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PROPERTIES OF SYNTHETIC AND NATIVE LIVER GLYCOGEN [1]

A. J. PARODI [2], C. R. KRISMAN, L. F. LELOIR AND J. MORDOH [3]

Instituto de Investigaciones Bioquímicas, Fundación Campomar and Facultad de Ciencias Exactas y Naturales, Obligado 2490, Buenos Aires, Argentina

The properties of high molecular weight glycogen extracted from rat liver and of that prepared *in vitro* with muscle phosphorylase and liver branching enzyme have been compared. The stability at different pH values was measured spectrophotometrically for liver, corn, and synthetic glycogen. The former is more labile, but the shape of the pH-stability curve is very similar for all of them. Borate, copper, and iron accelerate the decomposition of the three types of glycogen. Sonication produces breakdown but affects in the same way synthetic and liver glycogen. After shortening the outer chains with β-amylase, native liver glycogen becomes slightly more stable to acid treatment and decomposes giving smaller molecules than the untreated glycogen. Glycogen extracted from livers of toad and pigeon was similar in molecular weight distribution and acid lability to that of rat liver. Rat muscle glycogen had a molecular weight of about 8 million.

High molecular weight liver glycogen (particulate glycogen) has been studied by several methods. Estimates of the sedimentation coefficients give values which range from less than 100 up to 10,000S (1, 2). These values would correspond approximately to molecular weights of less than 10 to more than 3000 million. Observations with the electron microscope show large particles of 60-200 mμ diameter composed of 20-40 mμ subunits. These were referred to as α-and β-particles, respectively, by Drochmans (3). On the basis of the action of detergents, urea, and enzymes, Orrell *et al.* (4) reached the conclusion that the subunits are not held together by protein, hydrogen bonds, or nucleic acids.

Mordoh *et al.* (5) obtained preparations of high molecular weight from glucose 1-phosphate with purified phosphorylase and branching enzyme. Observations with the electron microscope showed that these synthetic preparations were similar to those of native liver glycogen. However, further work (1) showed that the latter is more labile to heat, alkali, and acid. The type of breakdown is also different because the molecular weight of synthetic samples decreases progressively while native liver glycogen gives preferentially molecules of 8 million daltons. Mordoh *et al.* (1) suggested that native liver glycogen might have one "labile" bond for every 50,000 glucose residues. This work has been continued, and a more detailed analysis of the breakdown of both glycogens has been carried out.

EXPERIMENTAL PROCEDURE

Synthetic glycogen was prepared as described previously (5). Native liver glycogen was obtained by the $HgCl_2$ method (1) followed by dialysis. Rat muscle glycogen was extracted by homogenizing in 8 volumes of neutralized 3 % $HgCl_2$ for 2 minutes in a blender. After two extractions the yield was about 70 % of that obtained with the usual KOH method (6). It was verified that with this treatment the molecular weight of native liver glycogen did not change. Corn glycogen was obtained as previously described (1).

The sedimentation rate of glycogen in sucrose gradients was measured according to Mordoh *et al.* (1), In some cases larger scale separations were performed in order to obtain glycogen of more homogenous molecular weight.

Sonication was carried out with a Raytheon sonicator at 10 kilocycles per second.

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2 Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas (R. Argentina).

3 Career investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (R. Argentina).

The analytical methods used were as follows: glycogen according to the iodine method of Krisman (7), and nonreducing end groups with periodate as described by Fales (8). The degree of β-amylolysis was determined by incubating glycogen and a commercial preparation of β-amylase in 0.1 M citrate buffer of pH 6.4. Aliquots were taken at several intervals until the reducing power reached a plateau. The product was characterized as maltose by paper chromatography in butanol-pyridine-water (6:4:3) (9). The initial amount of glycogen was measured by the phenol-sulfuric acid method (10), and the reducing power by the Somogyi (11)-Nelson (12) method. The absorbance of glycogen solutions was measured at 500 mμ.

RESULTS

Breakdown of glycogen measured by changes in absorbance. The degradation of glycogen can be easily followed with a spectrophotometer. The absorbance per unit weight is theoretically proportional to the molecular weight, and for polydisperse materials it is proportional to the weight average molecular weight (13). Therefore for the same number of bonds hydrolyzed, the percentage decrease in absorbance should be larger for heavier fractions than for lighter ones.

