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On the Equilibrium State of a Branched Molecule

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It appears at the present time to be rather unanimously assumed that the molecules of amylopectin and glycogen have a ramified structure, in which the glucose residues are united by α-glucosidic 1,4- or 1,6-linkages. The latter are in a minority, constituting only 5—10 per cent of the total number and representing the branching points of the chain molecules. As to the pattern of ramification (whether quite irregular or possessing some sort of regularity) opinions differ somewhat [1].

Recent investigations have proved that the biological synthesis of the polysaccharides in question is effected by two phosphorylases, one reversibly synthesizing 1,4-linkages, the other 1,6-linkages, the starting material being in both cases glucose-1-phosphate [1–3]. The first enzyme is the phosphorylase studied by Hanes and others which, when acting alone, yields unbranched polysaccharide molecules of the amylose type. This enzyme is called »*P*-enzyme» by Haworth *et al.* [4] Bernfeld[5] and Meyer *et. al.* [6] use the name »phosphorylase» for this enzyme. The enzyme synthesizing 1,6-linkages is termed »isophosphorylase» by Meyer. The isophosphorylase seems, on the whole, to be identical with the Haworth group's »Q-enzyme» and with Cori's »branching factor».

The present authors tried some years ago to calculate the pattern of a polysaccharide formed by the simultaneous action of both enzymes [7]. It was assumed that the two enzymes act at random, causing a ramified molecule to grow from an original »germ». As a simplication of the problem an *irreversible* synthesis was assumed. The calculation yielded, among others, two results which could be compared with experimental data: it predicted that β, the »degree of ramification» (see below), should for large molecules be independent of the molecular weight, and it gave — as a function of β only — values for ζ, the fraction of the glucose units of the polysaccharide,

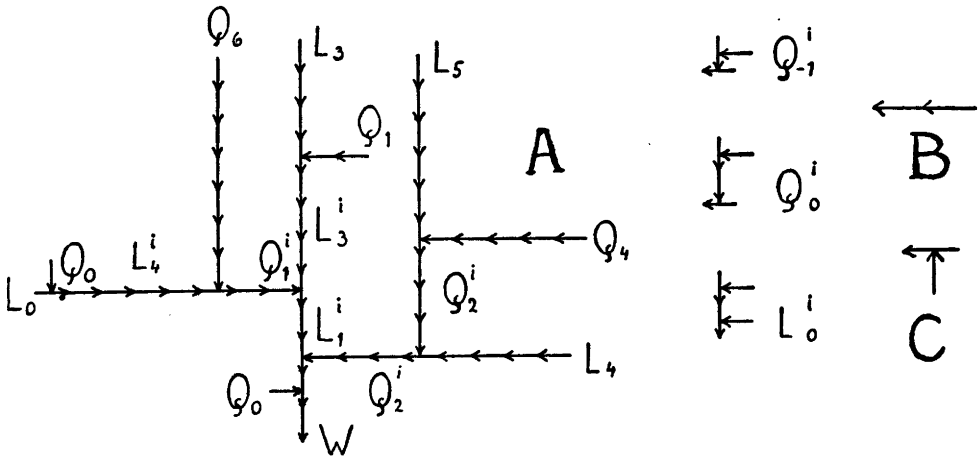

Fig. 1. A, part of a branched molecule, showing the different types of chains, Q_n, L_n, Q_n^i, and L_n^i. B, maltose; C, isomaltose.

which are split off as maltose on treatment of the polysaccharide with β-amylase; this enzyme attacks the end chains only [1]. The calculated values proved to agree reasonably well with the experiments.

Bernfeld [5] and Meyer [6] *et al.* emphasize the reversible nature of the synthesis and seem to assume that the final state of the polysaccharide molecules formed is really one of equilibrium. In the following we shall try to calculate the pattern of a molecule formed through a *reversible* synthesis by joint action of phosphorylase and isophosphorylase in such a way that the polysaccharide is in equilibrium with a solution containing simple glucose units.

