*ACTION OF AMYLO-1,6-GLUCOSIDASE ON LOW MOLECULAR WEIGHT SUBSTRATES AND THE ASSAY OF THIS ENZYME IN GLYCOGEN STORAGE DISEASE**

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In 1956 Illingworth *et al.* described a type of glycogen storage disease which was characterized by the accumulation of liver and muscle glycogen with abnormally short outer chains and the absence of amylo-1,6-glucosidase.[1] This enzyme splits off as free glucose previously exposed residues in α-1,6 linkage at branch points and thereby allows further degradation of the glycogen molecule by phosphorylase. When the glucosidase is absent, phosphorylase can degrade only the outer chains of glycogen, leaving a structure which approaches a phosphorylase limit dextrin. Observations made on 18 cases of "limit dextrinosis" (Type III glycogen storage disease) have recently been summarized.[2] Hers has described[3] additional cases and has developed a method for the detection of glucosidase activity in tissue homogenates which is based on the slight reversibility of the reaction: glycogen-α-1,6 unit $\rightleftarrows$ glucose-C^{14}. The specificity of this method has been demonstrated by adding highly purified rabbit muscle glucosidase to tissue homogenates from cases of limit dextrinosis. Incorporation of glucose-C^{14} could be detected only if glucosidase was added.[2]

In this paper we wish to report a quantitative and specific assay of glucosidase activity which is applicable to liver biopsy material. The assay is based on the observation that among the products of digestion of glycogen by α-amylase there are several branched oligosaccharides which can serve as substrates for glucosidase. Among those shown in Figure 1, the one designated as "Fast B_5" (in reference to its chromatographic behavior) proved most suitable. It yields one mole of glucose and one mole of maltotetraose when acted upon by the glucosidase.

Materials and Methods.—Amylo-1,6-glucosidase has been purified from rabbit skeletal muscle by a series of protamine sulfate and ammonium sulfate fractionation steps similar to those already described[4] followed by DEAE cellulose column chromatography.[5] The specific activity of the final preparation has been as high as 82,000 units per milligram of protein. Thus, it is more than ten times as pure as the best preparations previously available.[6] The details of purification of this enzyme will be the subject of a later communication.

The low molecular weight, singly branched glucose oligosaccharides used in this work were obtained by degrading 5 gm of rabbit liver glycogen or of corn amylopectin with 10 mg of twice-crystallized pancreatic α-amylase (Worthington)[7] in a 0.01 *M* glycylglycine buffer, pH 6.5, for 21 hr at 30° under toluene vapor. After deionization on a mixed-bed resin (Amberlite MB-3), the digest was fractionated on a 125 × 4.5 cm column of Sephadex G-25 (Fine) by elution with water. Only partial resolution of the oligosaccharides was achieved. The approximate degree of polymerization of the substances in the various fractions was calculated from reducing power[8] versus total glucose content.[9] Suitable fractions were pooled and complete separations of the component oligosaccharides were achieved by descending chromatography on Whatman No. 3 paper using a butanol-pyridine-water solvent (3:2:1.5). The compounds obtained were characterized by chromatographic mobility, degree of polymerization, and by use of the flavazole derivative method of Nordin and French.[10] Further, the assignment of structures was confirmed by various transformations of one substance into another produced by the actions of amylo-1,6-

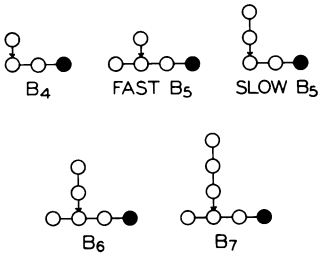

FIG. 1.—Structures of some oligosaccharides produced by α-amylase action on glycogen. ○, a glucose residue; ●, a reducing-end glucose unit; ——, the α-1,4-glucosidic bond; ↓, the α-1,6-glucosidic bond.

glucosidase and by phosphorylase as described below and in a following paper.[11] The principal singly branched oligosaccharides which we obtained had the structures shown in Figure 1. In general these products correspond to those reported by Nordin and French,[10] and Whelan and co-workers.[12-14]

