A Study of the Reaction Catalysed by the Liver Branching Enzyme

By W. VERHUE AND H. G. HERS

Laboratory of Physiological Chemistry, University of Louvain, Louvain, Belgium

(Received 2 August 1965)

The mechanism of action of liver branching enzyme has been studied by using as substrate two polysaccharides in which the non-reducing ends had been labelled by incubation with phosphorylase and a trace amount of [^{14}C]glucose 1-phosphate. After these polysaccharides had been treated with branching enzyme, their structure was analysed by periodate oxidation, by degradation with phosphorylase and amylo-(1 $\rightarrow$ 6)-glucosidase and by degradation with pullulanase. All the results indicate that the branching enzyme catalyses the transfer from (1 $\rightarrow$ 4)- to (1 $\rightarrow$ 6)-linkage of a chain of glucose units, the minimum length of which is six glucose units. A maltodextrin containing 16 glucose units was not acted on by the branching enzyme.

The transfer of a (1 $\rightarrow$ 4)-linked glucosyl unit located in the outer chains of glycogen or amylopectin into a (1 $\rightarrow$ 6)-position by branching enzyme has been demonstrated by Larnier (1953). The substrate used by this author was a glycogen that contained (1 $\rightarrow$ 4)-linked [^{14}C]glucosyl units in the outer chains; when these chains were acted on by branching enzyme, radioactive (1 $\rightarrow$ 6)-linked glucosyl units were formed from glucosyl units previously (1 $\rightarrow$ 4)-linked (Fig. 1). The enzyme has been described as a transglycosylase, capable of transferring a terminal segment of a chain to the 6-position of a non-terminal glucosyl unit; there is a specific requirement for chain length before branching can occur, but the length of the segment that is transferred has not been defined. However, branching enzyme has not been extensively purified and the experiments performed by Larnier (1953) do not rule out the possibility that the (1 $\rightarrow$ 6)-linked [^{14}C]glucosyl unit originates from the peripheral position of the outer chains. This glucosyl transfer from (1 $\rightarrow$ 4)- to (1 $\rightarrow$ 6)-linkage could be followed by one or several transfers from (1 $\rightarrow$ 4)- to (1 $\rightarrow$ 4)-linkage of either an oligoglucan or of single glucosyl units, under the action of either the same or of another enzyme (Fig. 1). A two-step mechanism would be somewhat analogous to that of the debranching of glycogen phosphorylase limit dextrin by amylo-(1 $\rightarrow$ 6)-glucosidase, which involves the transfer from (1 $\rightarrow$ 4)- to (1 $\rightarrow$ 4)-linkage of an oligoglucan followed by the hydrolysis of a (1 $\rightarrow$ 6)-glucosidic linkage (Walker & Whelan, 1960; Abdullah & Whelan, 1963; Brown, Illingworth & Cori, 1963; Hers, Verhue & Mathieu, 1964). Although different in nature, these two steps are apparently catalysed by a single enzyme (Brown & Illingworth, 1964).

Fig. 1. Schematic representation of two possible mechanisms for the branching of the outer chains of glycogen. On the left (a), part of a (1 $\rightarrow$ 4)-glucan chain (arbitrarily chosen as a maltohexaosyl unit) is transferred from a 4- to a 6-position. In this process, the peripheral unit (●) has remained in a peripheral position after branching; therefore it would still release formic acid on periodate oxidation and would appear as glucose 1-phosphate during degradation by phosphorylase and amylo-(1 $\rightarrow$ 6)-glucosidase. On the right (b), the peripheral unit is first transferred to a 6-position and is then covered by other glucosyl units through the transfer from (1 $\rightarrow$ 4)- to (1 $\rightarrow$ 4)-linkage of a maltopentaosyl unit or by repeated transfers of shorter oligosaccharide or even of single glucosyl units. The unit now in the 6-position would not release formic acid on periodate oxidation and would appear as glucose during degradation by phosphorylase and amylo-(1 $\rightarrow$ 6)-glucosidase. ○, Glucosyl unit; ⊙ and ●, radioactive glucosyl unit in the experiments performed by Larnier (1953); ●, radioactive glucosyl unit in the experiments described in this paper; —, α -(1 $\rightarrow$ 4)-glucosidic linkage; →, α -(1 $\rightarrow$ 6)-glucosidic linkage.

