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Enzymatic Breakdown of Glycogen

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The acid hydrolysis of glycogen was described in a previous paper [1]. In this paper we shall deal with the enzymatic breakdown of glycogen and of partly acid hydrolyzed glycogen. Experiments with periodate oxidation have also been carried out and the »formic-acid method» for determining the end-group content of glycogen applied.

ENZYMATIC INVESTIGATIONS

Myrbäck and Sillén [2,3] have attempted to calculate the constitution of amylopectin and glycogen from simple assumptions on the mechanism of the enzymatic synthesis. Lengthening of the chains occurs at carbon atom 4 at a certain rate and ramification at carbon atom 6 at another (lower) rate. Assuming that the ratio between these rates is constant, they have shown that even at moderately low degrees of polymerization the degree of branching assumes a constant value. It is also possible to calculate the distribution of the glucose units in chains of different lengths, as well as the average chain-length. The following two extreme cases are considered:

A. The ramification may occur at every glucose unit having a free hydroxyl group at carbon atom 6.

The problem of the actual position of the branching is, however, of no importance for these calculations.

B. The ramification may occur at the terminal group only.

The following calculations refer to glycogen assuming the end-group content to be 9 %. Provided that at least 1 or 2 units of the end-chain must remain at the branching point, β-amylase will yield 53 % or 45 % of maltose (case A) and 39 % or 33 % of maltose (case B). Meyer and Jeanloz [4] found the yield to be 43—47 %, while the experiments in this paper give slightly lower values: 38—46 % of maltose. The theoretical values for the average chain-length of

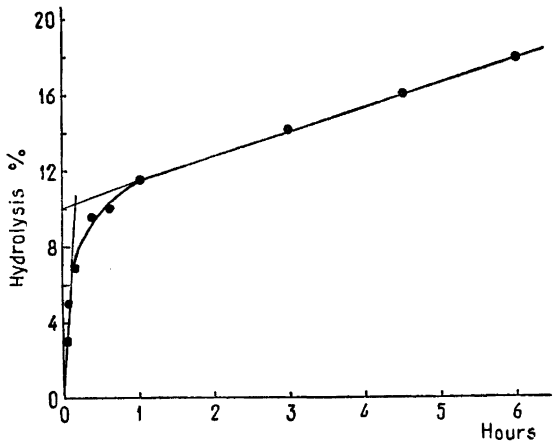

Fig. 1. The time course of the hydrolysis of glycogen with α-amylase.

the interior and the end-chains are 2.8 and 6.4 respectively (case A) and 4.3 and 4.8 respectively (case B). Meyer and Fuld [5] found that the β-dextrin contained 18 % of end-groups. Hence they concluded that the interior chains, *i. e.* the chains between two ramifications, have an average length of about 3 units and the end-chains of about 6 units.

From these results it is evident that the structure of a glycogen molecule corresponds to an intermediate state between the extreme states, but more close to A than to B. Similar results have been obtained in the case of starch (amylopectin) [3].

a) α-Amylase

In germinating barley there is formed or activated an enzyme, called dextrinogenic or α-amylase. According to Ohlsson [6], it is rather stable at increased temperatures and can easily be separated from β-amylase which is rapidly destroyed above 70°.

Experimental part

Preparation of α-amylase. 200 g of ground malt was extracted for 45 minutes with 1 l of cold water, and the suspension thus obtained was kept at 70° for 12 minutes. After filtration, alcohol was added so that the concentration became 70 % by volume. The mixture was kept at 0° for one hour. The precipitate was then centrifuged off, washed with 95 % alcohol and dried *in vacuo*. Finally, a water solution was prepared.

Performance of the experiments. 25 ml of 2—3 % substrate solutions, 5 ml of a phosphate buffer (1/15 *M*, pH = 5.2) and 1 ml of the enzyme solution were made up with water to 50 ml in a flask fitted with a glass stopper. A few drops of toluene were added, and the flask was kept at 30° in a thermostat. Just after adding the enzyme and at suitable intervals, 5 ml of the solution was pipetted into an iodine solution, and the reducing power was determined. A blank containing no substrate was treated in the same manner.

The result from an experiment with glycogen is shown in Fig. 1. The hydrolysis curve consists of two separate branches. This fact has been stated many times by Myrbäck and co-workers [7–10] in the case of starch and also of glycogen [3]. After about 10 % of loosened linkages, the reducing power increases very slowly. The practically straight lines are extended until they meet, and the point of intersection corresponds to 10.3 %. The mean value of four experiments was 10.1 ± 0.3 %.

