soluble fraction by centrifugation at 46,000*g* for 1 hr.

Ammonium Sulfate Fractionation of the Lysosomal Extract. The soluble extract was made 42% saturated in ammonium sulfate at 3° by the addition of 243 mg of the solid salt/ml of solution. Care was taken to stir the solution during the addition of ammonium sulfate, and the pH was maintained approximately at 7.0 by the addition of 1 M KOH as required. After standing for 10 min, a small precipitate was removed by centrifugation at 46,000*g* for 20 min. The clear supernatant was made 84% saturated in ammonium sulfate by the addition of 285 mg of the solid salt/ml. After adjusting the pH to 7.0 and allowing the mixture to stand for 10 min, the precipitate was collected by centrifugation at 46,000*g* for 20 min. It was dissolved in 10 ml of 5 mM Tris–2 mM EDTA–5 mM 2-mercaptoethanol–25 mM KCl (pH 7.0) and dialyzed overnight against 2 l. of this same buffer. The resulting solution could be stored for at least 1 month in the frozen state without significant loss of any of the glucosidase activities described in this paper.

Dextran Gel Filtration. Sephadex G-100 was pretreated with urea (see Materials), washed, and then suspended in a solution (pH 7.0) containing 5 mM Tris, 2 mM EDTA, 5 mM 2-mercaptoethanol, and 25 mM KCl. The gel was deaerated, and a 1.5 × 70 cm column of it was poured and washed with the buffer. After the height of the gel bed became constant, the flow rate was adjusted to 16 ml/hr. The ammonium sulfate fraction which has been dialyzed (15 ml, 60–80 mg of protein) was added to the top of the column and allowed to enter the gel bed. After connection to a reservoir of buffer, 4-ml fractions were collected at a flow rate of 16 ml/hr. The protein of the effluent was monitored by determining its absorbance at 280 mμ, and the α-1,4-glucosidase activity was measured in acetate buffer (pH 4.0) with maltose as the substrate. Auricchio and Bruni (1967) and Auricchio *et al.* (1968) described the fractionation on Sephadex G-100 of the α-1,-4-glucosidase activity, measured at pH 3.6, which is soluble after freezing and thawing a whole homogenate of rat liver tissue. They found that the void volume of such a column contained most of the soluble protein of the homogenate as well as some glucosidase activity. However, a second peak of glucosidase activity was markedly retarded and was eluted as a protein fraction with high specific activity. In agreement with these results, in the present purification procedure a large amount of protein containing a small peak of α-glucosidase activity passed through the column without much retardation. Then, as the protein content of the effluent continued to diminish, a second and much larger peak of glucosidase activity appeared. Results from one such column are shown in Figure 2. Of interest was the finding that the glucosidase profile was very nearly the same shape when it is measured with isomaltose as a substrate (α-1,6-glucosidase) as when it is measured with maltose or with glycogen as substrates (α-1,4-glucosidase). After the absorbance at 280 mμ of the effluent had decreased to 0.070–0.075, the next 90–100 fractions were combined to give a pool which contained from 50 to 75% of the total glucosidase activity which had been loaded on the column. It was found that exclusion in this way of the first glucosidase peak as well as the leading edge of the second peak made possible a further substantial purification of the enzyme in the following step.

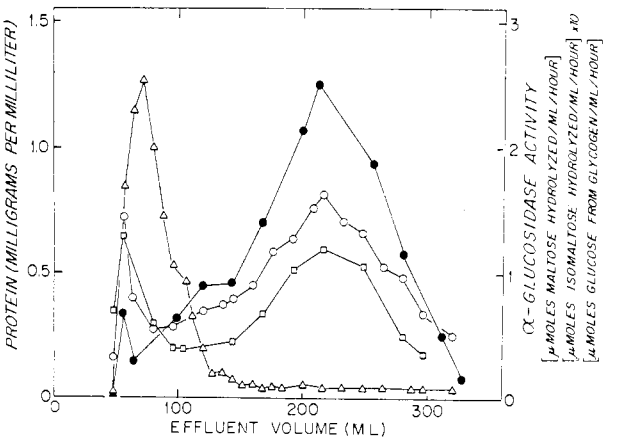

FIGURE 2: Separation on Sephadex G-100 of α-glucosidase activities. (△-△) Protein concentration determined by the method of Lowry *et al.* (1951). (○-○) α-1,4-Glucosidase activity toward maltose assayed as described in the text. (●-●) α-1,6-Glucosidase activity toward isomaltose (0.012 M) assayed as described in the text. (□-□) α-1,4-Glucosidase activity toward glycogen (1.25%) assayed as described in the text except that no KCl was added to the buffer. The column was prepared and eluted as described except that it was charged with 3 ml of ammonium sulfate fraction (44 mg of protein). Activities are expressed as units/ml of effluent from the column.

Chromatography on DEAE-cellulose. The pool of fractions from the Sephadex G-100 column was dialyzed overnight against 4 l. of 5 mM Tris–2 mM EDTA–5 mM 2-mercaptoethanol, pH 7.0, in order to reduce the KCl concentration of the solution containing the enzyme. A 1 × 20 cm column of DEAE-cellulose was prepared and equilibrated with this buffer at pH 7.0. The dialyzed solution of enzyme then was allowed to flow through the column at the rate of 40 ml/hr. Under these conditions all of the glucosidase activity but only about 15% of the total protein was retained by the column. After washing the column with 100 ml of the buffer, the glucosidase was eluted by buffer containing added 0.25 M KCl. Fractions of about 3 ml were collected slowly, and each of these was assayed for α-1,4-glucosidase, using maltose as a substrate. The protein content of these fractions was very low and could not be determined accurately. The enzyme was usually found in fractions 4 to 7 with the greatest amount being in 4 and 5. The four fractions containing the largest amount of activity were combined, and this pool was dialyzed overnight against a large volume of 5 mM Tris–2 mM EDTA–5 mM 2-mercaptoethanol, pH 7.0, containing 25 mM KCl. The resulting enzyme solution could be kept in the frozen state for short periods of time without any loss of activity. It retained about 50% of its original glucosidase activity after 1-month storage at −20°. The presence of KCl appeared to stabilize the preparation. For experiments in which a more concentrated solution of the enzyme was needed, such as those involving ultracentrifugation for the study of homogeneity and determination of molecular weight, the pool (12 ml) of fractions eluted from the DEAE-cellulose column by 0.25 M KCl was dialyzed against a large volume of the pH 7.0 buffer which contained no added KCl. The enzyme solution then was lyophilized.