When native liver glycogen is heated to 100° the absorbance decreases progressively as hown in Fig. 1. The rate of change depends on the pH and on the nature of the ions. Several of the usual analytical and graphical procedures were tried in order to express the rate of the reaction, but none of them was satisfactory.

In order to find out if the course of the reaction was similar under different conditions, the curves of decrease in absorbance were plotted against time. By changing the time scale all the points could be made to fall on the same curve. The results are shown in Fig. 1. The breakdown curve with acetate of pH 5 at 100° was plotted with the abcissa

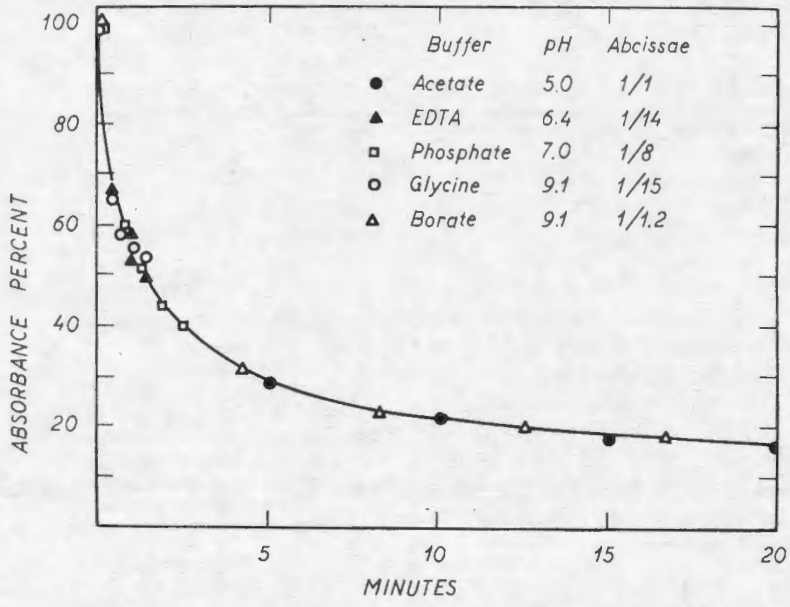

FIG. 1. — The decrease in absorbance of native liver glycogen heated to 100° in different buffers. The absorbance was measured at 500 mμ. The abcissa scale was reduced by the indicated factor. For instance the real scale for glycine would be 15 times larger The molarity of the buffers was 0.1 M.

TABLE I

Effect of Heating Glycogens under Different Conditions [a]

Addition	pH	t_{50} native	t_{83} synthetic	t_{70} corn
Sodium acetate	5	2	5.5	3
Sodium citrate	6.4	10.5	20	12
Citrate + 10^{-3} M EDTA	6.4	30	—	—
Potassium phosphate	7	11	8	8
EDTA	7	20	7.5	10
Tris-HCl	7[b]	2	—	—
Glycine-NaOH	9.1	25	30	7.5
EDTA	9.1	30	13.5	15
Diethyl barbiturate	9.1	11	11	5
2-Amino, 2-ethyl 1,3-propanediol	9.1	21	13.5	6
Sodium tetraborate	9.1	2	3.5	2
Sodium tetraborate + EDTA	9.1	2.5	3.5	—
Sodium carbonate	10	12	3 5	4
Sodium hydroxide (0.1 M)	13	4	3	2.5
Sodium hydroxide (0.05 M)	12.7	8	—	—

[a] The absorbance at 500 mμ was measured before and after heating to 100° for 5, 10, 15, and 20 minutes. The values of t_{50}, t_{83}, and t_{70} were calculated as described in text. Concentration was 0.1 M, unless otherwise indicated. The pH values are those of the solutions at 25°.

[b] pH decreases to about 5 on heating to 100°.

scale in minutes. The results obtained with other buffers are plotted with contracted abcissa scales. The results can be expressed either in t_{50}, i.e., the time at which absorbance becomes half, or as relative stability factors, i.e., the number by which the time scale has to be contracted in order to obtain superposition of the curves. For instance, in Fig. 1, if the results with acetate of pH 5 are taken as equal to one, the relative stability for glycine buffer is 15. Superposition of the curves was also obtained with synthetic and corn glycogen.