In the present paper we describe experiments similar to those of Larnier (1953), except that only the peripheral glucosyl units of the outer chains in the substrate polysaccharide were labelled. After treating this substrate with branching enzyme, two types of analysis on the newly branched product

allow it to be determined whether the branching has occurred by the single transfer of an oligoglucan or by repeated transfers. First, the amount of radioactivity present in the formic acid released on periodate oxidation would be unchanged by the first mechanism but markedly lowered by the second; secondly, during degradation by phosphorylase and amylo-(1→6)-glucosidase the glucose formed would be unlabelled according to the first hypothesis and labelled according to the second (Fig. 1). Both types of analysis gave results in favour of a single transfer.

To determine the length of the oligosaccharide that had been transferred, the newly branched polysaccharide was submitted to degradation by pullulanase. This enzyme is known to hydrolyse the outermost (1→6)-glucosidic linkages and to liberate the side chains that are longer than a single glucose unit (Bender & Wallenfels, 1961; Brown, Illingworth & Kornfeld, 1965). It will therefore yield a series of labelled oligosaccharides identical with those that have been transferred to a (1→6)-linkage. By this method the preferential transfer of a maltoheptaose unit has been demonstrated. A preliminary report of this work has been published (Verhue & Hers, 1965).

MATERIALS AND METHODS

Enzymes. Branching enzyme was prepared as described by Krisman (1962). It had the specific activity reported by this author and was free of α -amylase; it was, however, slightly contaminated with amylo-(1→6)-glucosidase (0.002 μ mole of glucose/min./mg. of protein formed from a phosphorylase limit dextrin) and with phosphorylase (0.09 μ mole of phosphate/min./mg. of protein). Phosphorylase *b* was prepared by the method of Fisher & Krebs (1962) and recrystallized 11 times; 1 mg. of protein formed 60 μ -moles of inorganic phosphate/min. The method of Bueding & Hawkins (1964) was used to prepare amylo-(1→6)-glucosidase; 1 mg. of protein formed 2 μ -moles of glucose/min. from a phosphorylase limit dextrin. Pullulanase was a gift of Dr K. Wallenfels; 1 mg. of protein formed 0.006 μ -mole of maltose or maltotriose/min. from a glycogen β -amylase limit dextrin. Phosphorylase and amylo-(1→6)-glucosidase activities were measured as described by Hers (1964).

Substrates. Two polysaccharides were labelled at their

non-reducing ends by incubation with a trace amount of [U-¹⁴C]glucose 1-phosphate and phosphorylase. The first (polysaccharide I) was made from rabbit-liver glycogen (100 mg.) that had previously been incubated with unlabelled glucose 1-phosphate (250 μ -moles) and phosphorylase (250 μ g.) with the result that the average chain length had been increased from 12 to 15.7 glucose units. This 'long-chain' glycogen was purified by repeated precipitation with ethanol and dialysed. The second substrate (polysaccharide II) was made from commercial amylopectin (L. Light and Co. Ltd., Colnbrook, Bucks.). In both cases 100 mg. of the polysaccharide and 0.5 μ mole of [U-¹⁴C]-glucose 1-phosphate were incubated for 15 hr. at 37° and pH 6 with 250 μ g. of phosphorylase in a total volume of 1.8 ml. The reaction was stopped by heating for 3 min. at 100° and the polysaccharide was isolated by repeated precipitation with ethanol. The specific radioactivities of polysaccharides I and II were 29 700 counts/min./mg. and 23 200 counts/min./mg. respectively. As shown in Table 1, the percentage of radioactivity present in the formic acid after periodate oxidation (see below) indicates that 80% of the labelled units were located at the periphery in polysaccharide I. For unknown reasons, the yield of [¹⁴C]formic acid from the amylopectin was one-quarter of that expected, suggesting that the radioactive units have covered each other to a large extent.