TABLE I: Purification of Lysosomal α-Glucosidase from Rat Liver.[a]

Fraction	Protein Concn (mg/ml)	Total Protein (mg)	α-1,4-Glucosidase (Maltose) Sp Act. (units/mg)	Total Act. (units)	Purification	α-1,4-Glucosidase (Glycogen) Sp Act. (units/mg)	Total Act. (units)	Purification	α-1,6-Glucosidase (Isomaltose) Sp Act. (units/mg)	Total Act. (units)	Purification
Cytoplasmic extract	18.7	7760	0.088	683		0.052	402		*b*		
Lysosomal fraction (L)	10.2	828	0.379	313	1	0.435	360	1	0.076	62	1
Soluble extract of L	1.68	133	2.23	296	5.9	2.44	325	5.6	0.432	57	5.7
Ammonium sulfate fraction	5.04	75.6	3.05	230	8.1	4.63	350	10.6	0.694	52	9.1
Sephadex G-100 pool	0.037	14.3	11.3	162	30	19.6	280	45	2.61	37	34
DEAE-cellulose eluate, dialyzed	0.012	0.160	374	60	985	693	111	1590	89.3	14	1175
Lyophilized and redissolved	0.031	0.082	464	38	1220	680	56	1560	101	8.3	1330

[a] The preparation described was from 30 g of liver. The cytoplasmic extract is that part of the whole homogenate which remains after the nuclear fraction (N) has been removed (see Methods). The three glucosidase activities are identified by the substrate with which the assay data were obtained. The purification is calculated as the ratio of the specific activity at each step relative to that of the lysosomal fraction (L). [b] The assay of α-1,6-glucosidase activity in the extract is not analytically satisfactory because the formation of glucose from endogeneous substrates is large compared with that from added isomaltose.

The powder was dissolved in 1–1.5 ml of buffer containing added 25 mM KCl and then dialyzed against 1 l. of this solution. The final enzyme preparation usually contained less than 100 μg/ml of protein. It could be kept in the frozen state for at least 2 weeks without loss of activity. After thawing the solution, its activity was lost on being kept overnight at 4°.

A summary of the purification of lysosomal α-glucosidase from 30 g of rat liver is given in Table I. By this procedure the enzyme was obtained in a yield of 0.001% of the total protein of the cytoplasmic extract. The final preparation contained about 13% of the total units of glucosidase activity which were present in the lysosomal fraction, and the data in Table I show that the specific activity of the enzyme was increased about 1300 times over that of the lysosomal fraction. Data obtained from numerous preparations show that the degree of purification of the enzyme at each step of the procedure is nearly the same whether the substrate used for its assay is maltose, glycogen, or isomaltose. Such small differences as appear to exist in relative activity toward these three substrates are due to the apparently greater recovery of activity toward glycogen than toward either of the disaccharides in the ammonium sulfate fractionation step. Although the data in Table I suggest that this difference may be substantial, it has not been a constant finding in all preparations. The similarity between the degree of purification of the α-1,4-glucosidase activity and that of the α-1,6-glucosidase activity at each step of the procedure is the most conspicuous feature of the results of purification. This finding suggests that a single enyme may have these two different activities. Other data bearing on this point will be discussed below and in the accompanying paper (Jeffrey *et al.*, 1970). The numerical values for the specific activities of the final preparation determined with each substrate and given in Table I are not the maximum values which can be obtained for the purified enzyme. This is especially true of its activity toward isomaltose, since, during the purification procedure, assays were made using a concentration of this substrate which was only about equal to its K_m (Jeffrey *et al.*, 1970). This was done in order to conserve the substrate. As will be discussed in more detail below, some increase in the activity of the glucosidase toward maltose and isomaltose could also be obtained by making changes in the ionic composition of the buffer used in the assay. Because of the fact that substrate inhibition by maltose is prominent at high concentrations of this substrate (Jeffrey *et al.*, 1970), the apparent specific activity of the glucosidase can be increased still further by assaying it at a lower maltose concentration than that which was used routinely. However, it was found that the numerical value for the degree of purification at each step of the procedure was not changed thereby. In addition to the influence of these factors, the absolute value of the specific activity of the enzyme toward any of the substrates is made somewhat uncertain by the fact that the protein concentration of the preparation was quite low at each of the last three steps in the procedure and was difficult to determine accurately because of the high blank in the protein assay due to the buffer alone.

Figure 1 shows that the lysosomal fraction (L) contained protein of mitochondrial and microsomal origin, as well, of course, as enzymes known to be localized within lysosomes. The effectiveness of the glucosidase purification procedure in removing some of these enzyme contaminants was studied. Only 2.7% of the glutamate dehydrogenase which was present in the L fraction was active in the soluble preparation ob-

tained after repeated freezing and thawing of the fraction followed by centrifugation at 46,000*g*. No glutamate dehydrogenase activity could be detected in the principal peak of glucosidase activity which emerged from the Sephadex G-100 column. Similarly, 3.8% of the glucose 6-phosphatase activity in the L fraction was found in the soluble extract of this fraction. Of this small amount, about 60% was present in the ammonium sulfate fraction which contained the glucosidase. Separation of the two activities was possible, however, since the initial protein peak from the Sephadex G-100 column contained all of this microsomal enzyme contaminant, while the glucosidase peak emerged later and was free of the phosphatase.