Our calculations seem to show that an equilibrated branched molecule would give much lower yields of maltose on treatment with β-amylase than those actually found for amylopectin; the assumption of *irreversible* synthesis seems thus to be nearer the truth for *amylopectin*. On the other hand, it seems quite possible that *glycogen* represents an *equilibrium* state. The usual yields of maltose from glycogen, when treated with β-amylase, are of the calculated magnitude, and the larger yields occasionally reported by Meyer *et. al.* (which were once taken by us as a check for the irreversible synthesis mechanism) may possibly have been caused by previous degradation of the polysaccharide.

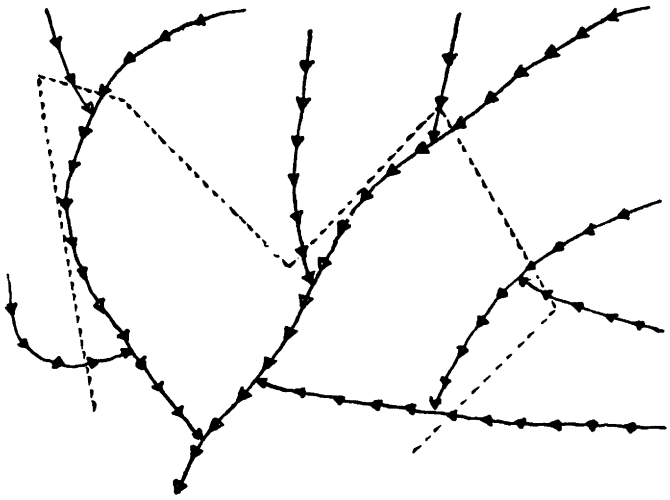

Fig. 2. Part of a ramified molecule of the amylopectin-glycogen type. Parts outside dotted line are split off as maltose by β-amylase.

THEORETICAL

Notations

Fig. 1 A gives a schematic picture of a small branched molecule. The glucose units are represented as arrows, the arrow point representing the 1-position. The 4-position is at the rear, the 6-position at the middle of the arrow shaft. Thus Fig. 1 B represents maltose (with a 1,4-linkage) and Fig. 1 C isomaltose (with a 1,6-linkage).

If the total number of glucose units in a molecule is N, and the number of 1,6-linkages (ramifications) is B, the ratio $\beta = B/N$, the *degree of ramification*, is a characteristic of the molecule, which can be estimated in several ways. Another characteristic quantity is ζ, the fraction of the total number of glucose residues that can be cut off by the action of β-amylase. This enzyme is believed to attack end chains only and to cut off the glucose units in pairs, yielding maltose (Fig. 2).

In the subsequent discussion it is convenient to introduce symbols for the different types of glucose chains: end chains of types Q_n and L_n, and inner chains of types Q_n^i and L_n^i. The numbers n are so chosen (see Fig. 1 A) that all glucose units are counted except those taking part in 1,6-links (two for every branching point). If the number of each type of chains is represented by the corresponding small letter, then

$$B = \beta N = \sum_0^\infty q_n + \sum_0^\infty l_n \tag{1}$$

$$N = \sum_0^\infty n q_n + \sum_0^\infty n l_n + \sum_{-1}^\infty n q_n^{\mathrm{i}} + \sum_0^\infty n l_n^{\mathrm{i}} + 2B \tag{2}$$

If it is assumed that the β amylase can split off maltose from Q_n and L_n so that finally only Q_0 or L_0 is left for even n and Q_1 or L_1 for odd n, then the yield will be

$$\zeta_2 N = 2\,(l_2+q_2+l_3+q_3) + 4\,(l_4+q_4+l_5+q_5) + 6\,(\ldots\ ) = \sum_1^\infty 2m\,(l_{2m}+ q_{2m}+l_{2m+1}+q_{2m+1}) \tag{3}$$

