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Rat Liver Glycogen Structure as a Function of Glycogen Level

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The average chain length of glycogen and the incorporation rate of radioactive glucose into it were measured during its accumulation in rat liver. Increasing levels of glycogen in liver were produced by various treatments of rats. The measured parametres increase with the increase in the liver glycogen content until this reaches the concentration of approximately 2.5% and then remain more or less constant. At this stage of glycogen accumulation in rat liver an agglomeration of glycogen subparticles seems to take place. It is concluded that the rat liver glycogen structure is related only to glycogen concentration and is not influenced specifically by the agents which affect glycogen level.

INTRODUCTION

Along with many papers on changes in the liver glycogen concentration under various experimental conditions, several papers have described the molecular weight distribution pattern for polydisperse glycogen preparations, isolated by a mild procedure, for a given species, tissue and physiological state[1–3]. However, only few data have been available in the literature on the changes of the average chain length of glycogen particles under different experimental conditions[4–6]. These investigations indicated that the average chain length of glycogen particles increases with increased glycogen concentration.

We have reported[7] that the average chain length of the rat liver glycogen particle first increases sharply with increased glycogen concentration and then gradually decreases with further increase in glycogen concentration. The experiments presented in this paper were performed in order to analyse this finding in greater detail and to determine what changes in the average chain length of the rat liver glycogen accompany changes in the glycogen concentration under different metabolic conditions. A preliminary report of this work appeared[8].

EXPERIMENTAL

Wistar male rats bred at the Institute were used. They were 12—16 weeks of age and weighed 210—250 g. Six groups of rats were treated to obtain increasing hepatic glycogen concentration in the following ways: *Group 1:* Fasting for 14 hours; *Groups 2 and 3:* Administration of glucose (50 g./100 ml. aqueous solution) by a stomach tube to 14-hours fasted rats (1 g. glucose/100 g. body wt.). Animals were killed at 2 and 6 hours after gavage. Glycogen concentration was observed to increase rapidly up to 2 hours and to reach a peak after 6 hours[9]; *Groups 4 and 5:* Intramuscular injection of cortisol acetate (Prolek, Beograd) to 3-days fasting and to *ad lib.* fed rats (3 treatments per day for 3 days at a dose of 1 mg./100 g. body

wt.). During the treatment the body weight of the animals was reduced by 48 and 16 g., respectively; *Group 6:* Fasting for 72 hours followed by feeding *ad lib.* for 24 hours, at which time the rats were killed.

The rats of all groups had free access to drinking water. They were killed by stunning, then decapitated and exsanguinated. The liver was excised, frozen on dry ice (in a Petri dish; 35—50 sec. after stunning) and homogenized in 18 ml. ice-cooled 6% trichloroacetic acid[10] in either a Potter-Elvehjem homogenizer D (Kontes Glass Co., Vineland, N. J.) or in an Ultra-Turrax blender (Janke und Kunkel KG, Staufen i. Br.). Glycogen was isolated from the supernatant obtained after centrifugation of the homogenate for 30 min. at 4°, 2200×g (Janetzky refrigerated centrifuge), with added ethanol and some LiCl crystals. The glycogen was purified by repeated precipitation from water by ethanol, with the addition of LiCl, dried by ethanol and ether, and stored in a vacuum desiccator over silica at room temperature. A trichloroacetic acid isolation procedure was used because it was assumed[4,11,12] that decreasing the average size of the particles would not greatly alter structural pattern of the glycogen. One hour before killing all rats were given an intraperitoneal injection of approximately 10^6 dpm D-glucose-^{14}C(U) of high specific activity (Amersham). The rate of radioactive glucose incorporation was used as the indicator of activity of rat liver enzymes engaged in glycogen synthesis in given conditions.