Separation of the lysosomal enzyme, β-*N*-acetylglucosaminidase, from the α-glucosidase was achieved for the most part by taking advantage of the fact that the former enzyme was found to be soluble to the extent of only about 2% after freezing and thawing the lysosomal pellet. The firm binding of β-*N*-acetylglucosaminidase to lysosomal membranes has also been described recently by Weismann *et al.* (1967). After the two-column fractionation steps in the glucosidase purification procedure, 0.1% of the activity of β-*N*-acetylglucosaminidase originally present in the L fraction remained as a minor contaminant in the most highly purified glucosidase preparation.

Lysosomal acid phosphatase was a prominent contaminant of the glucosidase preparation until the step in the procedure when the protein was subjected to fractionation on Sephadex G-100. At this point, all of the phosphatase emerged from the column in the initial protein peak. The glucosidase peak, which was retarded and emerged later as a dilute protein solution, contained no detectable acid phosphatase, and none could be found in the final glucosidase fraction which had been concentrated by lyophilization.

Disc Gel Electrophoresis. Although polyacrylamide disc gel electrophoresis at either pH 4.2 or 8.3 could be used to follow the removal of many contaminating proteins from the α-glucosidase preparation during the purification of the enzyme, the principal band due to the enzyme itself (see below) was best resolved from other proteins at pH 4.2. Accordingly, several highly purified enzyme preparations were subjected to electrophoresis at pH 4.2 according to the procedure described above (see Methods). In every case only two bands were visible and these were well separated. A faint band was located near the top of the gel column after 1-hr electrophoresis at 2.5 mA/tube. A second band, containing about 90% of the stainable protein, had moved more than halfway down the column in the same time. Elution of segments of an unstained gel column and assay of the eluates for each of the enzymic activities attributed to the glucosidase showed that only the faint, more slowly moving band had enzymic activity. Both α-1,4-glucosidase and α-1,6-glucosidase activities were demonstrable in this band. However, only about 10% of the total glucosidase activity which had been loaded on the gel column could be recovered in this protein band. Although these observations taken together might seem to indicate that the purified enzyme was not a homogeneous protein preparation, it is possible that dissociation of the protein had occurred during electrophoresis at acid pH, and that only the undissociated, slower moving form of the protein has enzymatic activity. Because of the small amount of protein available, it was not possible to test this hypothesis by subjecting the enzyme which had been eluted from the gel to repeated electrophoresis. In various experiments, the intensity of staining of both protein bands was found to increase in direct proportion to the number of enzyme units added to the column and in proportion to the specific activity of the preparations being examined.

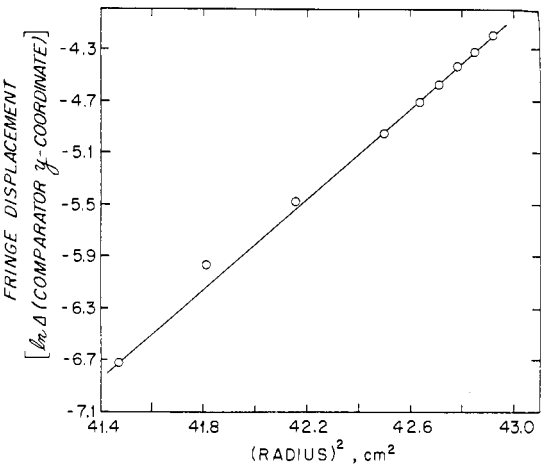

FIGURE 3: Equilibrium ultracentrifugation of α-glucosidase at 16,200 rpm at 5.6°. See the text for other details. The fringe displacement at each radial distance was calculated from the average of the measurements for five fringes. The straight line shown is that calculated by the method of least squares using a computer program.

Molecular Weight and Homogeneity. The purified lysosomal α-glucosidase was not obtained in sufficient quantity to permit a study of its homogeneity in the ultracentrifuge using schlieren optics. A dilute solution of the protein (86 μg/ml) was subjected to equilibrium ultracentrifugation by the meniscus depletion method of Yphantis (1964) using a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. The protein was centrifuged in 5 mM Tris–1 mM EDTA–2 mM 2-mercaptoethanol, pH 7.1, containing added 0.1 M KCl to minimize convection effects. Figure 3 shows the results of this experiment, plotted so that the slope of the resulting line can be used to estimate the weight-average molecular weight of the preparation. The partial specific volume of the protein is unknown. If a value of 0.730 ml/g is assumed for $\bar{v}$, the calculated molecular weight of the glucosidase is 107,000, while the assumption that $\bar{v}$ = 0.740 ml/g leads to a value of 111,000. The points in Figure 3 which were obtained from measurements of fringe displacement in the region of greatest protein concentration seem to lie on a straight line. This fact suggests that the preparation examined was reasonably homogenous. Of course, more complete centrifugal studies carried out at higher protein concentrations would be necessary before such a conclusion could be fully justified.

A sample of the most highly purified α-glucosidase was mixed with a solution containing three reference enzymes of known molecular weight, and the mixture was centrifuged in a linear sucrose gradient (5–20% sucrose) so that the distribution of α-1,4-glucosidase and of α-1,6-glucosidase activity could be compared and an apparent molecular weight for each calculated according to the procedure of Martin and Ames (1961). Because of the stabilizing effect of KCl on the lysosomal enzyme, the sucrose gradient was prepared in a dilute Tris buffer containing 25 mM KCl. Figure 4 shows the results