The results of heating native liver, corn, and synthetic glycogen to 100° in different buffers are shown in Table I. The decreases in absorbance were much smaller for corn and synthetic glycogen, so that results are given in t_{70} and t_{83}, respectively, instead of t_{50} used for liver glycogen. This procedure allowed comparison of the breakdown of each glycogen under different conditions but not of one type of glycogen with the others. Native liver glycogen is most stable between pH 7 and 11 and decomposes rapidly at pH 5 and 13. Sodium tetraborate accelerates the decomposition as compared with other buffers of the same pH. A rapid decomposition was also observed with Tris-HCl of pH 7; however, it was found that on heating to 100° the pH as measured with indicators decreases to about 5. The same acidification on heating occurs with buffers of other organic bases such as ethanolamine and triethylamine, but not with the other buffers. In the previous paper (1, Fig. 8) a curve is shown in which the decrease in absorbance at pH 7 is represented. At the time, it had not been noticed that such large changes in pH occurred on heating Tris buffer. The actual pH must have been near 5.

It was found that complexing agents such as glycine, EDTA, and citrate decreased the rate of decomposition, and therefore the action of some metals was tested. As shown in Table II, Cu^{++} and Fe^{3+} increased the rate of decomposition of both native liver and synthetic glycogen. Hg^{++} had the opposite effect. Citrate was used as buffer in these experiments, and the amount of free Cu^{++} can be calculated to be of the order of 10^{-3} M.

Effect of the pH on the breakdown of different glycogens. It was expected that information on the nature of the "labile" bonds of native glycogen might be obtained from the effect of pH on the breakdown. For this

TABLE II

Effect of Metals [a] *on Glycogen Stability*

Metal	t_{50}		t_{90}	
	Native	Change %	Synthetic	Change %
None	10.8	—	6.7	—
Fe^{3+}	4.0	−63	2.5	−63
Cu^{++}	5.5	−49	1.5	−78
Pb^{++}	7.5	−31	3.0	−55
Zn^{++}	8.6	−20	5.0	−25
Co^{++}	11.3	+5	5.3	−21
Hg^{++}	15.0	+39	13.3	+100

[a] The samples were heated to 100° in 0.1 M citrate plus 0.01 M of metal.

purpose samples of glycogen were heated to 100° for 5, 10, 15, and 20 minutes at different pH values. An EDTA buffer was used throughout in order to minimize the effect of metals. The relative stability was calculated as described for Fig. 1. The rates of change in absorbance for the different glycogens could not be compared directly, because in most cases the initial molecular weight was not the same and the breakdown products were different. However, the results can be expressed relative to changes at a certain pH. In this case the stability factor of each glycogen at pH 7 was taken as 100, and the respective values at different pH values were proportionally modified. The results are shown in Fig. 2. It may be observed that the curves for native liver, synthetic, and corn glycogen are similar although native liver glycogen degrades faster.

Effect of temperature on acid breakdown. The action of acid on native liver and synthetic glycogens at different temperatures was studied. When the decrease in absorbance was plotted against time it seemed that at 0° the curve became horizontal at a higher level than at 28°. But if the time scale for the 0° series is contracted, all the points fall on the same curve (Fig. 3). In order to obtain superposition of the points it was necessary to contract the 0° abcissae scale 180 and 75 times for native liver and synthetic glycogen,

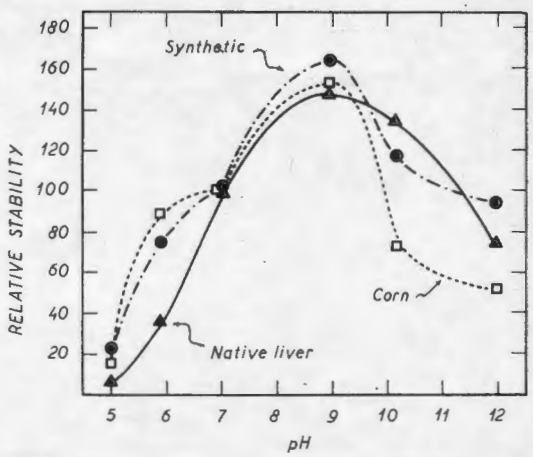

FIG. 2. — Effect of pH on the stability of synthetic, corn, and native liver glycogen. The pH values indicated correspond to the solution at 25°. They did not change more than 0.2 pH unit on heating to 100° as judged by the color of indicators. The relative stability was calculated as described in text.