The liver glycogen concentration was determined in a 100—200 mg. sample of the right lateral liver lobe[13] using a modified procedure of Seifter *et al.*[14]. The liver sample was digested in 10 ml. 30% KOH (w/v) on a boiling water bath for 20 minutes, after which the glycogen was precipitated in an aliquot by ethanol, heated to boiling, cooled, centrifuged, and the sediment dissolved in water and reprecipitated by ethanol. Glycogen content was determined by anthrone (0.2 g. anthrone/100 ml. sulphuric acid, obtained by mixing 750 ml. concd. acid with 300 ml. water). 1 ml. of aqueous glycogen solution (up to 300 μg. of glycogen) was used to overlay the 10 ml. of ice-cooled anthrone reagent. The tube was stoppered, mixed gently twice, and heated on a boiling water bath for 10 min. The OD was read at 620 nm using a red photocell.

The average chain length was determined by the periodate oxidation procedure[15]. A sample of the solution (5 ml.) containing about 50 mg. glycogen was cooled to 4° and oxidized with 4 ml. of pre-cooled 0.2 *M* $NaIO_4$ for 30 hours in the dark. Actual concentration of the glycogen solution was determined by anthrone. It was because we found in 15 samples the water content ranging from 1.1 to 18%, in average 13.5%. The liberated formic acid was titrated with 0.01 *N* NaOH prepared from a solution of 75 g. NaOH in 75 ml. water, by dilution with glass redistilled water which was boiled previously to expell carbon dioxide. The blank value was reduced to minimum by washing test tubes with chromium-sulphuric acid and then with warm (40°) mixture of equal parts of 6 *N* acetic acid and 6% H_2O_2[16]. Tubes were subsequently dried in a drying oven. Sucrose was employed as a reference standard for measurement of the extent of oxidation. The mean oxidation percentages are seen in the Table:

Number of measurements	mg. sucrose	Mean oxidation percentage
3	10	77.8
2	20	80.0
3	30	79.6
2	40	77.1
17	50	74.6

For the 50-mg. sample the values ranged from 70.5 to 79.5% of oxidized sucrose. Commercial oyster glycogen (British Drug Houses, Ltd.) was used for checking the method during each series. Its average chain length determined by the methylation technique[17] was 10.7 and, by the periodate oxidation, its mean value was 11.0 ± 0.13 (range in 16 measurements 10.1—11.8). The S-value determined by the analytical ultracentrifuge was 65 S_0 (range of values from 22 to 130 S).

The counting of labelled samples was carried out in a liquid scintillation counter after having hydrolyzed samples dissolved in the Herberg[18] solution, or the glycogen (2 mg. in 0.2 ml. water) was dissolved in 2 ml. of Nuclear Chicago Solubilizer to which the toluene solution of PPO and POPOP was added to 15 ml.[19]. The channels ratio method was used for the efficiency determination.

The arithmetic mean, the standard deviation, and the standard error of the arithmetic mean were calculated. The Student's *t*-test was used for testing differrences between the two arithmetic means of small independent samples. The level of significance used for calculations was 5% ($P < 0.05$).

RESULTS AND DISCUSSION

The results summarized in Table I indicate that the average chain length and the percentage of ^{14}C-glucose incorporated into glycogen show a similar trend of changes. These parametres increase with liver glycogen concentration and then gradually decrease. Statistical treatment of data in this study has shown that the values obtained in Group 1 deviate significantly at the level 1% from those obtained in all other groups for both measured parametres. Control values in six intact animals were as follows: relative liver weight, 3.9 ± 0.10 g./100 g. body wt., hepatic glycogen level, 5.5 ± 0.7%, average chain length, 12.7 ± 0.10.

The rats from Group 6 of this experiment were part of a separate study[20] in which rats were fasted for 72 hours and killed after feeding for differing times up to 24 hours. The results observed were similar to the results presented in this paper, where quite different metabolic conditions exist. Our previous experiments[21] in which different sugars were given to rats (glucose, fructose, galactose, and sucrose, respectively) demonstrated that the average chain length was in relation with the glycogen concentration only. Further experiments[22] were performed in which tryptophan was used to block gluconeogenesis, what is just opposite to the Group 4 of the present paper in which glycogen is synthetized entirely from glucose formed by gluconeogenesis. On considering all findings it has become clearly apparent that glycogen structure is related only to glycogen concentration and is not dependent upon the metabolic state.