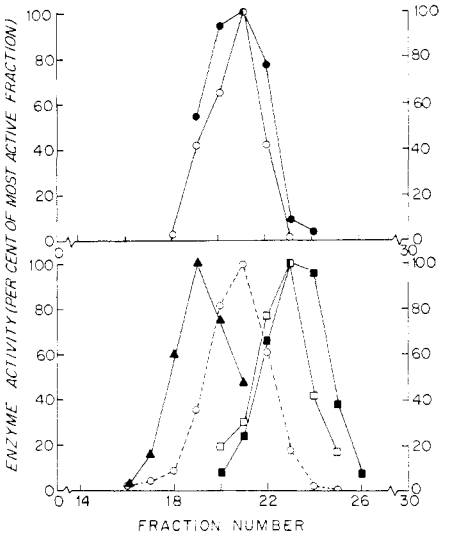

FIGURE 4: Sucrose density centrifugation of α-glucosidase in the presence of added reference enzymes. The glucosidase activity was determined using three substrates and the results for each substrate are plotted separately. For clarity two of these sets of data are shown in the upper part of the figure. All curves are from data obtained from 30 fractions of a single gradient tube: (O---O) α-glucosidase activity toward maltose; (O-O) α-glucosidase activity toward glycogen; (●-●) α-glucosidase activity toward isomaltose; (▲-▲) lactic dehydrogenase activity; (□-□) L-α-glycerophosphate dehydrogenase activity; (■-■) phosphoglucomutase activity. The glucosidase (5 μg; 650 units of activity toward glycogen/mg of protein) was mixed with the reference enzymes in 5 mM Tris–2 mM EDTA–5 mM 2-mercaptoethanol–25 mM KCl, pH 6.6, and centrifuged in the SW 65 L Ti rotor at 60,00 rpm for 7 hr at 1°.

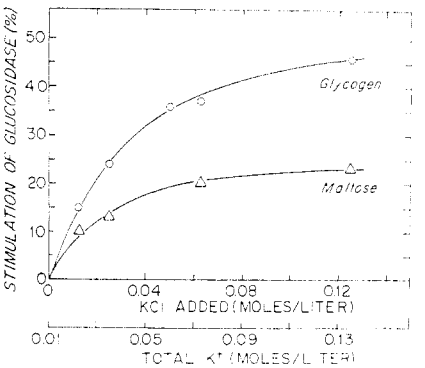

FIGURE 5: Effect of KCl on α-1,4-glucosidase activity. Standard assay conditions were used (see the text for details) except that, when glycogen was the substrate, the potassium acetate buffer (pH 4.2) was 0.05 M. Results are expressed as the per cent stimulation of the activity found without added KCl.

of such an experiment. It was found that the peak of α-1,4-glucosidase measured with either maltose or glycogen as substrate was in the same position in the gradient as the peak of α-1,6-glucosidase activity measured with isomaltose as substrate. Furthermore, the shapes of the three curves showing the distribution of the glucosidase activities in the gradient were similar. The seemingly greater width of the isomaltose activity curve near its peak can not be regarded as significant in view of the fact that the activity of the enzyme toward this substrate is only about one-seventh of that toward glycogen under the conditions used for assay. Thus, the accuracy of the isomaltase activity determinations was less than that of the other enzyme assays. From the data of Figure 4 the molecular weight of the lysosomal glucosidase was calculated to be 113,000 using L-α-glycerophosphate dehydrogenase (molecular weight 78,000) as the reference protein, 115,000 using phosphoglucomutase (molecular weight 67,100) as the reference protein, and 114,000 using lactic dehydrogenase (molecular weight 150,000) as the reference protein. These closely similar values for the molecular weight of the glucosidase are also in reasonable agreement with values calculated from the sedimentation equilibrium studies described above. The results from sucrose density centrifugation support the conclusion drawn from the results of the purification studies that it is probable that a single lysosomal protein has both α-1,4-glucosidase and α-1,6-glucosidase activities.

Dependence of Glucosidase Activity on pH and Salt Concentration. In preliminary work a partially purified preparation of the enzyme appeared to be maximally active toward maltose and isomaltose when incubated at 37° in acetate buffer, pH 4 (Jeffrey *et al.*, 1967). In view of the fact that KCl subsequently was found to increase the stability of the more highly purified enzyme during storage, the influence of this salt on enzyme activity toward glycogen and maltose was studied at pH 4, preliminary to making a more complete study of the dependence of activity on pH. The results are shown in Figure 5. It was found that addition of KCl to a potassium acetate–acetic acid buffer produced a greater stimulation of glucosidase action on glycogen than on maltose. Data from other experiments not included in this figure showed that the initial rate of glucose formation from glycogen was 28% greater in acetate buffer at pH 4.1, which contained 0.2 M KCl, than in a buffer of the same pH containing 0.1 M KCl. Hence, a comparison was made of the pH dependence of glucosidase activity of a purified enzyme preparation acting on each of the three substrates at 37° in 50 mM potassium acetate–acetic acid buffers which contained various amounts of KCl added so that the total concentration of K^+ was 0.2 M at every pH. The results are shown in Figure 6. The fact that the pH optimum for activity toward maltose appeared to be lower than that for the other two substrates prompted an investigation of the reaction in other buffers. When citrate was substituted for acetate and the concentration of K^+ maintained at 0.2 M, the glucosidase had about 20% more activity toward glycogen and about 15% more activity toward maltose over the entire pH range investigated. Thus, the shapes of the pH–activity curves were essentially the same as those shown in Figure 6. Similarly, the use of a mixed citrate–phosphate buffer containing added KCl had little influence on the dependence of activity on pH.