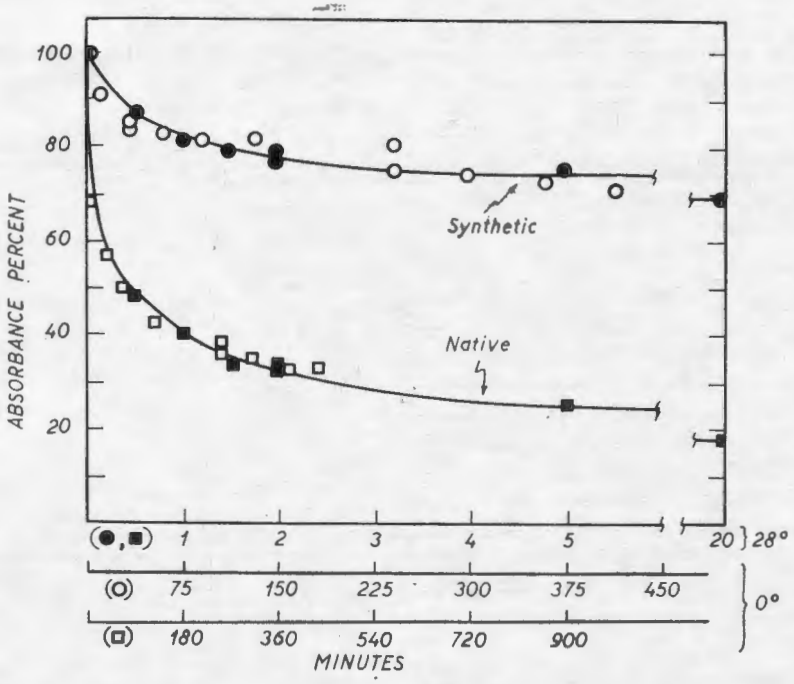

FIG. 3. — Effect of temperature on the breakdown of native liver and synthetic glycogen. The samples were maintained at the indicated temperatures in 0.1 N HCl and neutralized with a slight excess of dipotassium phosphate at different times. Three abcissa scales are drawn: one for the results at 28° and two for the values obtained at 0°.

respectively. It may be concluded that the reaction follows a similar course at different temperatures and that only the rate is changed. It was checked by gradient centrifugation that the type of breakdown of native liver glycogen at 0° in 0.1 N HCl is the same as at room temperature.

Breakdown products. It was observed previously (1) that 0.1 N HCl at 37° degrades native liver glycogen with preferential formation of molecules of 100 S which correspond to a molecular weight of about 8 million. The same type of experiments has now been carried out under other conditions. The changes in molecular weight were followed by gradient centrifugation after heating at 100° in 0.1 N NaOH, 01 M sodium tetraborate, water, or 0.1 N NaOH-8 M urea. In every case the first change was the appearance of 100S peak at the expense of the heavier fractions so that a two-peak curve was obtained. On a prolonged treatment, the heavy peak disappeared and the 100S peak was more slowly displaced to lower values.

Synthetic samples treated with 0.1 N HCl (37°), 0.1 M sodium tetraborate (100°). 0.1 N NaOH-8 M urea (100°) behaved in a different manner from native glycogen. The heavy peak was progressively displaced but there was no formation of 100S molecules.

Sonication. In the previous paper (1) it was reported that sonication affected native liver and synthetic glycogen in a different way, but the two samples used were of very different molecular weight distribution. Therefore the effect of sonication has been tested again with samples of the same molecular weight, and as shown in Fig. 4 it was found that the type of breakdown is very similar for both glycogens. The heavy fractions give rise to lighter molecules and a two-peak curve is formed. The sedimentation coefficient of the light fraction ranges between 100 and 1000S. On a prolonged treatment the heavy peak disappears and the light one becomes lighter and more homogeneous. Sonication does not lead to the preferential formation of the 100S-peak as occurs with acid or heat treatment on native liver glycogen.

