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PREPARATION AND PROPERTIES OF THE GLYCOGEN-DEBRANCHING ENZYME FROM RABBIT LIVER

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SUMMARY

The glycogen-debranching enzyme, oligo-α-1,4-glucan: α-1,4-glucan-4-glycosyltransferase–amylo-1,6-glucosidase has been purified about 500-fold from rabbit liver. The enzyme preparation has been shown to have both transferase and glucosidase activities. In view of the fact that the protein moves as one band in disc-gel electrophoresis and possesses the two activities in constant ratio throughout its purification, it is concluded that one protein in fact possesses both activities. The molecular weight of the enzyme has been found to be about 179 000 by sucrose-density gradient centrifugation. The enzyme is strongly inhibited when assayed in Tris buffer and somewhat less so in imidazole buffer as compared with citrate or phosphate buffers in which the optimum for activity is at about pH 6 when a limit dextrin of glycogen, prepared by prior phosphorylase action, is the substrate. Enzyme activity is also reversibly inhibited by urea at concentrations below 2 M. Guanidine at 0.15 M produces 50% inhibition of glucose formation from a phosphorylase limit dextrin.

INTRODUCTION

The glycogen-debranching enzyme, oligo-α-1,4-glucan:α-1,4-glucan-4-glycosyltransferase–amylo-1,6-glucosidase (hereafter referred to as "transferase–glucosidase"), has been purified previously from rabbit skeletal muscle and many of its properties have been studied[1-4]. It has been shown that this protein has two different enzymatic activities. Acting as a transferase, it preferentially moves maltotriosyl, and to a lesser extent, maltosyl residues from some donor oligosaccharides and from glycogen to other linear or branched acceptors of suitable structure[2,3]. However, the enzyme is unable to move single glucose residues from one α-1,4-glucosidically bonded position to another[1]. As a glucosidase, the enzyme has been shown to act on

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the single α-1,6-glucosidically bonded unit which becomes exposed at each outer branch point of glycogen following degradation of this polysaccharide to a limit dextrin by glycogen phosphorylase (EC 2.4.1.1) and subsequent maltotriosyl unit transfer by the transferase activity of the debranching enzyme[5]. The enzyme also has been shown[6] to act as an α-1,6-glucosidase on 6^3-α-glucosylmaltotetraose and on some other oligosaccharides of similar structure[1] as well as on α-glucosyl-substituted Schardinger dextrins[7]. The glycogen-debranching enzymes of human muscle and human liver may be under separate genetic control, since it has been found that about one-quarter of all individuals with Type III glycogen storage disease lack the glycogen-debranching enzyme activity in liver only, while the remaining group of affected individuals have this enzyme defect both in skeletal muscle and in liver[8]. Van Hoof and Hers[9] have compared different methods for the assay of transferase–glucosidase in human tissues and have found that in about one-quarter of their series of affected individuals there was enough disparity in the results of the different assay methods to conclude that in these cases of Type III glycogenosis the glycogen-debranching enzyme may have had some residual transferase or glucosidase activity in liver and muscle. Because of the possibility however, that the human liver and muscle enzymes may be products of different genes and, as such, might have different structures and might even differ in catalytic properties, an investigation of the rabbit liver enzyme was undertaken to allow its comparison with the corresponding rabbit muscle enzyme. The findings from this study are reported in the present paper.

MATERIALS AND METHODS

Materials

Glycogen isolated from rabbit liver was obtained from Mann Research Laboratories or from Sigma. [U-^{14}C]Maltose (5 μCi/μmole) was from Amersham-Searle. Purification of [U-^{14}C]maltose was by descending chromatography on Whatman No. 1 paper, using *n*-butanol–pyridine–water (6:4:3, v/v/v) as the developing solvent.

Sephadex G-200 (140–400 μm) was from Pharmacia, and before use it was treated in 6 M urea according to the procedure of Auricchio and Sica[10]. Urea and sucrose were both ultra-pure grades obtained from Schwarz-Mann. Enzymes used for analytical procedures were from Boehringer Corp. Twice crystallized pancreatic α-amylase (EC 3.2.1.1) was from Worthington. All other chemicals used were of analytical reagent grade.