The stimulation by KCl of α-1,4-glucosidase action on glycogen was strikingly dependent on pH when studied in either acetate or histidine buffer. This dependence is illustrated by the data of Figure 7 which show that glucose formation from glycogen (20 mg/ml) is stimulated fivefold by 0.2 M KCl at pH 4.0. In other experiments it was found that the much smaller effect of KCl on maltose hydrolysis (Figure 5) and isomaltose hydrolysis was evident at all pH values. Thus, the shift in pH optimum toward more acidic values which is produced by KCl and the large degree of salt activation of the enzyme are characteristic of its action on the polysaccharide substrate, glycogen, and are not seen with either disaccharide

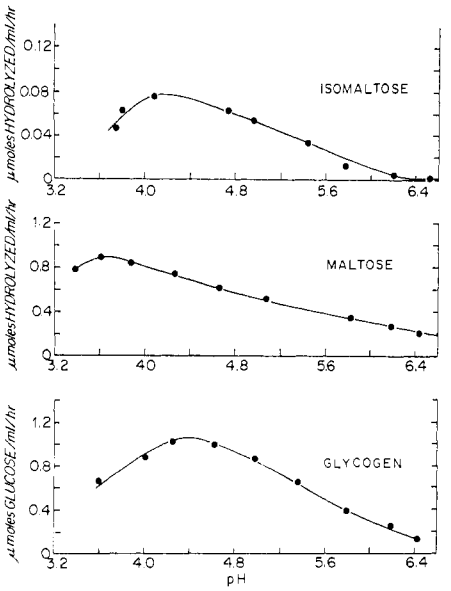

FIGURE 6: Dependence of α-glucosidase activity on pH. All reaction mixtures contained 2 μg/ml of enzyme in 0.05 M potassium acetate–acetic acid buffer to which was added KCl so that the total K^+ was 0.2 M. Otherwise the composition of the reaction mixtures was as described for the standard assays except that isomaltose was 8.1 mM. The linearity with time of the assays at 37° at various pH values was verified experimentally. The measurement of pH was at 37°.

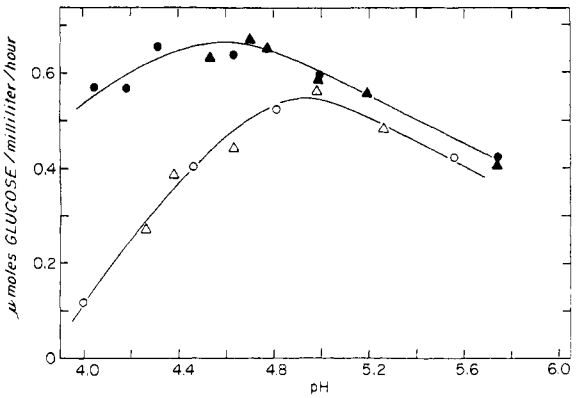

FIGURE 7: pH dependence of KCl stimulation of α-1,4-glucosidase action on glycogen. All reaction mixtures contained 2% glycogen and were incubated at 37° for 30 min with 1 μg/ml of enzyme: (○-○) 0.01 M potassium acetate buffer; (△-△) 0.01 M histidine-Cl^- buffer; (●-●) 0.01 M potassium acetate buffer + 0.2 M KCl; (▲-▲) 0.01 M histidine-Cl^- buffer + 0.2 M KCl. Measurement of pH was at 37°.

substrate tested. It was of interest to determine whether these effects of KCl can be attributed specifically either to the anion or to the cation, or whether the rate of hydrolysis of glycogen is influenced principally by the ionic strength of the medium. This question was studied by comparing the pH dependence of the reaction in 0.16 M histidine–HCl buffers with that in 4 mM acetate buffers containing added 0.16 M KCl. The pH–activity curve in the presence of a high concentration of histidine-Cl^- was found to be very broad and flat, and it showed little dependence on pH between pH 3.3 and 5.6. When compared with the rate of reaction in 10 mM histidine buffer, shown in Figure 7, the rate of the reaction in the 0.16 M histidine–HCl buffer at pH values above 4.1 was inhibited to the extent of more than 50%. The fact that a high concentration of histidine-Cl^- causes inhibition, while KCl addition to either acetate or histidine buffers produces a large stimulation of activity below pH 4.8, suggests that the stimulatory effect of KCl is due largely to K^+ rather than either to Cl^- or to a general effect of ionic strength. Additional support for this conclusion was provided by the finding that addition of KCl, KBr, KI, or K_2SO_4 to an acetate buffer (pH 4) containing 10 mM K^+ produced about the same amount of stimulation (25–30% at 25 mM added salt and 35–40% at 50 mM added salt) irrespective of the nature of the anion. Similarly, equimolar concentrations of KCl, NaCl, LiCl, and NH_4Cl (10–135 mM) were about equally effective in stimulating the initial rate of glucose formation from glycogen by the glucosidase. That such stimulation is not confined to univalent cations was shown by the finding that 0.1 and 0.2 M solutions of KCl, $CaCl_2$, and $MgCl_2$ were equally stimulatory at pH 4.0. In the light of all the data available, the α-1,4-glucosidase action of the enzyme on a high concentration of glycogen is best described as being especially responsive to stimulation by inorganic cations. Some additional observations on the effect of KCl on the kinetics of the reaction with glycogen are discussed in the following paper (Jeffrey *et al.*, 1970).

Specificity of Glucosidase Action. The purified enzyme had no detectable activity in catalyzing the hydrolysis of the following oligosaccharides: D-(+)-cellobiose, D-(+)-melibiose, lactose, D-(+)-turanose, D-(+)-melezitose, sucrose, D-(+)-raffinose, and stachyose. Each of these substances (0.05 M) was incubated in 0.05 M acetate buffer, pH 4.0, with 9 μg/ml of the enzyme for 1 hr at 37°. Afterward, assay for glucose was by the coupled enzymatic procedure described above. If fructose was a constituent of the oligosaccharide, assay for it also was made by adding phosphoglucoseisomerase (Boehringer) to the analytical system for glucose. The results showed that the lysosomal glucosidase appeared to have no action on the β-1,4-glucosidic bond or on the α-1,6-galactosidic or β-1,4-galactosidic bonds in disaccharides in which there is a terminal reducing glucose unit. Oligosaccharides of glucose in which there are both α-1,6- and α-1,4-glucosidic bonds, such as panose and the series of singly branched compounds isolated from an α-amylase digest of glycogen (Brown *et al.*, 1965), are substrates for the enzyme. In the case of these latter substances, the α-1,4-glucosidic bonds peripheral to the branch point glucose unit are hydrolyzed much more rapidly than the single α-1,6-glucosidic bond which each compound contains. When uniformly labeled ^{14}C compounds of this series were prepared as described by Brown *et al.* (1965) and incubated with the glucosidase, it could be shown (B. I. Brown and D. H. Brown, 1969, unpublished data) that the hydrolysis to [^{14}C]-glucose which had occurred was due to combined α-1,4-glucosidase and α-1,6-glucosidase action. It could also be shown that debranching of the oligosaccharides by α-1,6-glucosidase action was the rate-limiting step in their total hydrolysis to glucose by the lysosomal enzyme. The relevance of these observations to the total degradation of glycogen by the enzyme is discussed in the following paper (Jeffrey *et al.*, 1970).