The various dextrin preparations described in a previous paper [1] were also treated with α-amylase. The number of rapidly broken linkages was calculated from the curves of hydrolysis as the difference between the reduction value at the point of intersection and the original degree of hydrolysis (Table 1).

Table 1. Treatment of various dextrin solutions (hydrolyzates) with α-amylase.

No.	Original degree of hydrolysis %	Number of rapidly broken linkages %	No.	Original degree of hydrolysis %	Number of rapidly broken linkages %
1	4.0	8.0	6	57.0	—
2	8.0	5.9	I	5.6	7.4
3	11.9	3.5	II	14.8	4.0
4	22.0	1.9	III	31.8	1.5
5	30.1	1.0	IV	61.8	(0.5)

At degrees of hydrolysis > 30 %, the action of this amylase is insignificant. As described in a previous paper [1], the average chain-length of the unfermentable part of the dextrins can be estimated. Thus the value of $\bar{n} = 6.1$ is obtained for solution no. III, and according to these experiments, shorter chains contain no rapidly broken linkages. (Shorter chains can, of course, be ruptured during the slow, second phase, the saccharification). Myrbäck and Thorsell [10] found that the dextrins formed during the dextrination phase of *amylose* chiefly consisted of 4—7 units.

b) Fractionation of α-dextrins

In order to investigate the α-dextrins more closely, the reaction is interrupted when 10 % of the linkages are broken, and the dextrin mixture is fractionated with alcohol.

Method

2.81 g of glycogen was dissolved in 96 ml of water. 2 ml of the phosphate buffer (pH = 5.3) and 2 ml of the α-amylase solution were added. When the degree of hydrolysis

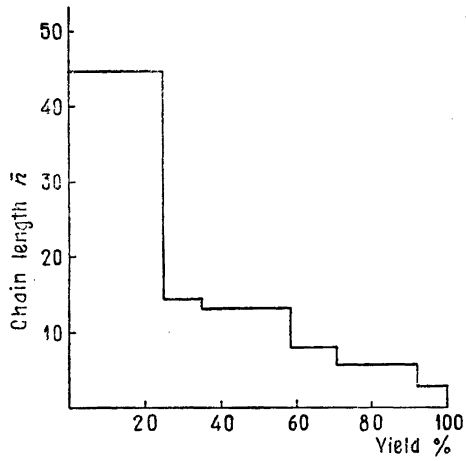

Fig. 2. Fractionation of α-dextrins. The corrected yield and the average chain-length of the various fractions.

was 10.6 % the solution was boiled for a few seconds. The dextrins were fractionated with alcohol of concentrations between 40 and 90 % by volume. The precipitates were centrifuged off, dried *in vacuo* and then dissolved in water. The concentrations of these solutions were determined in three ways: by means of total hydrolysis, by weighing of the dried substance and by the optical rotation. The reducing power was also determined by means of an iodometric method (see the previous paper[1]), and the average degree of polymerization, $\bar{n}$, could then be calculated. The loss of substance was 7 %, and the yields of the fractions were corrected for this.

Most of the dextrins have chain-lengths between 3 and 15 units, but there is also a fraction consisting of rather high-molecular dextrins, about $\bar{n} = 45$ (see Fig. 2). In the corresponding experiments with starch [9], no higher dextrins than $\bar{n} = 14$—19 occur. From this fact, it must be concluded that many interior chains in glycogen are so short as to remain intact; this is also proved later on in this paper. (On the other hand, Myrbäck and Sillén [3] have shown that the interior chains in starch [amylopectin] are ruptured about once.)

Part of the fraction $\bar{n} = 2.7$ was fermented, and the amount of glucose and of maltose + maltotriose was 1 % and 4 % respectively of the total amount of glycogen used. The formation of fermentable sugar during the course of dextrination is thus little or none as these saccharides can also be formed from the primary dextrins.