Phosphorylase limit dextrin

Glycogen phosphorylase limit dextrin was prepared by the repeated action of muscle glycogen phosphorylase (formerly known as phosphorylase *a*) on rabbit liver glycogen according to the method of Larner *et al.*[11]. The preparation of glycogen phosphorylase was free of glycogen-debranching enzyme activity.

Preparation of oligosaccharide substrates

The two substrates, 6^3-α-glucosylmaltotetraose ("Fast B_5") and 6^3-α-maltotriosylmaltotetraose (B_7) were prepared by the action of α-amylase on rabbit liver glycogen as described by Illingworth and Brown[6]. Following deionization on Amberlite MB-3, the digest was fractionated on a 2.5 cm × 100 cm water-jacketed column of

BioGel P-2 (Bio-Rad Laboratories) using water as eluant. The column was maintained at a temperature of 60 °C during chromatography by circulating water through the column jacket[12]. This procedure affords partial resolution of the oligosaccharides. Final separation was achieved by descending chromatography on Whatman No. 1 paper using *n*-butanol–pyridine–water (6:4:3, v/v/v) solvent. Usually, chromatography for 6–10 days was required to resolve the oligosaccharides. In this manner Fast B_5 could be satisfactorily resolved from Slow B_5 (6^3-α-maltosylmaltotriaose). Each oligosaccharide was compared chromatographically with standards. The preparations of Fast B_5 and B_7 were tested for their effectiveness as substrates using a purified preparation of rabbit muscle oligo-α-1,4→1,4-glucantransferase–amylo-1,6-glucosidase prepared by the method of Brown and Brown[3]. These substrates were obtained and used at a level of purity of from 85 to 100%.

Assay for transferase–glucosidase

The method of Brown and Brown[3] was used. The incubation mixture contained 40 mM sodium citrate–20 mM 2-mercaptoethanol buffer (pH 6.0), 1.0% (w/v) glycogen phosphorylase limit dextrin, and enzyme in a final volume of 0.25 ml. The enzyme was prediluted in 10 mM sodium citrate–5 mM 2-mercaptoethanol buffer, pH 6.0. The reaction was started by adding the enzyme to the incubation mixture at 37 °C. Following 10 min of incubation the tube was immersed in a bath of boiling water for 1 min. The mixture was centrifuged to remove coagulated protein, if necessary, and the glucose content of the supernatant fluid was determined. Glucose was assayed enzymatically by the spectrophotometric measurement of NADPH in the presence of added $NADP^+$, Mg^{2+}, ATP, hexokinase (EC 2.7.1.1), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

Transferase–glucosidase was also assayed using the branched oligosaccharide, B_7, as substrate[3]. The reaction mixture contained 50 mM sodium citrate–25 mM 2-mercaptoethanol buffer (pH 6.0), 5 mM B_7 and enzyme in a final volume of 0.20 ml. Incubation was at 37 °C and, at 30-min intervals, 0.05 ml aliquots were removed and assayed for glucose as described above.

Specific assay for glucosidase activity

Both of the assays described above depend on the combined action of the transferase and the glucosidase. The activity of the glucosidase was also measured directly using the branched oligosaccharide "Fast B_5"[3,6]. The composition of the assay mixture was the same as described above for the B_7 assay except that this substrate was replaced by 6 mM "Fast B_5".

Preparative polyacrylamide-gel electrophoresis

Preparative gel electrophoresis was performed using a 4.8% gel slab of 6 mm thickness in an EC470 vertical cell (EC Apparatus Corp., Philadelphia). The gel was formed by mixing 50 ml of 24% acrylamide–0.8% *N,N'*-methylenebisacrylamide, 50 ml of 0.48 M Tris–HCl (pH 7.3), 0.25 ml of tetramethylenediamine and 25 ml of water. Oxygen was removed by bubbling N_2 through the mixture. 125 ml of 0.14% ammonium persulfate solution, also freed of oxygen as above, was added to the acrylamide solution and the resulting mixture was poured into the vertical cell and allowed to gel. The buffer used for electrophoresis was 34 mM asparagine–5 mM 2-mercaptoethanol–