The substance designated Fast B_5 has not previously been clearly identified as a component of an α-amylase digest. It is separable from the other principal branched 5-unit compound (Slow B_5) by the fact that it has a slightly greater chromatographic mobility. Accordingly, it has been possible to isolate Fast B_5 as a compound containing less than 10% of Slow B_5 as a contaminant. A flavazole derivative of the substance at this stage of purity was prepared and purified by paper chromatography (yield: 80%, calculated according to Nordin and Doty[15]). Partial acid hydrolysis of this derivative gave rise to the flavazoles of glucose, maltose, maltotriaose (trace), and B_4. No panose flavazole was formed. This finding, together with the observations on the action patterns of salivary and pancreatic α-amylases,[16] and the enzymatic results reported here, support the structure of Fast B_5 shown in Figure 1. The alternative structure in which the branch point glucose residue is located one unit closer to the reducing end would form a flavazole derivative whose acid hydrolysis products would include panose flavazole.[10]

Results.—A simple and specific enzymatic differentiation of Fast B_5 and Slow B_5 is possible because the former substance is cleaved by amylo-1,6-glucosidase to form one mole of glucose and one mole of maltotetraose and is not at all degraded by phosphorylase *a* in the presence of inorganic phosphate. Slow B_5, on the other hand, is not acted on at all by amylo-1,6-glucosidase, but is quantitatively degraded by phosphorylase *a* to one mole of glucose-1-phosphate (determined microenzymatically) and one mole of B_4. The action of glucosidase on Fast B_5 is represented in Figure 2*a* where proportionality to enzyme concentration is shown. The rate of liberation of glucose from Fast B_5 is slow when compared with the rate of action of glucosidase on a phosphorylase limit dextrin (Fig. 2*a*). There is, however, some uncertainty about the structure of this limit dextrin. According to Walker and Whelan[14] the branch units are covered by 3 glucose residues which must be moved by a transglycosylase before the 1,6-glucosidase can act. According to Hers both free and covered branch units are present.[17] The use of Fast B_5 as a substrate makes possible the assay of amylo-1,6-glucosidase without any question of the prior action of a transglycosylase. Accordingly, it has been used to investigate the presence of the glucosidase in samples of human liver tissue taken by biopsy or at autopsy from children suspected of having glycogen storage disease.

The method depends upon the fact that the enzyme is present in a high-speed centrifugal pellet which contains glycogen and other subcellular elements.[18] The pellet is prepared by grinding by hand approximately 200 mg of liver in 9 volumes of ice-cold 0.15 *M* KCl, using an all-glass Potter-Elvehjem homogenizer. The homogenate is spun in 2 ml tubes in the No. 40 rotor of the Model L Spinco centrifuge for 60 min in the cold at 100,000 × *g*. The supernatant fluid is decanted, fat is removed by wiping out the tubes carefully, and the pellet is washed by resuspending it in 0.15 *M* KCl. After centrifugation for 45 min at 100,000 × *g*, the pellet is drained well. It is suspended in 1.0 ml of 0.05 *M* glycylglycine-0.001 *M* mercaptoethanol buffer, pH 6.6, and, after thorough disintegration, aliquots of the milky suspension are taken for glucosidase assay.

