induced frog enzyme did not increase its specific activity at 55° in up to 10 min and decreased sharply to about 30% of the original activity in another 5 minutes. It should be noted here that the fact that L-histidine and hydrocortisone had no effect on the tadpole liver tryptophan pyrrolase activity[4] also suggests a difference in the system between rat and tadpole.

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Reinvestigation of the pH optimum in terms of the action and properties of rabbit muscle amylo-1,6-glucosidase–oligo-1,4 → 1,4-transferase

Amylo-1,6-glucosidase (dextrin 6-glucanohydrolase, EC 3.2.1.33) and oligo-1,4 → 1,4-transferase (α-1,4-glucan: α-1,4-glucan 4-oligoglucantransferase, EC 2.4.1.24) comprise the glycogen debranching system in rabbit muscle. Both glucosidase and transferase activities appear to be associated with each other throughout purification[1] and it has been suggested that they may represent a "two-headed" enzyme[2].

Although glucosidase–transferase has been extensively studied, there have been discrepancies between the properties of various preparations. The pH optimum for hydrolysis was found to be 7.2 in one case[3] and 5.8–6.5 in others[1,4–7]. Likewise, the pH optimum for glucosyl incorporation has been reported as 7.4 (ref. 8) and 6.4 (ref. 7). In the latter two cases neither optimum coincided with that of the hydrolytic reaction.

Several suggestions have been put forth to explain these differences. The incorporation reaction has been suggested to proceed by a mechanism different from simple reversal of the hydrolysis by the glucosidase[6]. In the case of hydrolysis, evidence has been obtained suggesting that glucosidase–transferase activities with differing pH optima may be present in partially purified preparations[9].

In this communication we wish to present evidence, obtained with a highly purified preparation, which indicates that the incorporation of glucosyl groups into polysaccharide catalyzed by the glucosidase transferase is due mechanistically to the slight reversibility of the hydrolytic reaction of the glucosidase. This evidence explains the buffer effects noted in the literature[1,5,6,11] and indicates that the enzyme proceeds *via* a glucosyl enzyme intermediate in both the hydrolytic and synthetic directions, rather than as has been proposed[6], through a different type of intermediate in the synthetic direction.

The glucosidase-transferase from rabbit muscle was purified to a specific activity higher than that obtained by previous methods[1,10] and its properties reinvestigated. The enzyme was purified from fresh rabbit muscle by extracting in dilute $KHCO_3$–EDTA solution followed by acid precipitation of extraneous protein at pH 5.3 in the presence of glycogen, precipitation with ammonium sulfate, and chromatography on a DEAE-cellulose column. The enzyme preparation at this point contained approx. 50% phosphorylase *b* (EC 2.4.1.1) as a contaminant. The phosphorylase *b* was converted to the apo-enzyme and removed along with other proteins by ammonium sulfate precipitation followed by isoelectric precipitation and column chromatography on phosphocellulose. The specific activity of the initial muscle extract was 0.07–0.08. The final specific activities of the preparation ranged from 6.5 to 8.3, with overall purifications in the range of 90–100-fold and yields of 2–6%. The preparation migrated as a single component on polyacrylamide gel electrophoresis at pH values from 5 to 8. In addition, the preparation contained no detectable α-amylase (EC 3.2.1.1), maltase (EC 3.2.1.20) UDP glycose: α-1,4-glucan 4-glucosyltransferase (EC 2.4.1.11) or phosphorylase.

The pH optimum for hydrolysis of glycogen phosphorylase limit dextrin was determined from pH 4 to 9 using both anionic and cationic buffers. All of the anionic

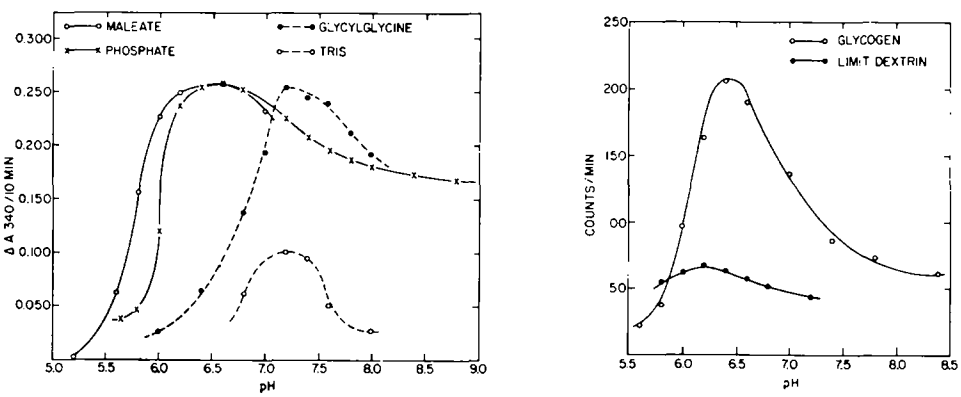

Fig. 1. Hydrolysis of glycogen phosphorylase limit dextrin by purified glucosidase–transferase in various buffers. Incubations were conducted for 10 min at 30° in 0.01 M buffer containing 0.5% limit dextrin and 0.021 unit (ref. 1) of enzyme per ml. The release of glucose was determined spectrophotometrically[1] after heat denaturation of the enzyme.