1 mM EDTA; the pH was adjusted to 7.3 with Tris. Pre-electrophoresis was carried out at 250 V for 2.5 h to remove ammonium persulfate. Following pre-electrophoresis fresh buffer was placed in the cell and then the enzyme sample (1–2 mg of protein) was applied to four slots 2 cm wide formed in the gel. Samples were carefully layered underneath the buffer in a mixture containing 15% glycerol and 0.0015% bromophenol blue as a tracking dye. A voltage of 150 V was applied for 15 min until the sample had entered the gel and then the voltage was increased to 230–250 V (current, 125 mA). Electrophoresis was continued for 4.5–6 h by which time the tracking dye had migrated 10 cm. Throughout the electrophoresis the apparatus was maintained at 0–4 °C using a refrigerated water bath and circulating pump. The electrode buffer was also recirculated during electrophoresis.

Sucrose-density gradient centrifugation

The method of Martin and Ames[13] was used to determine the molecular weight of the enzyme. The purified transferase–glucosidase was dialyzed overnight at 5 °C in 5 mM Tris–5 mM 2-mercaptoethanol–2 mM EDTA–25 mM KCl, pH 6.6. A mixture of reference enzymes from commercial sources (Sigma and Boehringer) was dialyzed against the same buffer. Following dialysis the transferase–glucosidase and the reference enzymes were mixed and layered on the top of a 5–20% linear sucrose gradient which had been formed in the pH 6.6 buffer described above. Centrifugation was at 60 000 rev./min for 11.5 h in the SW 65 rotor of the Spinco Model L2-65 centrifuge. Fractions from the gradient (0.15 ml) were collected from the bottom of each tube, and appropriate fractions were assayed for transferase–glucosidase and other enzyme activities. The transferase–glucosidase was assayed by both the limit dextrin and the B_5 assays described above. The reference enzymes were assayed as follows: phosphoglucomutase (EC 2.7.5.1) by the method of Klenow and Emberland[14], glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) by the method of Beisenherz *et al.*[15], lactate dehydrogenase (EC 1.1.1.27) by the method of Kornberg[16], and pyruvate kinase (EC 2.7.1.40) by the method of Bucher and Pfleiderer[17].

Disc-gel electrophoresis

Analytical disc-gel electrophoresis was done using a 6% separating gel and a 3% stacking gel and the alkaline buffer system of Davis[18], or by using the same buffer system as that described above for the preparative gel separation. In the latter case, no stacking gel was used. In some experiments the buffer system of Hedrick and Smith[19] was used without a stacking gel.

Protein was determined by the method of Lowry *et al.*[20] using bovine plasma albumin as a standard.

RESULTS

Purification

Frozen rabbit liver (200 g) from rabbits which had been fasted for 48 h (Type 1B from Pel-Freez Biological Inc., Rogers, Ark.) was partially thawed in ice-cold 5 mM sodium phosphate–5 mM 2-mercaptoethanol–1 mM EDTA buffer, pH 7.0. The liver was homogenized in 4 vol. of fresh buffer using a Sorvall Omnimixer and a stainless steel vessel cooled in ice. The homogenization was done in three 30-s periods

at 70 V with a 30-s interval for cooling between each period of grinding. The homogenate was adjusted to pH 7 using 2 M Tris. This and all subsequent operations were carried out at 0–5 °C unless otherwise specified.

The homogenate was centrifuged at 12 000 × *g* for 30 min and the supernatant carefully decanted through glass wool. The residue was reextracted with an amount of fresh buffer equal in volume to two times the original weight of liver, and the mixture then was centrifuged as described above and the two supernatants were combined.

First $(NH_4)_2SO_4$ fractionation

$(NH_4)_2SO_4$, saturated at 4 °C and adjusted to pH 7, was added slowly to 0.25 satn (assuming additive volumes) and the mixture was allowed to stand for at least 1 h at 4 °C. The loosely packed precipitate was removed by centrifugation at 12 000 × *g* for 30 min and discarded. The supernatant was made 0.40 satd in $(NH_4)_2SO_4$. The precipitate was removed by centrifuging as described above, dissolved in 20 mM sodium phosphate–5 mM 2-mercaptoethanol–1 mM EDTA, pH 7.0, and dialyzed overnight against 8 l of this buffer.