[U-¹⁴C]Glucose 1-phosphate was obtained by exhaustive action of purified phosphorylase and amylo-(1→6)-glucosidase on radioactive starch (25 μ C/mg.; The Radiochemical Centre, Amersham, Bucks.), AMP and potassium phosphate buffer, pH 7.4. No more than 15–30% of the polysaccharide is converted into glucose 1-phosphate by this procedure. To facilitate the purification of the ester and to make use of unchanged material, the reaction mixture was brought to pH 3.5 with *N*-acetic acid and incubated with a solution of a crude α -glucosidase (Takamine diazyme powder; Miles Laboratory, Clifton, N.J., U.S.A.). By this procedure all the unchanged dextrans were converted into glucose. Glucose 1-phosphate was then separated from glucose and from phosphate by paper chromatography in ethanol-*m*-ammonium acetate, pH 7.5 (Paladini & Leloir, 1952).

Linear oligosaccharides of the maltose series were obtained by heating a 10% suspension of amylose in 0.1 *N*-H₂SO₄ for 1 hr. at 100°. After neutralization, 1.5 vol. of 95% (v/v) ethanol was added and the precipitate was removed by centrifugation and discarded. A weight of charcoal (Darco) equivalent to the weight of the initial amylose was added and the suspension was centrifuged again. The supernatant solution was deionized with Amberlite MB-3 (mixed-bed resin) and concentrated *in vacuo*, and placed on Whatman 3MM paper, which was irrigated for

Table 1. Radioactivity of formic acid obtained by periodate oxidation of the radioactive polysaccharides

The oxidation was performed as described in the Materials and Methods section, except that the duration of the reaction with periodate varied. The results are expressed as percentages of the total radioactivity recovered after the oxidation.

Oxidation time ...	Radioactivity of formic acid (% of total)					
	16 hr.	24 hr.	40 hr.	5 days	12 days	16 days
Polysaccharide I	10.6	11.3	12.0	13.1	13.7	13.7
Polysaccharide II	3.3	3.9	3.5	3.9	4.4	4.3

1–3 weeks with water–ethanol–nitromethane (23:41:35, by vol.) (Robyt & French, 1963). Sixteen components were separated by this method and they are assumed to correspond to glucose and the first 15 polymers of the maltose series (Glc₂ to Glc₁₆).

Periodate oxidation. Approx. 100 μ g. of radioactive polysaccharide was mixed with 10 mg. of unlabelled polysaccharide, precipitated twice with ethanol and dissolved in 0.3 ml. of cold water. Then 0.2 ml. of 0.46 M-NaIO₄ was added and the mixture was left for 40 hr. at 0°. The unchanged periodate was then destroyed by the addition of 0.02 ml. of 10 M-ethylene glycol. After 10 min. the mixture was transferred to a flask for the determination of radioactivity in the Packard Tri-Carb liquid-scintillation spectrometer, together with 0.1 ml. of 0.05 N-H₂SO₄ which was used to rinse the incubation tube. The formic acid was extracted four times with 6 ml. of ether and the washings were transferred to another Packard flask containing 0.5 ml. of 0.1 N-NaOH. This second flask was heated at 80° to evaporate the ether. To each flask 10 ml. of a scintillation mixture containing 10% (w/v) of naphthalene, 1% of 2,5-diphenyloxazole, 0.025% of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 20% (v/v) of ethanol dissolved in dioxan was added. All radioactivity determinations were made with a Packard Tri-Carb liquid-scintillation spectrometer. The radioactivity of the formic acid is expressed as a percentage of the total radioactivity (sum of radioactivity of the formic acid plus radioactivity of the aqueous residue). If all the radioactive glucosyl units were in a peripheral position in the polysaccharide, they would all yield C-3 as formic acid, which would then contain 16.6% of the total radioactivity when the oxidation is complete.