If, however, the end chains with even n are broken down only to Q_2 and L_2 the yield is

$$\zeta_3 N = \zeta_2 N - 2(l_2+q_2+l_4+q_4+\ldots) = \zeta_2 N - 2\sum_1^\infty (l_{2m}+q_{2m}) \tag{4}$$

Irreversible synthesis

The present authors have tried previously the following simple assumptions [7]. The branched molecule grows from a solution containing glucose, in the form of glucose-1-phosphate, and the enzymes. The probability that between the times t and $t+\mathrm{d}t$ a certain end chain will grow by the linking of a new glucose unit at the free 4-position is $k\mathrm{d}t$. The probability that in the same time a new branch will be formed on a certain glucose residue with a free 6-position is $\gamma k\mathrm{d}t$. The quantities k and $k\gamma$ are the same for all units with free 4- or 6-positions in the macromolecule. Even if k varies with time (*e. g.* because of irregular input of fresh glucose-1-phosphate) the ratio γ is assumed to be constant.

Under these assumptions we found that β tends to the value for which

$$\gamma = \beta^2\,(1-\beta)^{-2} \tag{5}$$

and, moreover,

$$q_n = N\,c_n\,(1-\beta)$$

$$l_n = N\,c_n\,(n+1)\,\beta$$

$$q_n^{\mathrm{i}} = N\,c_n\,(1-\beta)^3\,(1+n\beta)^{-1}\,[1+(n+1)\,\beta]^{-1}$$

$$l_n^i = q_n^i [(n+1)^2 \beta^2 + (n-1)\beta + 2] (1-\beta)^{-1} [1 + (n-1)\beta]^{-1} \quad (6)$$

where

$$c_0 = \beta^2 (1 - \beta + \beta^2)^{-1}; \; c_n = c_{n-1} (1-\beta)^2 [1 - \beta + (n+1)\beta^2]^{-1} \quad (7)$$

For the yield of maltose with β-amylase we found

$$\zeta_2 = 0.6557 - 1.4371\beta + 0.3151\beta^2 + 0.3154\beta^3 + \ldots . \quad (8)$$

if the end chains are broken down to $n = 0$ or 1, and

$$\zeta_3 = 0.6557 - 2.4371\beta + 1.8151\beta^2 + 1.8154\beta^3 + \ldots . \quad (9)$$

if they are broken down to $n = 2$ or 1. *E.g.* for amylopectin with $\beta = 0.056$ we calculated $\zeta_2 = 0.576$; $\zeta_3 = 0.525$.

Reversible synthesis

We shall consider a branched macro molecule which is in prolonged contact with a solution containing glucose-1-phosphate and the two phosphorylases. We assume, as is also done by Bernfeld and Meyer *et. al.*, that the enzymes can act only by linking new glucose units from the solution, one by one, to free 4- or 6-positions in the macromolecule, or by the reversal of these reactions: splitting off the outermost glucose units, one by one, by breaking a 1,4- or 1,6-link. Thus we assume that the enzymes cannot split off molecules with two, three or more glucose units, nor can they synthetize such molecules in the solution. (Haworth *et. al.*[4] assume that the Q-enzyme is able to attach ready made »unit chains» of considerable length to the polysaccharide.)

As an approximation it is moreover assumed here that all free 4-positions in the macromolecule are equally accessible so that the same velocity and equilibrium constants apply to all. Similarly all free 6-positions are assumed to be equivalent.

Now the problem is to find the distribution of the glucose units over the various types of chains in the equilibrium state of the molecule which is obtained when the reversible processes have been acting on the macro molecule for a very long time.

The problem can be attacked in several ways. It can be treated kinetically, as was the irreversible synthesis in our previous papers; terms for the reversed reactions are included in the time derivatives for the numbers of different types of branches, and the derivatives are equated to zero at equilibrium.