Heat treatment

The pH of the dialyzed solution was adjusted to 6.2 by the addition of 0.5 M citric acid. The solution was placed in a water bath at 65 °C and stirred rapidly, using a magnetic stirring bar, while its temperature increased to 50 °C during a period of approximately 3 min. The temperature of the solution was held at 50(± 0.5) °C for exactly 1 min, following which the solution was cooled quickly in an ice–water bath. Approximately 4 min was required for the temperature of the solution to drop to 9 °C. The denatured protein was removed by centrifugation at 27 000 × *g* for 30 min.

Second $(NH_4)_2SO_4$ fractionation

The supernatant was brought to 0.20 satn in $(NH_4)_2SO_4$ by the slow addition of a cold, neutral saturated solution of the salt. The mixture was allowed to stand 30 min. If there was a significant precipitate, it was allowed to stand for another 30 min and then centrifuged at 12 000 × *g* for 30 min to remove the precipitate which was discarded. In some preparations, depending upon the exact conditions of the preceding heat treatment, it has been necessary to make the mixture 0.25 satd in $(NH_4)_2SO_4$ in order to obtain a visibly coagulated precipitate which has then been removed as described above and discarded. The supernatant then was made 0.30 satd in $(NH_4)_2SO_4$ (if the first precipitate had been removed at 0.20 satn), or 0.33 satd (if the first precipitate had been removed at 0.25 satn). The precipitate was removed by centrifugation, dissolved in 5 mM Tris–5 mM 2-mercaptoethanol–1 mM EDTA, pH 7.2, and dialyzed for 16 to 24 h against several changes of this buffer.

DEAE-cellulose chromatography

The dialyzed enzyme was centrifuged if necessary to remove denatured protein and then concentrated to 15–20 ml by ultrafiltration using an XM-50 membrane filter (Amicon Corp.). The solution, clarified again, if necessary, by centrifugation, was loaded on a 2.5 cm × 25 cm column of Whatman DE-52 which had been equilibrated previously with the buffer used for dialysis. After loading, the column was well washed and the transferase–glucosidase was eluted with a linear salt gradient formed by 500

ml of the buffer in the mixing chamber and 500 ml of the buffer containing added 0.3 M NaCl in the source bottle (flow rate, 20–30 ml/h; fraction volume, 7 ml). Fractions containing the enzyme (limit dextrin assay) were pooled and concentrated to 3 ml by ultrafiltration (XM-50 membrane).

Sephadex G-200 chromatography

The concentrated enzyme solution from the DE-52 column was dialyzed against 5 mM Tris–5 mM 2-mercaptoethanol–1 mM EDTA–50 mM NaCl, pH 7.0. Then it was chromatographed on a 2.5 cm × 100 cm column of Sephadex G-200 using the buffer containing 50 mM NaCl to elute the enzyme. Active fractions were pooled and concentrated to a volume of less than 1 ml by ultrafiltration as described above.

Preparative polyacrylamide-gel electrophoresis

The enzyme obtained from chromatography on Sephadex G-200 was subjected to slab electrophoresis as described in Methods. Following electrophoresis the gel slab was quickly removed from the apparatus and cut transversely to the direction of electrophoresis into 3-mm strips. Each gel strip then was disrupted by homogenization in 7 ml of 50 mM sodium phosphate–5 mM 2-mercaptoethanol–1 mM EDTA, pH 7.0, using a Potter–Elvejhem homogenizer with a motor-driven pestle. Each gel homogenate was stored overnight in ice to allow for more complete enzyme extraction, and then was transferred to a dialysis casing and dialyzed for 6 h against the pH 7.0 phosphate buffer with several changes. The mixtures were centrifuged to pack the gel particles and the supernatant fluids were withdrawn and assayed for enzyme activity and for protein content.

Table I shows a summary of the procedure by which a 500-fold purification of the transferase–glucosidase from rabbit liver has been achieved. The figures reported in the right hand side of Table I show that the relative activities of the transferase and the glucosidase remained in constant ratio throughout the purification. Such a preparation gives one band on acrylamide-gel electrophoresis at pH 7.3 and pH 8.9.

TABLE I

PURIFICATION OF TRANSFERASE–GLUCOSIDASE FROM RABBIT LIVER

The results described are for a preparation from 200 g of liver.