Another experiment which showed that sonication breaks down native liver glycogen in a different manner than acid was carried out as follows. Two samples of native liver glycogen were degraded, one by sonication

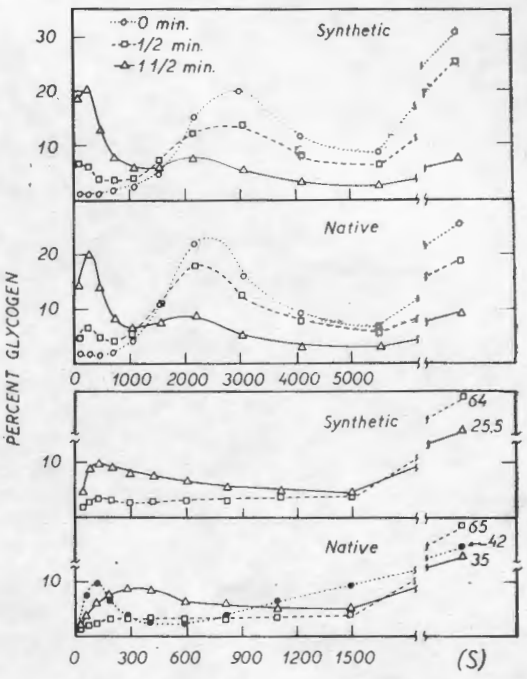

FIG. 4. — The effect of sonication Sonication was carried out at 25° in water for the times indicated in the figure. The two upper diagrams correspond to analytical runs of 30 minutes. The time for the two lower ones was 2 hours. In the latter, undegraded natives and synthetic glycogens were omitted because nearly all of the material appeared in the pellet. In the lower diagram, the full circle (●) curve corresponds to a 100 S preparation obtained by acid treatment.

and another by acid, so as to produce samples of approximately the same molecular weight. As shown in Fig. 5, although the range of sedimentation coefficients was similar for both preparations, the acid one is less polydisperse. Both samples were then treated by 0.1 N HCl (5 minutes, 37°). Figure 5 also shows that the glycogen pretreated with acid was hardly affected, whereas that obtained by sonication gave rise to the formation of a considerable amount of a lighter component of 75S.

It therefore seems that the bonds broken down by acid are different from those affected by sonication.

End group measurement and β-amylolysis. Samples of synthetic and native liver glycogen of approximately the same molecular weight were used for measurements of the number of end groups with periodate and of the degree of β-amylolysis. As shown in Table III, the two samples did not differ signi-

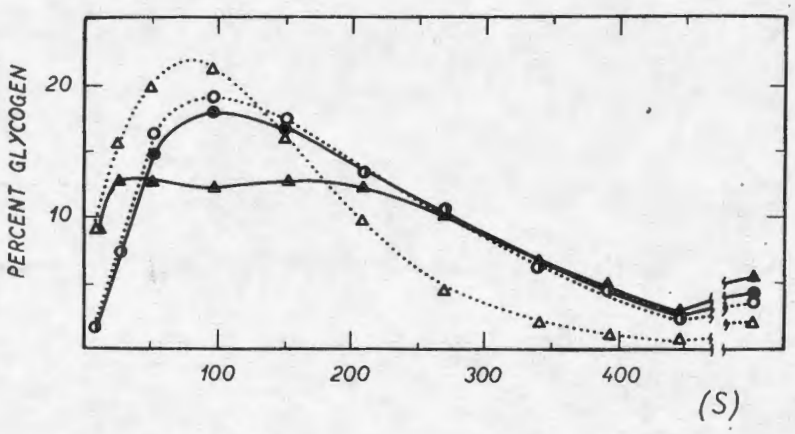

FIG. 5. — The effect of acid after sonication. Native liver glycogen was degraded with 0.1 N HCl at 37° for 10 minutes or by sonication for 12 minutes in 8 M urea at room temperature. so as to obtain a population of about 100S. Both samples were then treated with 0.1 N HCl at 37° for 5 minutes, and the rate of sedimentation in sucrose gradients was measured (15,000 rpm for 300 minutes). (▲) 100S obtained by sonic treatment ("100S sonic"); (Δ) "100S sonic" after acid treatment; (●) 100S obtained by acid treatment ("100S acid"); (o) "100S acid" after acid treatment.

ficantly. After an acid treatment which lowered the molecular weight to about the same extent, the degree of β-amylolysis increased equally in both samples. Presumably this increase was due to the fact that after acid treatment some long internal or "buried" external branches became available to enzyme action.