Part of the fraction $\bar{n} = 13.3$ was treated with β-amylase for 5 days. The reducing power remained constant after a breakdown of 39.4 %, calculated as maltose. The corresponding experiment on the fraction $\bar{n} = 5.8$ gave the value 89 % (as maltose). Obviously the lower dextrins consist to a large extent of unbranched chains, whereas the higher dextrins are more ramified since these are saccharified only partly. Myrbäck [11] has shown that most of the

α-dextrins from starch with chain-lengths of 6—7 units are normally built, while the larger dextrin molecules (about $\bar{n} = 12$—18) probably contain one branching point. The normal α-dextrins from both glycogen and starch are evidently very similar.

c) β-Amylase

Experiments with β-amylase from barley show that the enzyme splits off maltose from the non-reducing end of a chain with 1,4 - α-glucosidic bonds (summary by Myrbäck [8]). Thus only the end-chains of ramified molecules can be attacked, leaving behind a high-molecular residue, the β-dextrin, which contains the interior and fractions of the end-chains and the branching units.

Preparation of β-amylase from ungerminated barley

200 g of ground barley was extracted for 45 minutes with 1 l of cold water. After filtration, alcohol was added until the concentration became 75 % by volume, and the mixture was kept cold for one hour. The precipitate was then centrifuged off, washed with 95 % alcohol, dried *in vacuo* and finally dissolved in water. According to Blom [13], the traces of α-amylase which are always present were inactivated by adding acetic acid until pH = 3.6. The solution was kept at this pH and at 0° for two weeks. It was then decanted and sodium hydroxide was added to pH = 5.3.

These experiments were carried out as described above for α-amylase. The course of hydrolysis of glycogen and starch is shown in Table 2.

Table 2. Enzymatic breakdown of glycogen and potato starch with β-amylase. (Calculated as percentage of loosened linkages.)

Time (hours)	0.25	0.5	2	5	24	72	96	192
Glycogen	10.2	11.5	14.4	15.9	17.5	19.1	20.2	21.2
Starch	10.3	18.5	24.0	28.5	30.2	30.9	30.6	31.4

A number of glycogen experiments proceeding for 8—15 days gave values between 19 and 23 %. At first the rate of hydrolysis of glycogen is as high as that of starch, but when about 10 % of the linkages are broken it is much lower. As there are about 9 end-chains per 100 units, this fact means that one maltose group is rapidly split off from each branch, while the second or third one is loosened rather slowly.

The chains must be packed more closely in glycogen than in amylopectin (starch), and Meyer and Jeanloz [4] assume that the voluminous enzyme molecule cannot easily reach the interior parts of the glycogen molecule so

that certain end-chains remain unaffected. Measurements of osmotic pressure and of viscosity show that the glycogen molecule has a more or less spherical form, while the amylopectin molecule is more elongated. It is possible that this difference may also contribute to the low final velocity of hydrolysis in the case of glycogen.

The various hydrolyzates were treated with β-amylase for 5 days. The reducing power and the amounts of fermentable sugar were determined as previously described [1], and the values before treatment with the enzyme were obtained from that paper. In Table 3 it is shown that the glucose values

Table 3. Action of β-amylase on the hydrolyzates. Comparison between the increase in the reducing power and that of the maltose fraction.

No.	Before treatment with the enzyme	After treatment with the enzyme		
	Glucose %	Glucose %	Increase in the degree of hydrolysis, %	Increase in the maltose fraction, %
1	1.0	1.1	19.8	39.2
2	2.0	1.6	15.5	32.9
3	1.9	2.3	13.9	30.1
4	11.1	11.4	7.5	16.0
5	19.0	20.5	5.9	13.1
6	43.1	45.7	2.8	6.3

remain constant, while the increase in the maltose fraction is about double the increase in the reducing power. Thus only maltose can have been formed on action of β-amylase. This result is in conformity with what is known from starch experiments [7].

d) β-Dextrins

These dextrins were prepared by treatment with β-amylase for 5 days, after which the enzyme was inactivated by boiling for a few seconds. The dextrins were precipitated from a 90 % alcoholic solution, centrifuged off, dried *in vacuo* and dissolved in water. The reducing power was determined by iodometric titration and the concentrations of the solutions were measured in three ways as described in a previous paper [1], where the relation between the specific rotation, $[\alpha]_D$, and the degree of hydrolysis was also studied. The average degree of polymerization, $\bar{n}$, could then be calculated (Table 4).

Table 4. Comparison of the results from different methods for determining the concentration of β-dextrin solutions. The calculated values of $[\alpha]_D$ *and of* $\bar{n}$.