Glucosyltransferase Activity. The occurrence of transglucosylation reactions in mammalian tissues has been studied by a number of investigators using partially purified enzyme preparations (Giri *et al.*, 1955; Stetten 1959; Lukomskaya,

TABLE II: Glucosyltransferase Activity of Lysosomal α-Glucosidase.[a]

Preparation and Fraction	α-Glucosidase Activity			Glucosyltransferase Activity: Oligosaccharide Formation from [^{14}C]Maltose						Glucose Incorporation into Glycogen from [^{14}C]Maltose	
	(Maltose) Purification	(Glycogen) Purification	(Isomaltose) Purification	[^{14}C]Maltotriose		[^{14}C]Maltotetraose		[^{14}C]B_3^b			
				Sp Act. (units/mg)	Purification	Sp Act. (units/mg)	Purification	Sp Act. (units/mg)	Purification	Sp Act. (units/mg)	Purification
1. Cytoplasmic extract				0.0635		0.0056		0.0172		0.075	
1. Lysosomal fraction	1	1	1	0.121	1	0.0148	1	0.041	1	0.099	1
1. Ammonium sulfate fraction	5.0	8.7	8.1	0.354	2.9	0.0525	3.6	0.123	3.0	0.81	8.2
1. DEAE-cellulose eluate, dialyzed	1320	827	1008	69.9	579	10.2	687	33.4	820	94.6	959
2. DEAE-cellulose eluate, dialyzed, lyophilized	1055	1540	1470	61.5	*c*	6.4	*c*	32.2	*c*	*c*	*c*

[a] At several stages of purification the preparation was tested for its activity in catalyzing the intermolecular transfer of glucosyl units at pH 4 (see Methods). The degree of purification is the ratio of the specific activity of each fraction to that of the lysosomal fraction. The data are from two preparations which were different from the one described in Table I. [b] B_3 is a branched trisaccharide of unknown structure (see text). [c] Not calculated, since the specific activity of the lysosomal fraction in preparation 2 was not determined.

1960; Lejeune *et al.*, 1963; Hers, 1963; Torres and Olavarria, 1964; Brown and Brown, 1965). It has usually been concluded that various glucosidases are responsible for these reactions which often proceed *in vitro* concomitantly with the hydrolysis of oligosaccharide substrates or of glycogen. It was of interest to measure the glucosyltransferase activity in various ways during the purification of the rat liver lysosomal glucosidase. Table II contains the results expressed as the specific activity of the enzyme at each stage of the preparation with respect to its synthesis of each major transfer product from uniformly labelled [^{14}C]maltose. For ease of comparison the degree of purification of each activity is also calculated based on that of the whole lysosomal fraction taken as unity. The columns headed [^{14}C]B_3 contain data pertaining to the synthesis of an apparently pure trisaccharide of glucose which is not maltotriose, and which is designated as having a branch point (B_3) to distinguish it from the linear, maltosidically linked series of oligosaccharides having exclusively α-1,4-glucosidic bonds. Some information about the structure of this oligosaccharide is discussed below.

The data in Table II show that transferase activity at pH 4.0 was enriched in the lysosomal fraction and parallels the purification of the glucosidase thereafter. The correspondence in degree of purification as shown by the different assays is not perfect. However, the transferase assays are somewhat inaccurate, since their results show only the balance which exists at the end of incubation between the rate of formation of each compound by glucosyl transfer and the opposing rate of its hydrolysis in the presence of maltose which may act as a competitive inhibitor whose concentration is constantly decreasing during the time of reaction. In order to obtain sufficient transfer products to make feasible their accurate estimation, incubation was for 1 hr, and it is possible that the average rate which was measured was not a maximal initial rate. Alternatively, it is possible that the glucosidase and the glucosyl transferase reactions compete with each other, so that the latter might actually be more prominent after the maltose concentration has decreased by hydrolysis to a lower value than its initial one. With all of these factors in mind, it seems reasonable to conclude that the data of Table II support the idea that the purified rat liver lysosomal glucosidase has the intrinsic property of being a transglucosylase active on oligosaccharides, as well as being a hydrolytic enzyme. In human liver the lysosomal glucosidase has not been shown to catalyze simple transglucosylation reactions involving oligosaccharides alone (Hers, 1963; Brown and Brown, 1965), but this apparent difference from the rat liver enzyme may be due to the fact that more highly purified preparations of the human enzyme have not been studied in this regard (Auricchio *et al.*, 1968). Similarly, the α-glucosidase active at acid pH and prepared as a pure protein from bovine liver by Bruni *et al.* (1969) has not been studied with regard to any transglycosylation properties which it might have.