Degradation of polysaccharides by phosphorylase and amylo-(1→6)-glucosidase. A 0.1 mg. sample of polysaccharide, 5 μ moles of potassium phosphate buffer, pH 7.4, 0.05 μ mole of AMP, 25 μ g. of phosphorylase *b* and 13 μ g. of amylo-(1→6)-glucosidase were incubated for 2 hr. at 37° in a total volume of 0.11 ml. The reaction was stopped by the addition of 1.2 ml. of 95% (v/v) ethanol, 0.7 ml. of water and 20 mg. of charcoal (Darco). After centrifugation, the supernatant solution was concentrated *in vacuo* and chromatographed on Whatman 3MM paper in ethanol–m-ammonium acetate, pH 7.5 (Paladini & Leloir, 1952), to separate glucose 1-phosphate from glucose. For the determination of radioactivity, the paper was cut into small strips which were dipped directly in 10 ml. of the following scintillating mixture: 0.4% of 2,5-diphenyloxazole and 0.020% of 1,4-bis-(5-phenyloxazol-2-yl)benzene dissolved in toluene. This method enables the separate counting of the glucosyl units that were (1→4)-linked in the polysaccharide as glucose 1-phosphate and of those that were fixed by a (1→6)-linkage as glucose.

Degradation of polysaccharides with pullulanase. A 0.2 mg. sample of polysaccharide, 0.4 mg. of pullulanase and 50 μ moles of sodium citrate–citric acid buffer, pH 6.4, in a total volume of 0.15 ml. were incubated for 15 hr. at 40°. Then 0.65 ml. of water, 1.2 ml. of 95% (v/v) ethanol and 20 mg. of charcoal (Darco) were added. After vigorous shaking followed by centrifugation, 0.1 ml. of the solution was used for the determination of total radioactivity. The rest was deionized with a small amount of Amberlite MB-3 (mixed-bed resin), dried *in vacuo* and put on Whatman 3MM paper. The oligosaccharides were separated by multi-ascendent chromatography with water–ethanol–nitromethane

(23:41:35, by vol.) as solvent, as described by Robyt & French (1963), and their radioactivity was determined as described in the preceding paragraph. This method allows the radioactivity of side chains that are longer than one glucosyl unit to be counted. The upper limit of the length of the side chains that can be liberated by pullulanase is unknown, however.

RESULTS

A progressive decrease of chain length of the two radioactive substrates during incubation with branching enzyme is indicated by the decrease in the extinction of their iodine complex (Fig. 2). At various stages of the reaction, samples of polysaccharides I and II were isolated and submitted to chemical and enzymic analysis.

Periodate oxidation. Owing to the relatively low yield of [¹⁴C]formic acid obtained by oxidation of polysaccharide II, this type of analysis was performed only with polysaccharide I. The results indicated that, after the action of branching enzyme, the amount of radioactivity present in the peripheral glucosyl units of the polysaccharide was approximately the same as in the starting substrate.

Degradation by phosphorylase and amylo-(1→6)-glucosidase. When degraded by phosphorylase and amylo-(1→6)-glucosidase, polysaccharides I and II yielded practically all their radioactivity as glucose 1-phosphate and none as glucose. This was also true of polysaccharide I at various stages of the action of the branching enzyme. With polysac-

Fig. 2. Activity of the branching enzyme on the two substrates as a function of time. A 0.6 mg. sample of polysaccharide, 0.05 mg. of branching enzyme and 36 μ moles of sodium citrate–citric acid buffer, pH 6.5, were incubated at 37° in a total volume of 0.12 ml. At the end of the incubation period, 0.04 ml. of the mixture was heated at 100° for 2 min. and mixed with 2 ml. of 2 M-iodine. The extinction of the iodine–polysaccharide complex was read at 520 m μ . ○, Polysaccharide I; △, polysaccharide II.

Fig. 3. Distribution of the radioactivity in the oligosaccharides formed by the action of pullulanase on polysaccharide I before and after incubation with branching enzyme. The radioactivity of the substrate was 5500 counts/min. for each degradation. The time refers to the duration of the incubation with the branching enzyme. The numbers 1, 2, 3 etc. refer to glucose, maltose, maltotriose etc. The tenth block corresponds to maltodecaose together with longer oligosaccharides that were not separated from maltodecaose. The last block on the right shows the radioactivity of the compounds that stayed at the origin of the chromatogram.

charide II, the percentage of radioactivity recovered as glucose increased from 0.45 to 2.3% during 3 hr. incubation with the branching enzyme.