Step	*Volume (ml)*	*Total protein (mg)*	*Spec. act. (units/mg protein)*	*Yield (%)*	*Purification*	*Relative activities*	
						*LD/B₅**	*B₅/B₇**
360 000 × *g*·min supernatant	1200	23 100	0.0087	100	1	1.8	
$(NH_4)_2SO_4$ (25–40% satd)	360	6 120	0.016	48	1.8		
Supernatant from 50 °C treatment	310	1 485	0.055	41	6.4		
$(NH_4)_2SO_4$ (20–30% satd)	30	170	0.19	16	22	2.1	
DEAE-cellulose pool	3	5.8	2.1	6	240	2.4	12.5
G-200 pool	0.8	1.3	3.0	2	345	2.5	
Preparative acrylamide electrophoresis		0.7	4.5	1	520	2.5	12.1

* Assay conditions: citrate buffer, pH 6, 37 °C; 1% limit dextrin (LD); 5 mM branched pentasaccharide (B_5); 5 mM branched heptasaccharide (B_7).

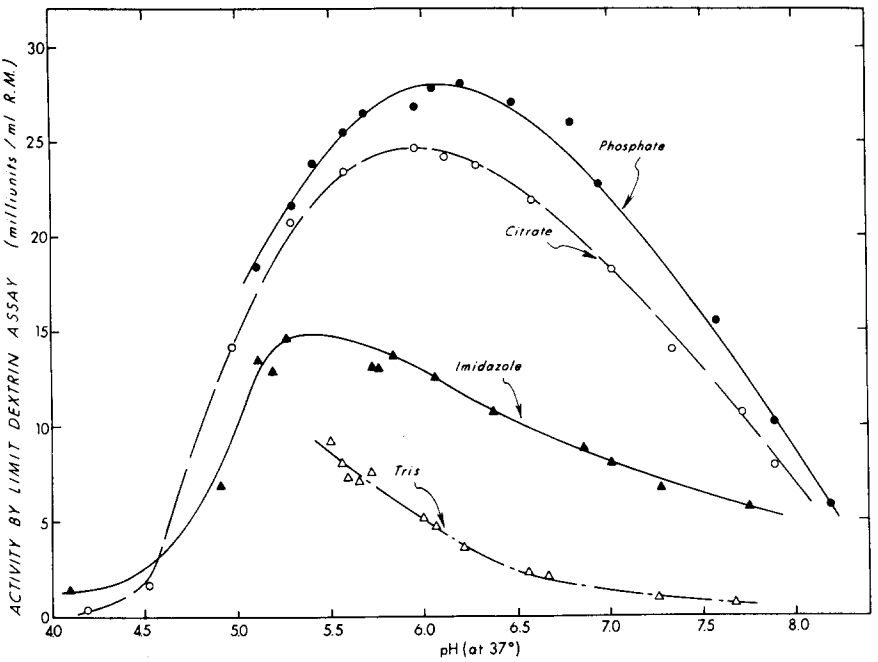

Fig. 1. Dependence on pH of the rate of formation of glucose from a limit dextrin. All reaction mixtures (R.M.) contained 1.25% limit dextrin, 5 mM 2-mercaptoethanol and 1 mM EDTA, in addition to 50 mM buffer as indicated. All pH measurements were made at 37 °C on the complete reaction mixtures including enzyme. Incubation was at 37 °C for 10 min in the presence of 0.025 unit/ml of transferase-glucosidase (spec. act., 4 units/mg). Citrate and phosphate buffers were prepared by mixing the appropriate sodium salts. Tris and imidazole buffers were mixtures of the free bases and of their hydrochloride salts.

Effect of pH

The pH–activity curve of the enzyme has a maximum near 6.0 at 37 °C in citrate or phosphate buffer when a phosphorylase limit dextrin of glycogen is used as substrate (Fig. 1). When only the glucosidase activity was assayed using the specific substrate, 6^3-α-glucosylmaltotetraose in sodium citrate buffer, the optimum pH was also found to be approximately 6. In imidazole buffer enzymatic activity is inhibited to the extent of about 50% and the pH optimum is shifted to about 5.4. An even greater inhibition is evident in the presence of Tris, and in this case the pH optimum appears to be shifted decidedly toward more acidic values. Strong inhibition by Tris of rabbit muscle transferase–glucosidase has been reported[2–4], and imidazole has also been found to be an inhibitor of the muscle enzyme[4]. It is interesting that these two inhibitory buffers have a qualitatively different effect on the pH optimum of the liver enzyme than on that of the muscle enzyme. They shift the pH optimum of the latter toward more alkaline values relative to that found in anionic buffers[4], while the pH optimum of the liver enzyme is shifted in the opposite direction. The inhibition of the liver enzyme by various buffers has not yet been studied extensively from a kinetic point of view.