Other samples of synthetic glycogen had longer outer branches and some of them gave β-amylolysis values of 50-55 % before the acid treatment.

Effect of acid on β-limit dextrins. Treatment of native liver glycogen with β-amylase led to the removal of about 40 % of the glycogen residues from the exterior branches. The β-limit dextrin obtained was found to be degraded more slowly by 0.1 N acid than native samples. Figure 6 shows the course of the breakdown of the β-limit dextrin compared with a sample of native liver and synthetic glycogen of the same molecular weight. The initial distribution of molecular weights appears in Fig. 6A. After treatment with 0.1 N HCl for 1 1/2 minute, the native sample gave rise to the formation of much more of the lighter component than the β-dextrin (Fig. 6B). After 10 minutes (Fig. 6C) the native sample was all converted to a light fraction, but about 75 % of the β-dextrin remained as heavy component. The light component formed from the β-dextrin had a sedimentation coefficient of about 70, and that formed from native glycogen was, as usual, the 100S component (Fig. 6D).

TABLE III

Number of End Groups and Degree OF β-AMYLOLYSIS

Glycogen	End groupe [a] (%)	β-Amylolysis [a] (%)	β-Amylolysis after acid treatment [b] (%)
Native liver	6.90	40	53
Synthetic	6.75	42	57

a Measured as described under *Experimental Procedure.*

b The acid treatment of native and synthetic glycogen was carried out in 0.1 N HCl at 37° and 100°, respectively. The time of treatment was sufficient to decrease the initial molecular weight of about 250 million to about 8 million.

Synthetic glycogen also decreased in molecular weight but did not give rise to a lighter component.

Glycogen from other sources. Glycogens from several other sources than rat liver were prepared by the $HgCl_2$ method. Toad and pigeon liver glycogens were observed to be very polydisperse and to give sedimentation curves similar to those from rat liver. On treatment with 0.1 N HCl at room temperature they were found to be degraded rapidly to 8 million dalton molecules.

The glycogen from rat muscle was quite different since it was nearly monodisperse and had sedimentation coefficients of 90, 100, and 110S in different samples. The sedimentation pattern of one of them is shown in Fig. 7. A preparation of human muscle glycogen

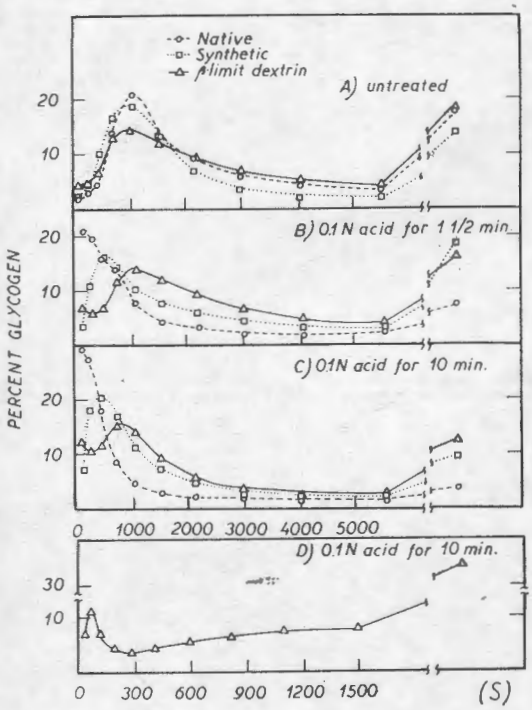

FIG. 6. — The breakdown of β-amylase limit dextrin of native liver glycogen. The three samples shown in *A* had been prefractionated so as to obtain similar molecular weigths. Acid treatment (0.1 N HCl) was carried out at 37° for the indicated times. The analytical gradients were run at 15,000 rpm for 30 minutes except for *D*, which was run 2 hours.

was examined by Bueding *et al.* (14). The sedimentation coefficient was about 130S, and observations with the electron microscope showed only small molecules.

Electron microscopy. It was previously reported that the aspect of native liver and synthetic glycogens was similar when they were stained with phosphotungstate and observed under the electron microscope (5). However, the pictures obtained by this method are not the same for different grids or for different zones of the same grid. Reissig and Bueding (personal communication) circumvented this difficulty by spraying the samples and observing them separately and in mixture. According to them the synthetic glycogen may offer very different aspects: some samples have no subunits at all, whereas others show subunits that are less defined than in native glycogen.