Method of determination	β-Dextrin from				
	glycogen	I	II	III	IV
	Concentrations, %				
Titration after total hydrolysis	1.60	0.71	0.98	1.25	0.73
Calculation from the dry weight	1.58	0.71	1.01	1.24	0.83
Calculation from the optical rotation	1.61	0.71	0.97	1.21	—
$[\alpha]_D$ (hydrates, x · $C_6H_{12}O_6$)	177°	175°	172°	158°	
$\bar{n}$	77	17.5	11.6	5.0	3.0

The results from the various methods do not differ more than ± 2 % (apart from no. IV). The values of $\bar{n}$ are surely too low, since all the traces of alcohol and other reducing substances cannot be removed, but the errors are considerable only at fairly low degrees of hydrolysis.

These β dextrins were then treated with α-amylase, and the percentage of easily hydrolyzable linkages was determined in the usual way (Table 5). On comparison with the values in Table 1, it is evident that α amylase has a lower affinity to the β-dextrins, which is quite natural as all the end-chains are lacking. As about 42 % of maltose is formed from glycogen, the yield of β-dextrin is 58 %, and the corresponding values for the dextrins from the hydrolyzates I, II and III are 55, 52 and 35 % respectively. By means of these figures the values in the last line of Table 5 are calculated. Assuming that

Table 5. Calculation of the number of rapidly broken linkages in the β-dextrins when treated with α-amylase.

Dextrin	Glycogen		I	II	III
Original degree of hydrolysis	1.3	1.0	5.7	8.6	19.8
The number of rapidly broken linkages in the β-dextrins, %	3.1	3.7	2.7	1.9	0.7
The number of rapidly broken linkages, calculated for the amount of glycogen started with, %	2.0		1.5	1.0	0.2

there are 9 interior chains per 100 units in glycogen, only about one fifth of these chains is thus rapidly broken with α-amylase. The β-dextrin III, the average chain-length of which is 5 (according to Table 4) contains only few rapidly broken linkages.

e) Calculations of the mechanism of α-amylase degradation

As mentioned before, Sillén and Myrbäck [2] have attempted to calculate the statistical distribution of different chain-lengths, and the following figures are extracted from that paper. Only case A is considered, *i. e.* the ramification can occur at every glucose unit.

Table 6. The number of interior and end-chains of a certain, minimum length, n, calculated for 100 units in glycogen. (According to Sillén and Myrbäck [2].)

$n \geqq$	2	3	4	5	6
Interior chains	4.71	3.57	2.72	2.08	1.57
End-chains	7.31	6.47	5.68	4.94	4.33
The sum of all chains	12.02	10.04	8.40	7.01	5.90

At first we can assume that every chain is ruptured once by α-amylase. The β-dextrin from glycogen contains 2 % of rapidly broken linkages (Table 5), and thus the best agreement is obtained if interior chains shorter than 5 units are assumed to be intact. When the sum of all chains is considered the best value of n is 3, as 10.1 % of the glycogen linkages can be split off rapidly.

Surely longer chains can be ruptured many times. As the normal, unbranched α-dextrins consist of 5—7 units, it may be assumed that chains containing 13—18 units are split twice and the longer ones three times. The values calculated in this way are given in Table 7.

Table 7. Calculation of the number of linkages which can be rapidly broken by α-amylase, assuming the longer chains to be broken many times.

Minimum chain-length, $n \geqq$	3	4	5	6
The number of broken linkages in the interior chains, %	3.81	2.96	2.32	1.83
The sum of all broken linkages, %	11.76	10.12	8.74	7.57

According to these calculations, α-amylase cannot rapidly split interior chains shorter than 5—6 units and end-chains shorter than 3—4 units.

The results mentioned above show that in dextrins with 5 or 6 units there are only a few rapidly broken linkages. Although *a*-amylase cannot rapidly split tri and tetrasaccharides, it could be assumed that the end-chains with 3 or 4 units in large molecules are broken by the enzyme. Myrbäck and Sillén's theories of branched molecules seem therefore to be consistent with the known facts concerning the enzymatic degradation of glycogen.