Demonstration of oligo-α-1,4→1,4-glucantransferase activity

A sample of the purified enzyme was tested for its ability to transfer oligosaccharyl units from amylopectin to maltose as an acceptor. The reaction mixture consisted of 0.44% (w/v) amylopectin in 20 mM sodium citrate–5 mM 2-mercaptoethanol–1 mM EDTA buffer, pH 6.1, containing 6 mM [U-^{14}C]maltose (117 000 dpm/μmole).

Transferase–glucosidase (spec. act., 5.86 units/mg protein) was added to a final concentration of 0.46 unit/ml and the mixture was incubated at 37 °C. Aliquots of 0.3 ml were removed at time intervals of 3, 6, and 12 h and heated in a boiling water bath for 1 min to stop the reaction. A blank reaction mixture containing no added enzyme was included and sampled after 12 h of incubation. Amylopectin was precipitated by the addition of 2 vol. of 95% ethanol and the supernatant was deionized by passage through a column of Amberlite MB-3 and then chromatographed as described under Methods.

The results shown in Fig. 2 are a direct demonstration that the purified enzyme possesses oligo-α-1,4→1,4-glucantransferase activity. Maltopentaose is formed at approximately three times the rate of maltotetraose indicating that the enzyme has a preference for maltotriosyl unit transfer. This specificity is qualitatively the same as for the rabbit muscle enzyme[1] but differs from that of the corresponding yeast enzyme (Lee *et al.*[21]). The latter authors found the purified enzyme from yeast to have a preference for maltosyl unit transfer.

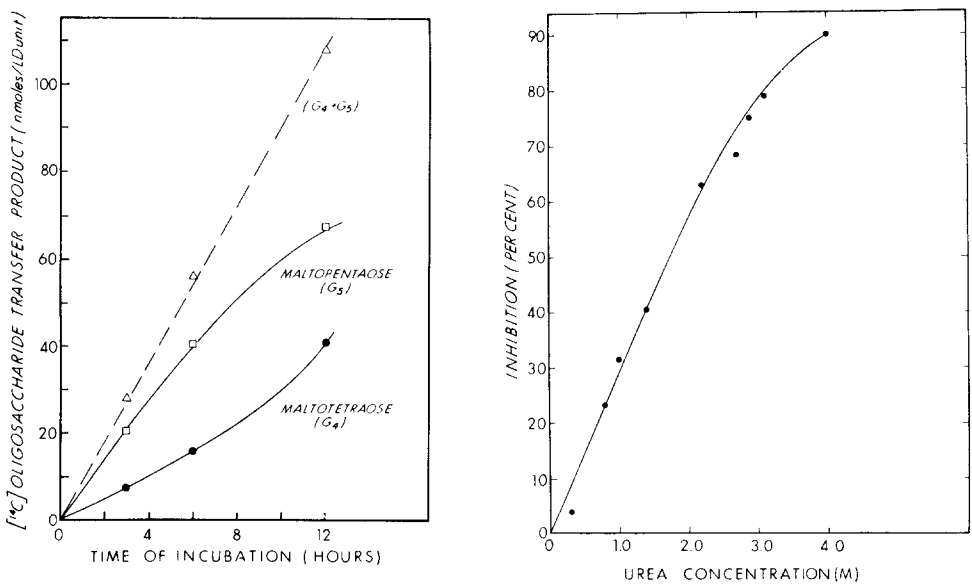

Fig. 2. Assay of oligo-α-1,4→1,4-glucantransferase. For details of this assay see the text. The initial velocity of formation of maltopentaose was calculated to be 7.0 nmoles/h per unit of enzyme activity (limit dextrin (LD) assay), and that of the formation of maltotetraose was calculated to be 2.5 nmoles/h per unit of activity. The dotted line shows the calulated sum of the total transfer products formed at each sampling time.