Degradation with pullulanase. Polysaccharides I and II were treated with pullulanase and the percentage of the radioactivity that appeared in the ethanol-soluble fraction (obtained by treatment with charcoal and ethanol) was only 7% for polysaccharide I but 45% for polysaccharide II. After incubation with branching enzyme, these percentages increased to 25 and 66% respectively. As shown by Figs. 3 and 4, these increases are due to the appearance of a large amount of radioactive oligosaccharides of the maltose series, ranging mostly from maltohexaose to maltononaose. Oligosaccharides longer than nine glucose units were also present but were not separated. In both experiments, maltoheptaose was the most abundant product, whereas practically no oligosaccharides shorter than maltohexaose were formed. It therefore appears that a maltoheptaose unit is preferentially transferred from a (1→4)- to a (1→6)-linkage by the liver branching enzyme and that oligosaccharides shorter than maltohexaose are not transferred.

Search for an action of branching enzyme on linear oligosaccharides. Linear oligosaccharides of the maltose series ranging from Glc₁₃ to Glc₁₆ were incubated with branching enzyme. This incubation did not cause any change in the spectrum of the iodine complex of the oligosaccharides. Further treatment with pullulanase did not cause the appearance of shorter oligosaccharides. One can therefore conclude that the branching enzyme does not act on these oligosaccharides.

DISCUSSION

The experiments presented in this paper give strong support to the theory proposed by Larner (1953) for the mechanism of action of liver branching enzyme. The fact that the peripheral units remain in a peripheral position during branching is proof that branching occurs by transfer of a single oligosaccharide chain and not by a succession of transfers. This conclusion is reinforced by the experiment with phosphorylase and amylo-(1→6)-glucosidase, which shows that, at least with polysaccharide I, none of the peripheral glucose units were put in a (1→6)-linkage by the branching

Bioch. 1966, 99

Fig. 4. Distribution of the radioactivity in the oligosaccharides formed by the action of pullulanase on polysaccharide II before and after action of the branching enzyme. The total radioactivity of the substrate was 5100 counts/min. for each degradation. For other explanations see Fig. 3.

enzyme. The experiment performed on polysaccharide II is less conclusive in this respect. The slight increase of radioactivity in the glucose liberated by amylo-(1→6)-glucosidase is, however, easily explained by the fact that, as shown by periodate oxidation, no more than 25% of [^{14}C]-glucosyl units were located at the periphery of the molecule. It therefore appears possible that some of them were in the sixth or seventh position in the outer chains of the substrate and were (1→6)-linked after the transfer.

The degradation by pullulanase has shown that the new branches are at least six glucosyl units long and that maltoheptaose is the preferential oligosaccharide transferred. Information on the structural requirement of the substrate that can be acted on by the branching enzyme is still indirect. We have shown that the enzyme does not ramify the Glc_{16} maltodextrin and this indicates that only longer chains can be used as a substrate. A similar observation has been made by Nussenbaum & Hassid (1952) and by Peat, Whelan & Bailey (1953) for the plant Q-enzyme. Although we do not know if this conclusion can be extended to the outer chains of glycogen, it fits an observation made by Brown *et al.* (1965) that glycogen synthetase (UDP-glucose-glycogen glucosyltransferase) tends

to make repeated transfers from UDP-glucose to the same chain, with the consequence that apparently some of the chains become very long and could therefore constitute an appropriate substrate for the branching enzyme. The lack of reactivity of the branching enzyme towards oligosaccharides made it impossible to determine whether or not the donor and the acceptor glucosyl units must be part of the same chain.