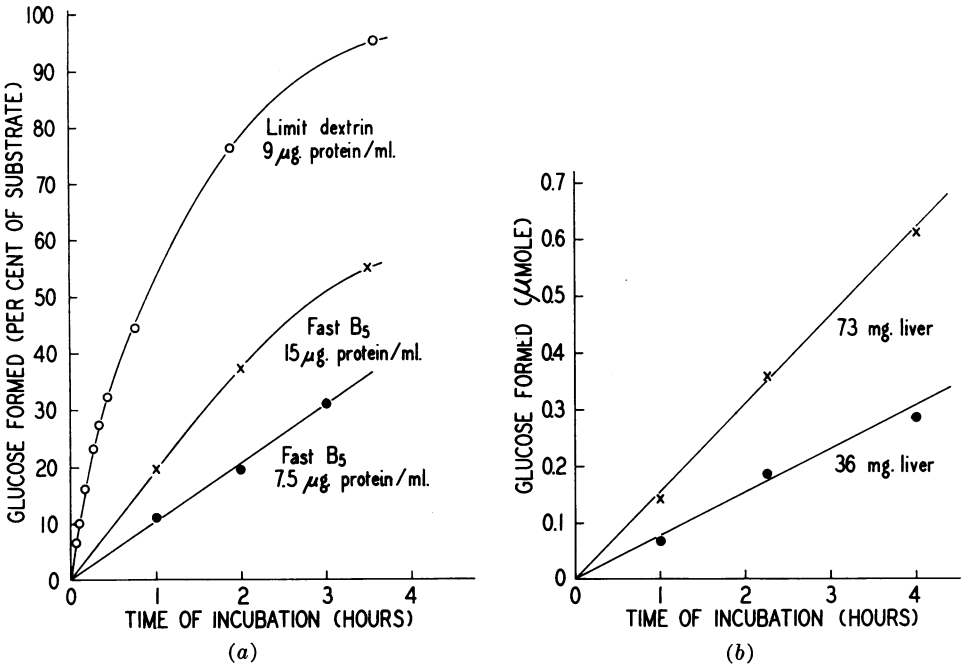

FIG. 2a.—Rate of action of amylo-1,6-glucosidase on two substrates. Phosphorylase limit dextrin (LD) of glycogen, 1 mg/ml; 0.05 *M* Tris and 0.002 *M* mercaptoethanol buffer, pH 7.2, 25°; sp. act. of glucosidase, 42,000 units/mg. Glucose formation is plotted as per cent of the amount formed at the end point (4.4% of the LD). Fast B_5, 8×10^{-4} *M*; incubated with glucosidase (sp. act., 50,400 units/mg) in 0.01 *M* Na citrate and 0.02 *M* glycylglycine and 0.001 *M* mercaptoethanol buffer, pH 6.6; 30°.

FIG. 2b.—Rate of action of a high-speed centrifugal pellet from human liver (Patient D. M., Table 1) on Fast B_5. Substrate concentration, 2.73×10^{-3} *M*; 0.007 *M* Na citrate and 0.009 *M* glycylglycine and 0.001 *M* mercaptoethanol buffer, pH 6.6; 30°.

The glucosidase assay is done by incubating 0.15 ml (equivalent to about 30 mg of original liver tissue) with 1 μmole of Fast B_5, in the presence of a sodium citrate buffer, pH 6.7, at a final concentration of 0.01 *M*. Incubation is at 30° for 2 to 3 hr in a final volume of 0.4 ml. The reaction is stopped by heating in boiling water; after dilution to 1.0 ml with water and centrifugation at 25,000 × *g* in the International Centrifuge, suitable aliquots are analyzed for glucose microenzymatically via hexokinase and glucose-6-phosphate dehydrogenase. Simultaneously, a tissue blank is incubated in the absence of Fast B_5 to measure the small endogenous production of glucose. Any net production of glucose due to the addition of Fast B_5 measures the rate of action of amylo-1,6-glucosidase. Figure 2*b* shows that the rate of formation of glucose from Fast B_5 by a pellet prepared from the liver of a patient with Type I glycogen storage disease (absence of glucose-6-phosphatase) is linear with time and proportional to enzyme concentration. This enzyme preparation, as well as ones from other types of cases, formed no glucose from B_4, Slow B_5, or B_6, showing the specificity of Fast B_5 as a substrate. Experiments in which Fast B_5-U-C^{14} (prepared from starch-U-C^{14}) has been used have shown that, after suitable chromatography, radioactive glucose and radioactive maltotetraose are present in equimolar quantities and in the amount expected from the net production of glucose measured enzymatically.