Fig. 3. Effect of urea on the activity of transferase–glucosidase (limit dextrin assay). Each reaction mixture contained 0.015 unit of enzyme (spec. act., 4.8 units/mg) per ml and 1% limit dextrin in 20 mM sodium citrate–10 mM 2-mercaptoethanol, pH 6.0. Urea was added as indicated and incubation was at 30 °C.

Molecular weight

Sucrose-density gradient centrifugation of the liver transferase–glucosidase showed that the enzymic activity detected by assay using a limit dextrin as the substrate coincided exactly with that found by assay using the glucosidase-specific substrate, 6^3-α-glucosylmaltotetraose. This finding supports the conclusion from the purification data in Table I that the protein has both transferase and glucosidase activities. From the sucrose-density gradient data, the molecular weight of the liver enzyme was found to be 178 000 using pyruvate kinase (mol. wt 237 000) as the reference protein, 181 900 using lactate dehydrogenase (mol. wt 144 000) as the reference protein, 176 200 using glycerol-3-phosphate dehydrogenase (mol. wt 78 000) as the reference protein, and 180 900 using phosphoglucomutase (mol. wt 67 000) as the reference protein. These values are in reasonably good agreement and give an average value of 179 250 for the molecular weight of liver transferase–glucosidase. A reinvestigation of the molecular weight of the pure rabbit muscle transferase–glucosidase has recently been completed, and it has been found that preparations of the muscle enzyme ($s_{20,w} = 7.73$) which have specific activities from 8–10 units/mg of protein have a mol. wt of 170 000 as determined by sedimentation equilibrium as well as by sucrose-density centrifugation[22]. Thus, the less extensive data which have been obtained for the rabbit liver enzyme indicate that it is similar in size to the muscle enzyme from the same species. However, whether the liver protein has, in fact, a slightly greater molecular weight is uncertain.

Inhibition by urea and guanidine and enzyme stability

The transferase–glucosidase activity as measured in the limit dextrin assay is inhibited by urea as shown in Fig. 3. Activity at 30 °C is 90% inhibited in 4 M urea. At 37 °C a similar degree of inhibition is produced by about 2.6 M urea (data not shown). The stability of the enzyme in urea at 30 °C is shown in Fig. 4. Samples of the purified enzyme were incubated for the times indicated at 30 °C in phosphate buffer, pH 6.6, containing either 2.1 or 3.2 M urea, as well as with no urea as a control. Aliquots of the reaction mixtures were diluted with citrate buffer, pH 6, such that the concentration of urea became 0.2 M or 0.3 M, respectively, and then activity toward a limit dextrin was determined. The transferase–glucosidase activity in 2.1 M urea declined by 20% during the first 15 min but then remained constant at 80% of its original value for up to 40 min. In 3.2 M urea over 60% of the activity appeared to be lost after 40 min.

Fig. 5 shows the activity of the enzyme toward a limit dextrin at 0 °C in the presence and absence of 2 M urea. The reaction proceeds slowly at this temperature and is inhibited to the extent of 65% in the presence of urea. This inhibition is comparable to a value of 57% in the presence of 2 M urea at 30 °C (Fig. 3). This comparison indicates that elevated temperatures are not required to demonstrate an effect of urea on the protein. Inhibition of the activity of the muscle enzyme by urea has also been observed, and, in fact, muscle transferase–glucosidase is even more sensitive than the liver enzyme to this agent[3]. Thus, while 2 M urea produces 57% inhibition of the latter enzyme at 30 °C, the muscle enzyme's activity is inhibited to an extent of greater than 90%. In both cases the inhibition is almost totally reversible by dilution. Although sedimentation velocity studies of the muscle enzyme in urea have sometimes shown a small change in the sedimentation constant of the protein,

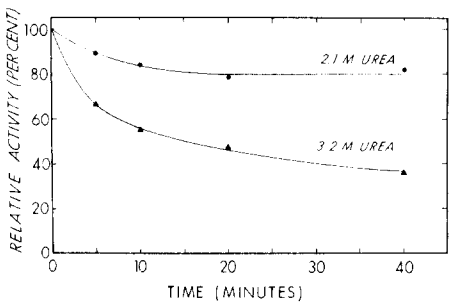

Fig. 4. Stability of transferase–glucosidase in urea. The enzyme (spec.act., 4.8 units/mg) was pre-incubated at 30 °C at a concentration of 20 μg/ml in 10 mM sodium phosphate–5 mM 2-mercapto-ethanol–1 mM EDTA, pH 6.6, containing urea as indicated. At various times 0.1-ml aliquots were removed and assayed for transferase–glucosidase activity as described.