Fig. 1 shows individual values for the average chain length in relation to the liver glycogen level. All available measurements are presented. Along with the increase of glycogen concentration to about 2.5% there is an increase in the average chain length from approximately 7 to 13 glucose units. This is followed by a slow decrease to about 12 glucose units as glycogen level increases further. It appears from the data that up to the concentration of 2.5% only one process actually occurs — the growth of the existing glycogen particles. Once this concentration is attained, some new small macromolecular structures, capable of acting as primers of a further synthesis, appear to develop. This can be seen in the mild falling slope of the curve in Fig. 1 and this portion of the curve is the result of superposition of the two series of values — the low values for the average chain length of particles in their initial growth, and the values relating to the preexisting large particles. After reaching an average chain length of about 12 glucose units, the glycogen concentration continues increasing as a result of the agglomeration of the already formed particles of a certain chain length, with constant recruiting of the new primers. Increase in concentration to the highest glycogen levels is likely to occur by continuation of the agglomerating process.

TABLE I

The Average Chain Length of Glycogen and the Glucose Incorporation Rate into it at its Varying Concentrations

Group and treatment	Number of animals	Body weight	Ratio of liver to body weight (g./100 g.)	Concentration of glycogen in liver (g./100 g.)	Average chain length	% of ^{14}C dose incorporated into liver glycogen
1. Fasting for 14 hours	6	223±4*	3.15±0.03*	0.9±0.24*	8.8±0.57*	1.5±0.3*
2. Fasting for 14 hours, glucose administration killed 2 hours later	6	208±7	3.14±0.04	2.1±0.09	13.3±0.44	9:5±0.8
3. Fasting for 14 hours, glucose administration killed 6 hours later	6	202±9	3.19±0.06	3.7±0.22	12.6±0.33	6.0±1.2
4. Fasting for 3 days with cortisol administration	6	184±3	3.80±0.09	5.1±0.47	11.9±0.15	5.4±0.9
5. Cortisol administration for 3 days to *ad lib.* fed rats	6	212±5	4.64±0.12	6.1±0.52	12.3±0.20	2.9±0.2
6. Fasting for 3 days, then food *ad lib.*	9	219±4	4.58±0.15	9.1±0.30	12.5±0.25	3.1±0.3

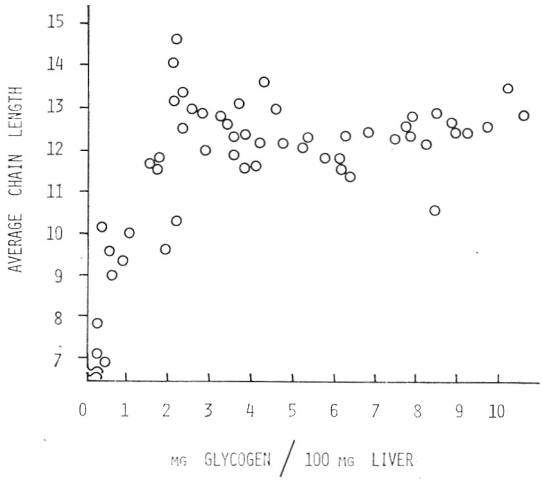

Fig. 1. The relationship between the liver glycogen concentration and the average chain length of glycogen particles.