PERIODATE OXIDATIONS

Periodate or periodic acid is able to rupture a carbon chains between two adjecent hydroxyl groups, and two aldehyde groups are formed. If three adjecent cabon atoms have unblocked hydroxyl groups, the middle one is oxidized to formic acid. Accordingly, all the C—C linkages of glucose are broken, and 1 mole of formaldehyde and 5 moles of formic acid are formed, while 5 atoms of oxygen are consumed (*cf.* Fig. 3a). The oxidation of maltose proceeds as in the case of glucose. Ahlborg [13] found, however, that 6-*a*-glucosido-glucose only consumes 6 atoms of oxygen per mole (*cf.* Fig. 3b). According to Jackson and Hudson [14], starch and cellulose consumed slightly more than one atom of oxygen per glucose unit (abbreviated to O/glucose). Hence the pyranose ring is split between the carbon atoms 2 and 3. The non-reducing end-group of a glucose chain contains free hydroxyl groups in the positions 2, 3 and 4, and it gives thus rise to one mole of formic acid. A source of error in all periodate experiments is due to the reducing chain. Little is known of the properties of this chain.

a) Determination of the consumption of oxygen

The oxidations are carried out in more or less acid solution. Periodate is reduced to iodate, and the most simple method for determining the excess of periodate has been devised by Rappaport *et al.* [15], consisting of iodometric titration at pH = 7 in a phosphate buffer. In acid solution iodate also reacts, which makes the volume of thiosulfate unnecessarily large.

Experimental

The experiments were carried out at 30°, for the reaction did not come to a standstill at higher temperatures. 0.1 *M* periodate solution was prepared from 21.4 g of $NaIO_4$, 25 ml of 4 *N* H_2SO_4 and water made up to 1 l. A certain volume of 2 % carbohydrate solutions and 10 ml of the periodate solution were mixed with water in a glass-stoppered flask so that the volume became 50 ml. The excess of periodate must be at least 25 %. pH ranged from 1.8 to 2.4. At suitable intervals, 5 ml was transferred to a flask contain-

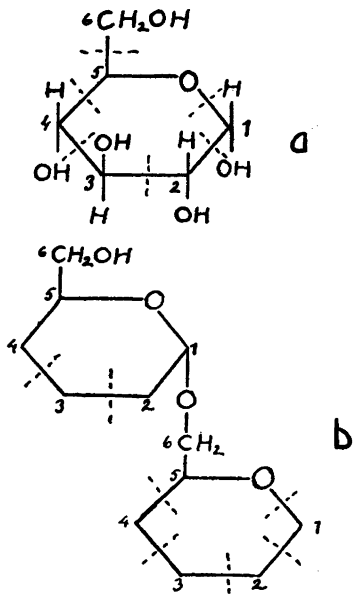

Fig. 3. The action of periodate on glucose (a) and on 6-glucosido-glucose (b).
(a) All C—C linkages in glucose are split by oxydation and the oxygen bridge is hydrolyzed.
(b) 6 C—C linkages in 6-glucosidoglucose are split. The oxygen bridge of the reducing glucose unit is hydrolyzed, whereas the two remaining ones are intact.

ing 20 ml of 1 % phosphate buffer (pH = 7.0). About 0.1 g of KI was added, and the iodine liberated was titrated with thiosulfate. A blank without carbohydrate was treated in the same way.

The results from oxidation of glycogen, starch and dextran are shown in Table 8. Further experiments on glycogen gave final values between 1.10 and 1.15 O/glucose. Apart from the reducing chain, the value would be 1.09

Table 8. Periodate oxidation of polysaccharides at pH about 2 and at 30°. The consumption of oxygen is indicated as O/glucose.

Time (hours)	0.5	1.0	3.0	5.0	10.0	24.0	48	96
Glycogen	0.53	0.66	0.82	0.87	0.92	1.05	1.09	1.12
Starch	0.60	0.75	0.89	0.94	0.96	1.00	1.08	1.07
Dextran	0.83	1.15	1.56	1.68	1.78	1.85	1.91	1.90

O/glucose for glycogen and 1.045 for starch if the interchain linkages are of the 1,6-type. Otherwise the calculated value would be 1.00 for both polysaccharides. The observed values seem therefore to indicate the presence of 1,6-linkages.

On complete oxidation of the amylose (about 20 % of starch) the calculated value is about $0.8 + 0.2 \cdot 5 = 1.8$ for starch.

It is evident that amylose is oxidized in almost the same way as amylopectin under these conditions. One would expect a 1,6-polysaccharide to consume about 2 O/glucose, and the observed value of 1.9 for dextran, which contains mainly such linkages, is therefore quite satisfactory.

b) Periodate oxidation of β-dextrins

Some β-dextrins were treated with periodate in the same way for 3 days. The end-gruop content of the dextrin from glycogen is about 15 %. The greater

Table 9. Periodate oxidation of β-dextrins for 3 days at 30°.