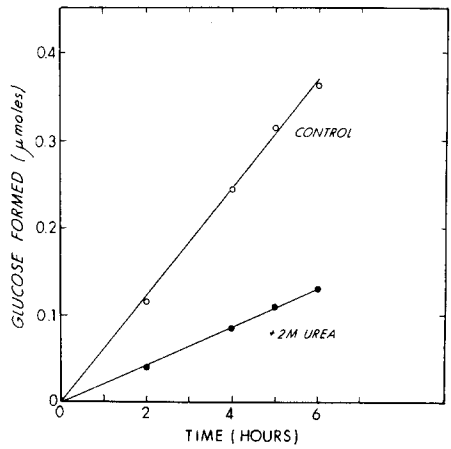

Fig. 5. Effect of urea on transferase–glucosidase activity at 0 °C. The enzyme (spec. act., 4.0 units/mg) was incubated at 0 °C at a concn of 50 μg/ml with 1% limit dextrin in 4 mM Tris–2 mM 2-mercaptoethanol–1 mm EDTA, pH 6.6. Urea was added as indicated, and at various times suitable aliquots were removed, heated in boiling water, and analyzed for their glucose content as described in Materials and Methods.

any such difference appears to be attributable to a change in shape of the protein rather than to its dissociation. In 2 M urea, where activity is almost totally inhibited, the sedimentation constant of the muscle enzyme is unchanged from the value characteristic of the native enzyme ($s_{20,w} = 7.7$)[22]. Although no comparable sedimentation velocity data are available for the liver enzyme, it is likely that this protein too is inhibited by urea without concomitant dissociation. This statement is based partly on the results of polyacrylamide disc-gel electrophoresis in various concentrations of urea, whereby it has been found that muscle transferase–glucosidase shows a tendency to aggregate rather than to dissociate in gels containing 3 to 8 M urea[22], and that the liver enzyme is similar to the muscle enzyme in this respect (Gordon, R.B. and Brown, D.H., unpublished).

Guanidine has been studied recently as an inhibitor of rabbit muscle transferase–glucosidase, and it has been found that much lower concentrations of this substance than of urea are required to produce marked inhibition of glucose formation from a limit dextrin[22]. A similar difference has been found for the effects of these agents on the liver enzyme. At 37 °C in sodium phosphate buffer, pH 6.3, as little as 0.15 M guanidine produces 50% inhibition in the limit dextrin assay, while 0.05 M guanidine inhibits to the extent of 20%. An adequate explanation of the effects of guanidine, as well as of urea, on enzyme activity will require extensive kinetic studies of assay systems in which the transferase and glucosidase activities of the enzyme are measured separately and specifically.

DISCUSSION

The preparative procedure described in this paper is suitable for obtaining a relatively pure sample of liver transferase–glucosidase for study of its catalytic and kinetic properties. However, the quantity of the pure protein which can be obtained in this way is quite small, and studies on the physical properties of the enzyme have been limited by its availability and by its somewhat greater lability on storage than the corresponding muscle enzyme. The most significant feature of this work is the demonstration that the liver enzyme, like its muscle counterpart, possesses both transferase and glucosidase activities. Of interest also is the fact that these two proteins have similar, although possibly not identical, molecular weights: about 179 000 for the liver enzyme and about 170 000 for the muscle enzyme[22]. It is interesting that the two debranching enzymes have now been found to be proteins which have approximately the same molecular weights as the active forms of the corresponding glycogen phosphorylases with which they can act together to bring about the rapid degradation *in vivo* of their macromolecular substrate, glycogen, whose molecular size exceeds that of the enzymes themselves by one to two orders of magnitude.

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