The idea of agglomeration of preformed or newly synthesized particles is by no means new. Orrell *et al.*[3] considered this possibility on the evidence of data on different specific activities of light and heavy glycogen fractions. Colucci *et al.*[4] found different incorporation rates of ^{14}C-glucose into different fractions of the glycogen sedimentation spectrum of the tapeworm *Hymenolepis diminuta*. They observed a more intense incorporation into larger particles at higher (about 8%) concentrations and, into smaller particles at lower (1.5%) concentrations. It means that during glycogen accumulation at lower concentrations smaller particles are formed; but, the accumulation at higher concentrations is a result of agglomeration. As a result, we cannot rule out either the possibility of the agglomeration of glycogen particles during the phase of the increasing average chain length, or the budding of the existing particles to permit development of new structures on the surface of the pre-existing ones. But, Parodi *et al.*[23] obtained the curves of the changes in the amounts of light and heavy UDPG-glycogen during *in vitro* synthesis that would be expected if light glycogen was formed first and then aggregated to form the heavy population. Scott and Still[24] gave evidence about uniform enlarging of glycogen particles during glycogen synthesis in leukocytes. Since the leukocyte glycogen is a rather uniform, low-molecular weight glycogen which does not form *alpha* particles, this finding is not easy to compare with the liver glycogen. Our data may be also interpreted in the light of results of Parodi *et al.*[23] so that in the first stage of glycogen accumulation a »random-type« addition of glucose molecules takes place. After reaching a critical concentration (and a certain size) another mechanism may dominate — agglomeration. According to the interesting findings of Kindt and Conrad[12] and Parodi *et al.*[25] only a part of the non-reducing ends is available to the action of glycogen synthetase. It was found by the latter group that the heavier glycogens had fewer available terminal glucose units than the lighter ones of the same preparation. These results are compatible with the above consideration.

In vitro experiments conducted by Smith *et al.*[26] indicated that the glycogen, synthesis induced by phosphorylase continued at a decreasing rate, independent of glycogen concentration, while the average chain length diminished. It would be, however, difficult to compare this finding with our *in vivo* studies.

Consideration of the number of particles of glycogen in the cell is actually a question about a glycogen particle being either an »organelle« in a state of constant turnover and varying in size depending on the relative rates of synthesis and degradation[24], or a particle which may be split by hydrolytic enzymes to give new primers for further glycogen synthesis[27]. Parodi[28] and Vaillant and Jost[29] support the view that there is an increase in the number of particles during the course of glycogen accumulation, while Scott and Still[24] and De Wulf and others[30], on the basis of measurement of the steadily increasing number-average molecular weight of mouse liver glycogen, consider the number of glycogen particles to be constant. The data presented here are compatible with Parodi's view. Further, several groups of investigators[31–33] observed low molecular weight of glycogen at high liver glycogen concentrations, what may be interpreted so that number of particles of glycogen in liver increases.

It is to be noted that at the present time it is neither possible to draw conclusions concerning the chemical nature of the inter-agglomerate bonds nor to consider the enzymes which may effect the agglomeration. Luzardo-Baptista[34] suggests that the glycogen *beta* particles could be kept joined by *gamma* particles which are seen in electron microphotographs with an average width of 1.8 nm.

Studies of the incorporation of radioactive glucose into glycogen show it to have a high specific activity until glycogen concentration reaches 2.5%, followed by a decrease of specific activity with further concentration increase. The activity of the enzymes taking part in the glycogen synthesis apparently decreases with the increasing glycogen concentration, when evaluated by the incorporation rate of ^{14}C-glucose into whole liver glycogen. Actually, measurement of the activity of glycogen synthetase[35] during glycogen accumulation from lowest to very high levels (effected by administration of glucose during 16 hours[36]) and subsequently to a low level (by fasting), indicated a pronounced increase in the glycogen synthetase activity, then decrease to a very low activity and an increase when fasting started.

The next aspect to be considered is the average chain length of the glycogen particles in the sedimentation spectrum. Molecular weights ranged[4] from 10^5 to 10^9, but the average chain lengths of all the fractions of any given preparation were identical[4,37–39]. Thus it is possible to suggest a process of subparticle agglomeration into larger particles in which the basic properties of the structure are preserved. From this, the problem of the glycogen structure becomes apparent: Which are the factors that regulate the average chain length in such a way as to make it equal in the whole population of a polydisperse preparation and simultaneously changeable with concentration change?