Our observations may be correlated with another conclusion reached by Brown *et al.* (1965) that the transfer of glucosyl from UDP-glucose to oligosaccharides and polysaccharides under the action of glycogen synthetase is mostly (up to 98%) on the main chains. The side chains, at least those that in glycogen and in its phosphorylase limit dextrin could be taken out by pullulanase, are very poor acceptors for new glucosyl units. It has also been demonstrated that, in the oligosaccharides, the main chains need not be longer than one glucosyl unit peripheral to the branching point to act as acceptors. It therefore seems possible that the polysaccharide that is formed after branching has short main chains (possibly one glucosyl unit) and, as shown in the present paper, side chains that are at least six units long (Fig. 5). It also appears that, to be elongated by glycogen synthetase, a side chain

Fig. 5. Minimal structure of the outer chains of a polysaccharide after action of the branching enzyme (see the text). $\circ$, Glucosyl unit; $-$, α -(1 $\rightarrow$ 4)-glucosidic linkage; $\rightarrow$, α -(1 $\rightarrow$ 6)-glucosidic linkage; the inner chain is four units long and could be fixed to the core of the molecule by either an α -(1 $\rightarrow$ 4)- or an α -(1 $\rightarrow$ 6)-glucosidic bond.

must first be converted into a main chain by accepting itself a new chain in a 6-position. As a side chain newly formed by branching is long enough to give birth to both a new inner chain and a short main chain, it constitutes an adequate acceptor for further transfer from (1 $\rightarrow$ 4)- to (1 $\rightarrow$ 6)-linkage. This could only occur if the branching enzyme can transfer a segment of an outer chain to a neighbouring chain. The parent chain would then be a main chain and could not be shorter than seven glucosyl units; the acceptor glucosyl unit would be either in a main chain or in a side chain. Larnier (1953) has already shown that, to be acted on by branching enzyme, the outer chains of glycogen must have an average length greater than six units.

As mentioned above, there is no proof that the transfer to a neighbouring chain can occur. If it

cannot occur, another mechanism should be proposed to account for the observations made by Brown *et al.* (1965).

This work was supported by the Fonds de la Recherche Scientifique Médicale and by the U.S. Public Health Service (Research Grant A-4053).

REFERENCES

- Abdullah, M. & Whelan, W. J. (1963). *Nature, Lond.*, **197**, 979.
- Bender, H. & Wallenfels, K. (1961). *Biochem. Z.* **334**, 79.
- Brown, D. H. & Illingworth, B. (1964). In *Control of Glycogen Metabolism*, p. 139. Ed. by Whelan, W. J. & Cameron, M. P. London: J. and A. Churchill Ltd.
- Brown, D. H., Illingworth, B. & Cori, C. F. (1963). *Nature, Lond.*, **197**, 980.
- Brown, D. H., Illingworth, B. & Kornfeld, R. (1965). *Biochemistry*, **4**, 486.
- Bueding, E. & Hawkins, J. T. (1964). *Analyt. Biochem.* **7**, 26.
- Fisher, E. H. & Krebs, E. (1962). In *Methods in Enzymology*, vol. 5, p. 369. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Hers, H. G. (1964). *Advanc. metab. Disorders*, **1**, 1.
- Hers, H. G., Verhue, W. & Mathieu, M. (1964). In *Control of Glycogen Metabolism*, p. 151. Ed. by Whelan, W. J. & Cameron, M. P. London: J. and A. Churchill Ltd.
- Krisman, C. R. (1962). *Biochim. biophys. Acta*, **65**, 307.
- Larnier, J. (1953). *J. biol. Chem.* **202**, 491.
- Nussenbaum, S. & Hassid, W. Z. (1952). *J. biol. Chem.* **196**, 785.
- Paladini, A. C. & Leloir, L. F. (1952). *Biochem. J.* **51**, 426.
- Peat, S., Whelan, W. J. & Bailey, J. M. (1953). *J. chem. Soc.* p. 1422.
- Robyt, J. & French, D. (1963). *Arch. Biochem. Biophys.* **100**, 451.
- Verhue, W. & Hers, H. G. (1965). *Biochem. J.* **97**, 10F.
- Walker, G. J. & Whelan, W. J. (1960). *Biochem. J.* **76**, 264.