Dextrin from	Glycogen		I	II	III	3	4	7
O/glucose	1.25	1.17	1.72	1.86	3.08	1.60	1.80	3.60
Average chain-length			17.5	11.6	5.1			2.7

consumption of oxygen of this dextrin (Table 9) as compared with that of glycogen may be due to the higher end-group content in the case of branching at position 6. Otherwise the calculated value for the β-dextrins would also be near 1. The dextrins from the solutions no. 3, 4 and 7 have been prepared by removal of the fermentable sugar, and, hence, they can contain low-molecular substances as *iso*maltose and branched trisaccharides. The theoretical consumption of oxygen has been calculated for these compounds (Table 10).

Table 10. Theoretical consumption of oxygen of different di- and trisaccharides.

Type of linkage *Iso*maltoses	O/glucose	Types of linkage Trisaccharides	O/glucose
1,6	3.0	1,4 + 1,6	3.7 2.3 2.0
1,3	5.0	1,4 + 1,3	5.0 5.0 2.0
1,2	1.5	1,4 + 1,2	2.7 1.3 1.3

The β-dextrin preparation no. 7 is evidently a mixture of unfermentable di- and trisaccharides (and probably small amounts of higher dextrins). The observed value of 3.6 O/glucose seems to exclude the presence of 1,2-linkages, but is consistent with both the 1,3 and 1,6-structures.

c) Determination of the end-group content

Hirst and co-workers [16, 17] have developed a method based upon the fact that each end-group gives rise to one mole of formic acid. They showed that

the reducing chain was normally oxidized at low temperatures (15°) and when the periodate concentration was decreased by precipitating the bulk of $NaIO_4$ with potassium chloride. A number of glycogen samples from different animals were examined, and, in most cases, they found, the end-group content to be about 9 %, but in certain samples from rabbit liver the value was only 6 %.

Experimental

The experiments were carried out at 15° or at room temperature (20—22°). In this case periodate solution alone was used so that the pH was higher (about 4) than in the previous experiments. 10 ml of 0.1 *M* $NaIO_4$ was mixed with 10 ml of 1 % solutions of glycogen and starch in a stoppered flask, and in one series potassium chloride wes added. When the reaction was to be broken, 1 ml of ethylene glycol was added to destroy the excess of periodate. The precipitate (potassium periodate) was dissolved after a few minutes and then destroyed. The formic acid was titrated with 0.01 *N* NaOH and phenolphthalein as indicator. Blanks, containing the same amounts of the solutions, were also treated, but in this case the periodate was first detroyed by glycol.

As may be seen from Table 11, the formation of formic acid is fairly slow at 15°, and the velocity is further decreased when KCl is added. Experiments on starch gave values between 5.1 and 5.5 (3 days, room temperature). The

Table 11. Determination of formic acid formed by oxidation of glycogen. Calculated as moles of formic acid per 100 glucose units.

Time (days)	1	2	4	6	8
Without KCl (room temp.)	9.8	10.9	11.9		
» » (15°)	7.5	8.2	9.2	9.6	
KCl added (room temp.)	7.8	8.0	8.8	9.5	
» » (15°)	4.0	5.2	6.7	8.4	9.2

preparation of glycogen used in these investigations seems to have an end-group content which corresponds to the higher value of 9 %.

SUMMARY

Enzymatic breakdown of glycogen and of partly acid hydrolyzed glycogen with α- and β-amylase has been carried out. Various dextrin fractions have been prepared and the average chain-length calculated. When treating glycogen with β-amylase, the terminal maltose group of every branch is split off more rapidly than the second or third one. As in the case of starch, lower dextrins consisting of less than 5—6 units cannot be rapidly split by α-amylase. Only about one fifth of the interior chains in glycogen is rapidly broken, thus

indicating that such chains must be rather short as compared with amylopectin. The results have been compared with Myrbäck and Sillén's theories of enzymatic synthesis of ramified molecules, and according to these calculations, α-amylase cannot rapidly attack end-chains shorter than 3—4 units and interior chains less than 5—6 units.

Periodate oxidation of glycogen and of its dextrins has been carried out. The results seem to exclude the presence of 1,2-linkages, but it is scarcely possible to decide whether the linkages are of the 1,6 or 1,3-type or a mixture of both. The formic acid liberated corresponds to about 9 % of end-groups.

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