It can also be treated by applying the law of mass action, thus by assuming one constant, K_q, for the formation of 1,6-links and one, K_1, for the formation of 1,4-links.

However, the simplest line of approach seems to be the statistical; of course it gives the same results as the others. Let us regard a macro molecule at equilibrium. The number of 1,6-links is $N\beta$, that of 1,4-links is $N(1-\beta)$ and that of end chains is $N\beta$. Then for any one glucose residue ➡ in the macro molecule the probability that its

1-position forms a 1,4-link ➡→ is $1-\beta$

» forms a 1,6-link ➡↓ is β

4-position forms no link ➡ is β

» forms a 1,4-link →➡ is $1-\beta$

↓

6-position forms a 1,6-link ➡ is β

» forms no link ➡ is $1-\beta$.

Now the numbers of the different types of branches can be calculated. Let us begin with q_n.

The number of glucose units, the C_1 of which forms a 1,6-link is $N\beta$. The probability that a chain of n residues, united by 1,4-linkages, is tied to a glucose unit of this kind with a 1,4-link is $(1-\beta)^n$; that the last glucose unit has a free 4-position is β; and that all the $(n+1)$ units have their 6-positions free is $(1-\beta)^{n+1}$. Thus we find the number of Q_n chains

$$q_n = N\beta\ (1-\beta)^n\ \beta(1-\beta)^{n+1} = N\beta^2\,(1-\beta)^{2n+1}$$

$$q_0 = N\beta^2\,(1-\beta) \tag{10}$$

In the same way we find, as can easily be controlled by means of Fig. 1,

$$l_n = N\beta\,(1-\beta)^n\,\beta(1-\beta)^n = N\beta^2\,(1-\beta)^{2n}$$

$$l_0 = N\beta^2 \tag{11}$$

$$l_n^i = N\beta\,(1-\beta)^{n+1}\,\beta\,(1-\beta)^n = N\beta^2\,(1-\beta)^{2n+1}$$

$$l_0^i = N\beta^2\,(1-\beta) \tag{12}$$

$$q_n^i = N\beta\,(1-\beta)^{n+1}\,\beta\,(1-\beta)^{n+1} = N\beta^2\,(1-\beta)^{2n+2}$$

$$q_{-1}^i = N\beta^2 \tag{13}$$

These formulas can by elementary summation be found to agree with (1) and (2).

For the yield of maltose with β-amylase we find using (3) and (4):

$$\zeta_2 N = \sum_1^\infty 2m(l_{2m} + q_{2m} + l_{2m+1} + q_{2m+1}) =$$

$$= N\beta^2\,(2-\beta)\,[1+(1-\beta)^2]\,2\sum_1^\infty m(1-\beta)^{4m} = \frac{2N\beta\,(1-\beta)^4}{1-(1-\beta)^4}$$

and

$$\zeta_2 N - \zeta_3 N = 2\sum_1^\infty (l_{2m} + q_{2m}) = 2N\beta^2\,(2-\beta)\sum_1^\infty (1-\beta)^{4m} =$$

$$= \frac{2N\beta^2\,(2-\beta)\,(1-\beta)^4}{1-(1-\beta)^4}$$

from which

$$\zeta_2 = \frac{2\beta\,(1-\beta)^4}{1-(1-\beta)^4} = 0.5 - 1.25\beta + 0.625\beta^2 + 0.3125\beta^3 + 0.03125\beta^4 + \ldots. \tag{14}$$

$$\zeta_3 = \frac{2\beta\,(1-\beta)^6}{1-(1-\beta)^4} = 0.5 - 2.25\beta + 3.625\beta^2 - 2.1875\beta^3 + 0.03125\beta^4 + \ldots. \tag{15}$$

(Incidentally these equations happen to be the same as those obtained if it is assumed that the synthesis is irreversible but that branching can take place only at the last units of the end chain/»Case E»/[7].)