TABLE 1

ASSAY OF AMYLO-1,6-GLUCOSIDASE IN LIVER TISSUE FROM CASES OF GLYCOGEN STORAGE DISEASE

Patient	Type*	% Glycogen in liver	Isolated glycogen % split by phosphorylase	C^{14}-Glucose incorporation (cpm/mg reisolated glycogen)	Rate of splitting of fast B_5 (μmoles glucose/gm liver/hr)
L. G.	I	10.	36		2.80
D. M.	I	6.4	37	60	2.06
M.	I	6.7	32		2.00
A. A.	II	7.0	34		1.25
R. I.	III	14.3	16	5	0
K. S.	III	16.5	17		0
C. B.	I–III	13.8	17	4	0
A. L.	III	14.8	19	3	0
J. J.	III	15.1	18		0
B. R.	?	9.5	40	106	3.14
B. S.	?	10.	35		2.93
D. P.	VI	11.7	38		2.27

* Type I, no glucose-6-phosphatase; Type II, generalized glycogenosis; Type III, no amylo-1,6-glucosidase; Type VI, hepatic storage disease with low liver phosphorylase.

Table 1 gives a summary of the results in 12 cases including a variety of types of glycogen storage disease. For comparison, data on the content of glycogen in the tissue as well as its outer chain length (measured by phosphorylase) are given. In some cases, measurements of the incorporation of glucose-C^{14} into glycogen according to Hers are given also. It may be seen that glucose formation from Fast B_5 has never been observed in any case in which other types of evidence favor a diagnosis of Type III glycogen storage disease (absence of the glucosidase). It is also important that the use of this substrate makes possible the certain detection of this type of disease in the liver from cases in which nutritional or other factors have resulted in the isolated glycogen having intermediate outer chain lengths such that a positive diagnosis of "limit dextrinosis" cannot be made from the glycogen structure alone.

Summary.—Amylo-1,6-glucosidase has been extensively purified from rabbit muscle and has been found to act directly on a singly branched oligosaccharide containing five glucose residues to yield one mole of glucose and one mole of maltotetraose. This low molecular weight substrate has been purified chromatographically from the mixed products of pancreatic α-amylase action on either glycogen or amylopectin and its structure has been investigated. It has been found suitable as a substrate for the assay of the glucosidase in small samples of human liver. This specific assay procedure has been applied to a number of cases of glycogen storage disease, and its usefulness in detecting that type of disease in which there is a lack of the glucosidase has been shown.

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*BIOLOGICAL VALIDITY OF AMINO ACID CODES DEDUCED WITH SYNTHETIC RIBONUCLEOTIDE POLYMERS**

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Nirenberg and Matthaei[1] discovered that polyuridylic acid specifically stimulates the incorporation of phenylalanine into peptide linkage ("protein") in cell-free systems derived from *Escherichia coli*. They suggested that the polyuridylic acid functions as a synthetic template or messenger RNA,† replacing normal messenger,[2] and that the nucleotide code for phenylalanine consists of one or more residues of uridylic acid. Later studies with a variety of synthetic ribonucleotide polymers made it possible to deduce some features of the presumed code for almost all of the common amino acids. The conclusions reached independently in the laboratories of Nirenberg and Ochoa have been summarized recently[3,4] and are almost in complete accord (Table 1). Reference to amino acid replacements in homologous proteins, in particular, the proteins of tobacco mosaic virus mutants[3, 4] and the human hemoglobins,[5] supports the belief that the codes are biologically valid. We wish to call attention to additional evidence of their validity.

Gross Composition of Bacterial DNA and Protein.—The messenger hypothesis[2] holds that genetically active DNA directly governs the synthesis of a messenger RNA, which mimics the DNA in nucleotide composition and sequence. At the level of individual polynucleotide strands, the structural relation appears to be one of complementarity (A → U, T → A, G → C, C → G).[6–9] In turn, the messenger, acting as a template for protein synthesis, is thought to dictate through its sequence the sequence of amino acids in polypeptide chains.

Sueoka,[10, 11] working with a set of bacteria that differ widely in DNA base composition, has recently verified one consequence of the hypothesis, namely, that the mole fraction of individual amino acids in protein should be correlated with DNA