Glucose incorporation from uniformly labeled [^{14}C]maltose into glycogen was measured at various stages of the purification of the rat liver lysosomal glucosidase, and the results are shown in the last two columns of Table II. In this case, the correspondence was good between the degree of purification of transglucosylase activity and the purification of glucosidase activity toward glycogen, and, thus, these two activities appear to fractionate together. This latter result might have been expected from the fact that transglucosylation from maltose

into glycogen at pH 4 has been shown by Hers (1963) to be characteristic of normal human tissues but not to be seen in tissues from patients with type II glycogen storage disease, in which the glucosidase active at acid pH is missing. However, Hers and van Hoof (1968) have recently reported cases of the disease in which there appeared to be some dissociation of hydrolytic and transfer activity of the glucosidase. Furthermore, they have presented data suggesting that the incorporation of glucosyl residues into glycogen at pH 3.5 by partially purified rat liver glucosidase is partly into α-1,4-glucosidic linkage and partly into α-1,6-glucosidic linkage. Since the latter action would seem to be consistent with our finding of isomaltose hydrolysis by the purified enzyme, we have re-examined the nature of the linkage in the polysaccharide of [^{14}C]glucose units which have been transferred from [^{14}C]-maltose to glycogen. Incubation of 48 mg of glycogen with 100 μmoles of uniformly labeled [^{14}C]maltose (1.2×10^7 cpm) and 86 μg of purified glucosidase in 2.6 ml of 0.04 M acetate–0.08 M KCl buffer, pH 4.2, for 4 hr at 37° resulted in the formation of radioactive glycogen containing about 1% of the ^{14}C which would have been available for transfer to it from maltose if none of the disaccharide had been hydrolyzed to glucose by α-1,4-glucosidase action. The [^{14}C]glycogen (20 cpm/μmole of polymeric glucose) could be degraded to the extent of more than 99% to glucose 1-phosphate and glucose by the combined action of phosphorylase and the debranching enzyme system as described above (see Materials and Methods). Paper chromatography of the deionized dialysate showed that the glucose which had been formed by amylo-1,6-glucosidase action on the branch points of the [^{14}C]glycogen contained less than 5% of the total ^{14}C of the starting polysaccharide. In another experiment a similar amount of ^{14}C was found in the 1.2% of glucose which was formed by the direct and prolonged action of amylo-1,6-glucosidase alone on the [^{14}C]glycogen. Thus, there appeared to be very little [^{14}C]glucose in the polymer in α-1,6-linkage susceptible to hydrolysis by amylo-1,6-glucosidase. The quantity of [^{14}C]glucose present in α-1,4-linkage was estimated by determining the amount of [^{14}C]glucose 1-phosphate formed when the glycogen was degraded by two successive treatments with phosphorylase. In the first incubation, 37% of the molecule was degraded, and the glucose 1-phosphate contained 48% of the total ^{14}C originally present. In the second incubation another 4.8% of the molecule was converted into glucose 1-phosphate and this contained 16% of the total ^{14}C. Thus, although 64% of the ^{14}C transferred from maltose to glycogen appeared as glucose units susceptible to phosphorylase digestion, one-quarter of this amount was surprisingly resistant to phosphorolysis. [^{14}C]Glucose units which are very close to the outer branch points of glycogen would be expected to be relatively slow in being removed by phosphorylase action. Approximately 35% of the total ^{14}C in the labeled glycogen preparation remained in a polymer wholly resistant to enzymatic degradation by the methods described. However, less than 1% of the total polymeric glucose of the labeled glycogen was associated with this 35% of the isotope. Thus, the specific activity of the resistant polymer was much higher than that of the total labeled glycogen. It is possible that in the course of [^{14}C]glucose transfer from maltose to glycogen by the lysosomal glucosidase some glucosyl units are attached in a linkage which is neither α-1,4 nor α-1,6. Alternatively, it is possible that some [^{14}C]glucose units are attached in α-1,6 linkage to the nonreducing terminal units of some outer chains of glycogen. The resulting structure might prevent enzymatic degradation of that part of the polysaccharide molecule. This is a possibility inferred from present knowledge of the specificity of amylo-1,6-glucosidase (Brown and Brown, 1966). Because it has been possible to obtain so little of the [^{14}C]polymer of high specific activity, no direct evidence has been obtained regarding its structure.

The formation of a radioactive oligosaccharide, B_3, by incubation of the glucosidase with uniformly labeled [^{14}C]-maltose has been described above. This substance was isolated by eluting it from paper chromatograms prepared and developed as described in Methods. The mobility of the compound in 1-butanol–pyridine–water (3:2:1.5) is such that it occupies a position almost exactly midway between maltotriose and maltotetraose and slightly ahead of the branched trisaccharide, panose. Cochromatography of panose and the ^{14}Ccompound clearly showed that they are not identical. The total glucose content of B_3 was determined enzymatically after hydrolysis for 2.5 hr in 1 M HCl at 100°. The reducing power of the unhydrolyzed substance was compared with that of several pure oligosaccharides of glucose by the method of Park and Johnson (1949). On the assumption that B_3 is, in fact, a trisaccharide, as its chromatographic mobility suggests, its reducing power was found to be significantly lower than that of maltotriose but exactly the same as that of panose. It was considered possible that B_3 could be the trisaccharide, 4,6-di-*O*-(α-D-glucopyranosyl)-D-glucose which was synthesized by de Souza and Goldstein (1964). Chromatographic comparison of B_3 with a genuine sample of this synthetic trisaccharide showed that the two substances were not identical. Further information about the structure of B_3 was sought by incubating this uniformly labeled trisaccharide of [^{14}C]glucose (75,000 cpm/μmole) at a concentration of 0.1 mM with the purified lysosomal glucosidase in 30 mM acetate buffer, pH 4.2, at 30°. At various times, aliquots of the solution were cochromatographed with a mixture of pure oligosaccharides of glucose in order to find whether any ^{14}C-containing compounds could be identified as intermediates in the course of hydrolysis of B_3 to [^{14}C]glucose. It was found that [^{14}C]maltose and [^{14}C]glucose were the only detectable substances present on the chromatograms prepared during an incubation which was continued until 44% of the ^{14}C was present as glucose. At this time, the mole per cent of each component in the mixture was: B_3, 16%; maltose, 18%; glucose, 66%. At earlier times, maltose was relatively more abundant and glucose relatively less so. It was particularly noteworthy that no ^{14}C was found in the isomaltose region of the chromatogram. These results strongly suggest that B_3 is a trisaccharide of glucose in which a glucose unit is attached to maltose by a bond which is more susceptible to hydrolysis by the lysosomal glucosidase than is the maltosidic bond itself. Direct comparison with known substances has shown that such a bond cannot be of the α-1,6- type, since B_3 is neither panose nor 4,6-di-*O*-(α-D-glucopyranosyl)-D-glucose. In view of the facts that biosynthesis of this compound appears to be by a glucosyltransferase reaction from maltose, and that maltose but not isomaltose is an intermediate in its enzymatic degradation by the lysosomal glucosidase, there also is little likelihood that B_3 is 6-α-maltosylglucose ("isopanose") which has been isolated from a partial acid hydrolysate of pullulan (Bouweng *et al.*, 1963) and also synthesized enzymatically (French *et al.*, 1964). Lukomskaya (1960) has reported that protein fractions derived