The equilibrium constants

The process of forming a new 1,4-link can be written:

$$C_6H_{11}O_5PO_4H^- + \overset{4}{H}OC \ldots\ldots \rightleftharpoons C_6H_{11}O_5\overset{4}{O}C \ldots\ldots + H_2PO_4^-$$

or

$$C_6H_{11}O_5PO_4H^- + \overset{\cdot}{\longrightarrow} x \rightleftharpoons \longrightarrow\overset{\cdot}{} x + H_2PO_4^- \tag{16}$$

Here $\overset{\cdot}{\longrightarrow} x$ stands for any *specified* type of end chain which is of course *the same* in both membra of (16). At the x at C_1 there may be a 1,6-link or a chain

of a certain number of glucose residues coupled by 1,4-links and ended by a 1,6-link. Similarly at the point at the 6 position there may or may not be a 1,6-link.

The equilibrium constant for the process is

$$K_1 = \frac{\left\{\longrightarrow\overset{\cdot}{\longrightarrow} x\right\}\left\{H_2PO_4^-\right\}}{\left\{\longrightarrow\overset{\cdot}{} x\right\}\left\{C_6H_{11}O_5PO_4H^-\right\}} = \frac{\left\{\longrightarrow\overset{\cdot}{\longrightarrow} x\right\}}{\left\{\longrightarrow\overset{\cdot}{} x\right\} g} \tag{17}$$

We shall consider the quantity g introduced here. If K is the thermodynamic equilibrium constant for the formation of glucose-1-phosphate from dihydrogen phosphate ion and glucose,

$$C_6H_{12}O_6 + H_2PO_4^- \rightleftharpoons C_6H_{11}O_5PO_4H^- + H_2O \tag{18}$$

then g is related to the activities of the reacting substances by

$$g = \frac{\left\{C_6H_{11}O_5PO_4H^-\right\}}{\left\{H_2PO_4^-\right\}} = K\frac{\left\{C_6H_{12}O_6\right\}^*}{\left\{H_2O\right\}} \tag{19}$$

The asterisk (*) signifies that the *glucose* activity is *virtual* (the activity glucose would have had in equilibrium with glucose-1-phosphate and phosphate at the prevailing concentrations) and need not bear any relation to the actual concentration of glucose, since the equilibrium (18) is generally not realized.

One might also consider g as a measure of the virtual activity $\left\{C_6H_{10}O_5\right\}^*$ of glucose residues in the solution.

Of course, one might equally well use one of the ratios $\frac{\left\{C_6H_{11}O_5PO_4H_2\right\}}{\left\{H_3PO_4\right\}}$ or $\frac{\left\{C_6H_{11}O_5PO_4^{2-}\right\}}{\left\{HPO_4^{2-}\right\}}$, which always bear a constant ratio to g.

From the foregoing (compare *e.g.* q_n and q_{n+1} in (10) or l_n and l_{n+1} in (11) and use (17)) it is evident that

$$K_1 = (1-\beta)^2 g^{-1} \tag{20}$$

The process of forming a new 1,6-link is

$$\cdot\longrightarrow x + C_6H_{11}O_5PO_4H^- \rightleftharpoons \quad \cdot\underset{\downarrow}{\longrightarrow} x + H_2PO_4^- \tag{21}$$

where $\cdot\longrightarrow x$ stands for any glucose unit with a free C_6. Now the number of such units in the macro molecule is $N - B = N(1-\beta)$. The group in

the right member of (21) is a Q_0 chain; using (10) we find for the equilibrium constant K_q of (21)

$$K_q = \frac{q_0}{(N - B)g} = \beta^2 g^{-1} \tag{22}$$

Because of (20) and (22) both β and g are uniquely determined at equilibrium by K_q and K_1. On the other hand, since β and g can both in principle be determined experimentally, it should be possible to measure K_1 and K_q separately.