In order to recognize with certainty which were the molecules of synthetic glycogen and which were those of native liver glycogen, some experiments were carried out on mixtures of light native and heavy synthetic glycogen, on the inverse mixture, and on the separate components. The results are shown in Fig. 8. The preparation of synthetic glycogen appears to have subparticles, but they are less clearly defined than in native glycogen; and the molecules have a more compact aspect.

Another point which must be considered in these observations is that synthetic samples may be more or less ramified acording to the preparation. Less ramified samples, that is, those prepared with a relative deficiency in branching enzyme and which give more purple color with iodine, give a more fluffy appearance in the electron microscope (Drochmans, personal communication).

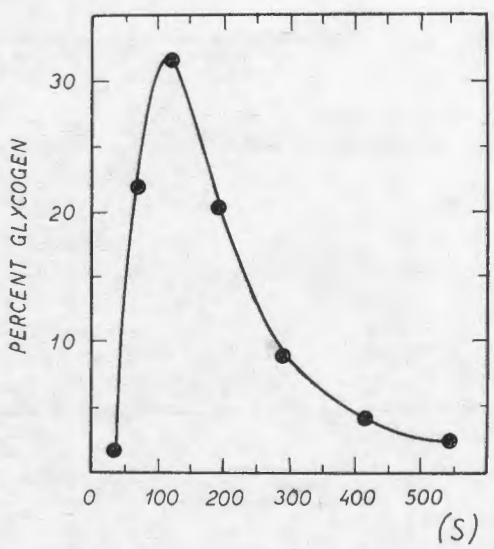

FIG. 7. — Sedimentation of rat muscle glycogen. Extraction was carried out as indicated in text, and gradient centrifugations at 15,000 rpm for 120 minutes.