Fig. 2. Incorporation of [^{14}C]glucose into glycogen and phosphorylase limit dextrin by purified glucosidase–transferase. Incubations were conducted for 10 min at 30° in 0.01 M phosphate buffer containing 10% glycogen or limit dextrin and 0.18 unit (ref. 1) of enzyme per ml. The concentration of glucose was 1.5 μmoles/ml with a specific activity of 20 μC/μmole. Radioactivity incorporated was determined by a filter paper method[11] in a liquid-scintillation counter.

buffers were used in the Na^+ form and all of the cationic buffers were in the Cl^- form. With anionic buffers an optimum at pH 6.6 was obtained, whereas with cationic buffers the optimum was shifted to 7.2. This is shown in Fig. 1. In the case of Tris, in addition to the shift in pH optimum, enzymatic activity was also inhibited as has been previously noted[1,11]. This inhibition was found to be non-competitive with substrate and related to the protonated form of the amine.

The pH optimum of the enzyme preparation for glucosyl incorporation into polysaccharide was also determined. This is shown in Fig. 2. Glycogen (on an equal weight basis) was a much better acceptor (Fig. 2) during reincorporation than limit dextrin. The pH optimum, however, was the same in either case. The optimum within experimental limits is the same as that measured during hydrolysis (Fig. 1), and was shifted and inhibited by Tris in a manner similar to that observed when Tris was used as a buffer for the hydrolytic reaction. The fact that Tris is a potent inhibitor of the reincorporation reaction indicates an action on the glucosidase. This was substantiated by showing that Tris inhibited the glucosidase when its hydrolytic action was measured directly (using glucosyl Schardinger dextrin as a substrate[4]). The inhibition was greater with glucosyl Schardinger dextrin than with limit dextrin.

Reincorporation was determined by adapting the filter paper assay method for UDPG glycogen transferase[12] based on the incorporation of [^{14}C]glucose into alcohol-insoluble polysaccharide. The presence of oligo-1,4→1,4-transferase does not interfere with this determination because, although the transferase can cover up the radioactive glucosyl group once it is incorporated as a (1→6)-substituent, it cannot remove it[5,6]. With glycogen this provides a convenient means of assay, since no special substrates are required and the method is rapid (T. E. NELSON, A. M. LOE AND J. LARNER, unpublished observations).

The incorporation reaction was further investigated by incubating the enzyme under synthesizing conditions, for 24 h at 30° in 0.01 M citrate (pH 6.0) in the presence of high concentrations of glucose (45%, w/v) and a suitable acceptor, either maltotetraose or α-Schardinger dextrin (5%). In each case the anticipated transfer product (a branched pentasaccharide or a branched Schardinger dextrin) was observed chromatographically (paper). These products were isolated by paper chromatography and identified by showing that after glucosidase action the original starting materials were re-obtained. In addition, incubation of the enzyme with glucose alone under the same conditions (45%) gave a product that was identical chromatographically (paper) with isomaltose. The isomaltose was isolated by paper chromatography in a 0,2% yield. This would be the product expected if the hydrolytic reaction were reversed in the presence of large quantities of glucose.

These results clearly demonstrate that with this highly purified glucosidase–transferase preparation, the pH optimum is a function of the type of buffer used. The similarity of the pH optima for both hydrolysis and synthesis as well as the similarity of the shift and inhibition in the presence of Tris indicate that glucosyl incorporation into polysaccharide is due mechanistically to the reversibility of the hydrolytic action of the glucosidase. Apparently, the glucosidase behaves under synthetic conditions as other glucosidases in reforming the original α-(1→6) linkage[13–15]. The synthesis of isomaltose (as well as branched pentasaccharide and glucosyl Schardinger dextrin) by this highly purified preparation demonstrates that the α-(1→6) linkage can be synthesized in the absence of polysaccharide, and suggests that the mechanism

proceeds *via* a glucosyl–enzyme intermediate rather than by a polysaccharide–enzyme intermediate as has previously been suggested[6].

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