Let us call the (bimolecular) velocity constant for the formation of new 1,4-links k_{1+} and that for new 1,6-links k_{q+}. The (bimolecular) constants for the breaking of the same links are denoted by k_{1-} and k_{q-}. The equilibrium constants for (18) and (21)

$$K_q = \frac{k_{q+}}{k_{q-}}\,;\; K_1 = \frac{k_{1+}}{k_{1-}} \tag{23}$$

are based on thermodynamics; these ratios are thus independent of the enzyme concentrations. On the other hand the ratio

$$\gamma = \frac{k_{q+}}{k_{1+}} \tag{24}$$

which occurred in our calculations on irreversible synthesis, may be different in different enzyme mixtures, and there is no reason why there should be any relation between γ, K_q and K_1.

DISCUSSION

We shall now see whether the equations derived for the equilibrium state can apply to the branched molecules of amylopectin and glycogen. If we insert the value $\beta = 0.056$ for amylopectin, we find $\zeta_2 = 0.432$ from (14) and $\zeta_3 = 0.385$ from (15). The experimental values are actually much higher, between 0.53 and 0.62, and agree rather well with those calculated from (9) for the case of irreversible synthesis. Thus it seems very improbable that amylopectin corresponds to an equilibrium state. Of course, the existence of long, almost entirely unbranched amylose chains is also quite incompatible with the assumption of equilibrium with a solution containing both 1,4- and 1,6-linking enzymes.

On the other hand, for glycogen with $\beta = 0.090$, we calculate $\zeta_2 = 0.393$ and $\zeta_3 = 0.325$ from (14—15). The experimental values of ζ are generally between 0.35 and 0.45. In a few exceptional experiments, Meyer obtained

ζ values for glycogen as high as 0.53. However, in these cases the glycogen had been treated repeatedly with alkali hydroxide solutions so that it does not seem improbable that a certain disruption of the inner chains had taken place which would of course increase ζ.

The hypothesis that amylopectin is chiefly formed by an irreversible process whereas glycogen is an equilibrium product is not incompatible with the different modes of formation. The starch is generally formed by a oneway process and stored for a long time, whereas the glycogen is formed as a reserve for short times of delivery and is continuously changing its amount in the animal (or yeast) organism. It is also possible that the differences between amylopectin, amylose and glycogen may be related to the growth of the starch granules [8].

It is possible that under certain conditions the organism can form glycogen so rapidly that there is no time for equilibrium. In such samples of glycogen the relation between β and ζ would deviate from (14 or 15) and instead tend towards (8 or 9). Thus at least one of β or ζ would differ from the values for normal, equilibrated glycogen.

If glycogen with $\beta = 0.09$ corresponds to the thermodynamic equilibrium between 1,4- and 1,6-links, we can then calculate

$$\frac{K_q}{K_1} = \frac{\beta^2}{(1-\beta)^2} = \frac{(0.09)^2}{(0.91)^2} \approx 0.01$$

SUMMARY

The paper deals with the pattern assumed by a branched macromolecule such as amylopectin or glycogen in equilibrium with a solution containing glucose-1-phosphate and the two phosphorylases causing the formation and breaking of α-glucosidic 1,4- and 1,6-linkages. Formulas (10—13) are derived for the partition of the glucose residues over different types of inner chains and end chains.

A quantity especially suited for comparison with experiments is ζ, the fraction of the glucose residues split off by the action of β-amylase.

For the case of equilibrium (reversible synthesis) equations (14, 15) are derived, giving ζ as a function of β, the degree of ramification. The authors have previously derived (8, 9), giving ζ (β) for the case of completely irreversible synthesis. When these equations are tested with experimental values of β and ζ, it seems that amylopectin is not in an equilibrium state but rather approaches the case of irreversible synthesis. The values for glycogen on the

other hand seem to agree rather well with the formula for equilibrium. The difference between amylopectin and glycogen may be understood from the different way in which they are formed.

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