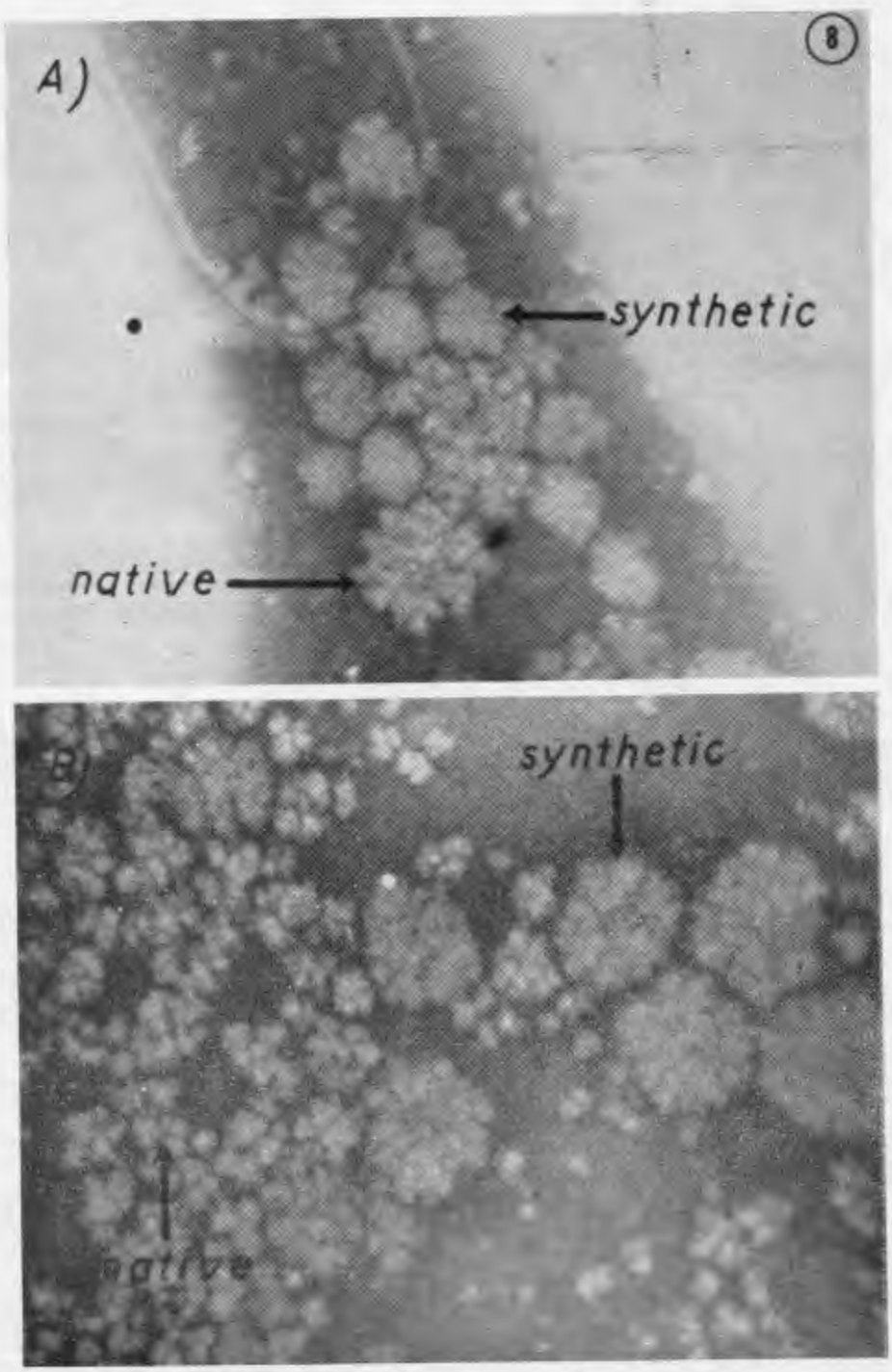

FIG. 8. — Electron microscope pictures of native liver and synthetic glycogen. Part A corresponds to a mixture of high molecular weight native glycogen and low molecular weight synthetic glycogen, and Part B to the inverse preparation. The observations were carried out as previously described (5). ×90,000.

DISCUSSION

A previous study (1) of the breakdown of native liver glycogen led to the conclusion that a small number of the bonds is more labile than the rest and than those of synthetic glycogen. The breakdown yields molecules of 8 million daltons so that the number of such bonds would be about one every 50,000 glucosidic residues. The lability of these linkages appears to be enhanced by a weight factor, that is, by the mechanical pull exerted on them. The fact that removal of the external branches by β-amylase reduces lability is in agreement with this idea. However, there must be some other cause of lability besides the weight factor. It seems that the β-limit dextrin has some bonds which, although they are not as labile as those of native glycogen, are cleaved preferentially and yield molecules of about 4.5 million daltons. Furthermore, synthetic glycogen can have molecular weights as high as the native, but is not as labile.

Several possibilities can be considered. One is that in native liver glycogen, the 8 million subparticles are joined by relatively long interconnecting chains and produce something similar to a bunch of grapes. The breakdown would then occur preferentially at the interconnecting chains. Observations with the electron microscope favor this possibility since native glycogen appears as a less compact structure with better defined subparticles. However, an observation difficult to reconcile with the grape structure is the effect of sonication. The latter would be expected to produce breakdown to the 8 million subparticles, but this is not the case. In fact the effect of sonication is nearly the same for native and synthetic glycogen.

Another explanation of the lability would be that some parts of the molecule contain residues different from α-1,4 − α-1,6 glucosyl It is known that under certain conditions, galactose (15) or glucosamine (16) residues can be introduced into the glycogen molecules. If residues such as fructofuranosyl were present they would confer acid lability to the glycogen molecule. Still another possibility is that some glucosyl residues are distributed in space so as to reduce stability by a different conformation or by decreased hydrogen bonding. The latter, however, do not seem to play an important role because the type of breakdown of native and synthetic glycogen under conditions in which no hydrogen bonds are present (100°, 8 M urea, pH 13) is respectively the same as at room temperature at pH 1, where this type of bonds should be present.

The fact that the pH-stability curve is so similar for native, synthetic, and corn glycogen indicates that a very similar type of bond is broken down in all of them. The same is true for the effect of tetraborate, copper, and iron, which accelerate decomposition of both native liver and synthetic glycogen.

If the labile bonds are glycosidic it should be possible to label the corresponding residues by reduction with tritium-labeled sodium borohydride. Many attempts carried out in this direction failed because a considerable amount of tritium became fixed, apparently in an unspecific manner. This difficulty has been found also by De Wulf and Hers (personal communication).

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