from rabbit muscle and liver can catalyze the synthesis from maltose at pH 6.8 of isomaltose, nigerose, maltotriose, panose, and a trisaccharide which was stated to have possibly an α-1,3-glucosidic bond linking two of its glucose units. However, at pH 4.8 only oligosaccharides with α-1,4-glucosidic linkages were formed. Torres and Olavarria (1964) found that a protein fraction from dog liver was able to effect the synthesis of oligosaccharides with α-1,3- or α-1,6-glucosidic linkages when it was incubated at neutral pH with maltose and [^{14}C]-glucose. Another enzyme fraction from dog liver which was more active as a glucosidase at acid pH was found to catalyze transglucosylation reactions in which only α-1,4-glucosidic bonds were formed. Taken together these reports indicate that liver may contain an enzyme capable of the synthesis of the α-1,3-glucosidic bond by a transglucosylation reaction from maltose at neutral pH. Because of the very small amount of B_3 available it has not yet been possible to obtain conclusive evidence as to the possible presence of an α-1,3-glucosidic bond in this trisaccharide which, however, is formed at pH 4.0 by a transglucosylation reaction from maltose catalyzed by the purified lysosomal glucosidase.

Discussion

The primary aim of the present work was to investigate whether liver lysosomes contain an enzyme system capable of the debranching of glycogen. Such an enzyme would be needed if glycogen were to be totally degraded to glucose. Lejeune *et al.* (1963) clearly showed that an α-1,4-glucosidase is present in lysosomes from rat liver. The action of such an enzyme, if it were specific, would stop after all of the glucose units present in maltosidic linkage were removed from the outer chains of glycogen. Since there is no evidence that any such limit dextrin of glycogen accumulates within lysosomes in normal mammalian liver, either debranching of the polysaccharide must occur, or the fraction of total intracellular glycogen catabolism which takes place within the lysosomal compartment of the cell is very small. Initial experiments (Jeffrey *et al.*, 1967) indicated that an α-1,6-glucosidase activity ("isomaltase") with an acid pH optimum seemed to be isolable from lysosomes. However, so many glucosidase activities have been reported to be present in liver that only a rigorous purification procedure could establish whether the isomaltase activity which had been detected could be assigned with certainty to the lysosomes.

An α-glucosidase has now been purified approximately 1300-fold from the lysosomal fraction as isolated by differential centrifugation of a sucrose homogenate of rat liver. This enzyme has both α-1,4-glucosidase and α-1,6-glucosidase activities at acid pH. These activities band together in a sucrose density gradient and, using appropriate reference proteins, the molecular weight of the enzyme has been calculated to be 114,000. Equilibrium sedimentation of a dilute solution of the protein showed that it was substantially homogeneous and that its molecular weight is about 109,000. In addition to this evidence of homogeneity, it was found that the two kinds of hydrolytic activity fractionated together during the purification procedure. Kinetic studies of the action of the enzyme on several oligosaccharide and polysaccharide substrates are reported in the following paper (Jeffrey *et al.*, 1970), together with evidence that the enzyme is able to catalyze the total degradation of glycogen to glucose. In an accompanying paper (Brown *et al.*, 1970), it is shown too that both α-1,4-glucosidase and α-1,6-glucosidase activity at acid pH are simultaneously absent from the tissues of children with type II glycogen storage disease, in which it has been found that there is intralysosomal storage of glycogen in the liver (Baudhuin *et al.*, 1964a) as well as in other tissues.

It has been found that the activity of the lysosomal glucosidase toward glycogen can be greatly stimulated by monovalent and divalent cations, and that the degree of stimulation is dependent upon pH. The effect is so pronounced at pH 4 (Figure 7) that the possibility might be considered that intralysosomal glycogen catabolism *in vivo* could be regulated to some extent by changes in the pH and/or salt concentration within the organelle. Further speculation on this point is not possible without some knowledge of the ionic composition of the lysosomal contents in the region where glycogen and the glucosidase might be in contact. In this connection, it is of interest that the glucosidase activity can be made wholly soluble by repeated freezing and thawing of the particles. Hence, there is no evidence that it is bound to the lysosomal membrane. The shape of the pH–activity curve for maltose as substrate is different from that for glycogen. In this respect, the preparation studied here seems to differ from the enzyme recently isolated from whole bovine liver (Bruni *et al.*, 1969). Other differences are discussed in the following paper (Jeffrey *et al.*, 1970).

The purified glucosidase has been shown to possess the ability to catalyze various transglucosylation reactions. That this activity is not the result of contamination by other enzymes is suggested by the fact that the specific activity of each transglucosylase activity which was measured increased parallel with the increase in glucosidase activity during enzyme purification. However, there is no evidence as to whether these transglucosylation reactions should be regarded as having any physiological importance within the lysosome.

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