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REFERENCES

1. S. A. Orrell and E. Bueding, *J. Biol. Chem.* **239** (1964) 4021.
2. E. Bueding and S. A. Orrell, Jr., *J. Biol. Chem.* **236** (1961) 2854.
3. S. A. Orrell, Jr., E. Bueding, and M. Reissig, in W. J. Whelan and M. P. Cameron, *Control of Glycogen Metabolism,* Churchill, London 1964, p. 29.
4. A. V. Colucci, S. A. Orrell, H. J. Saz, and E. Bueding, *J. Biol. Chem.* **241** (1966) 464.
5. A. D. Antoine and B. S. Tepper, *Arch. Biochem. Biophys.* **134** (1969) 207.
6. B. Illingworth, J. Larner, and G. T. Cori, *J. Biol. Chem.* **199** (1952) 631.
7. V. Fišter and A. Lutkić, *Radovi Med. fak.,* Zagreb **15** (1967) 185.
8. A. Lutkić and V. Fišter, *Abstracts of the 5th FEBS Meeting,* Prague, 1968, Comm. No 387.
9. V. Fišter and A. Lutkić, *Jugoslav. Physiol. Pharmacol. Acta* **2** (1966) 91.
10. M. R. Stetten, H. M. Katzen, and D. Stetten, Jr., *J. Biol. Chem.* **222** (1956) 587.
11. M. R. Stetten and H. M. Katzen, *J. Am. Chem. Soc.* **83** (1961) 2912.
12. T. J. Kindt and H. E. Conrad, *Biochemistry* **6** (1967) 3718.
13. A. Lutkić and V. Fišter, *Jugoslav. Physiol. Pharmacol. Acta* **3** (1967) 335.
14. S. Seifter, S. Dayton, B. Novic, and E. Muntwyler, *Arch Biochem.* **25** (1950) 191.
15. W. J. Polglase, E. L. Smith, and F. H. Tyler, *J. Biol. Chem.* **199** (1952) 97.
16. P. I. Voskresenskij, *Tehnika laboratornyh rabot,* 8th Edition, Himija, Moskva, 1967, p. 136.
17. W. Z. Hassid and S. Abraham, in *Methods in Enzymology,* Vol. III, Academic Press, New York, 1597, p. 40
18. R. J. Herberg, *Anal. Chem.* **32** (1960) 42.
19. D. L. Hansen and E. T. Bush, *Anal. Biochem.* **18** (1967) 320.
20. A. Lutkić and V. Fišter, *Jugoslav. Physiol. Pharmacol. Acta* **5** (1969) 399.
21. A. Lutkić and V. Fišter, *Jugoslav. Physiol. Pharmacol. Acta* **7** (1971) 361.
22. A. Lutkić, *Ph. D. Thesis,* University of Zagreb, 1968.
23. A. J. Parodi, C. R. Krisman, and J. Mordoh, *Arch. Biochem. Biophys.* **141** (1970) 219.
24. R. B. Scott and W. J. S. Still, *J. Clin. Investigation* **47** (1968) 353.
25. A. J. Parodi, J. Mordoh, C. R. Krisman, and L. F. Leloir, *Eur. J. Biochem.* **16** (1970) 499.
26. E. E. Smith, in W. J. Whelan, *Control of Glycogen Metabolism, Preceedings of the 4th Meeting of the FEBS,* Oslo, 1967, Universitetsforlaget, 1968, p. 203.
27. W. J. Whelan, *Biochem. J.* **122** (1971) 609.
28. A. J. Parodi, *Arch. Biochem. Biophys.* **120** (1967) 547.
29. R. Vaillant and A. Jost, *Biochimie* **53** (1971) 797.
30. H. De Wulf, N. Lejeune, and H. G. Hers, *Arch. Intern. Physiol. Biochim.* **73** (2) (1965) 362.
31. J. Mordoh, C. R. Krisman, and L. F. Leloir, *Arch. Biochem. Biophys.* **113** (1966) 265.
32. R. Willstätter and M. Rhodewald, *Z. Physiol. Chem.* **225** (1934) 103.
33. R. Vaillant, *Bull. Soc. Chim. Biol.* **52** (1970) 751.
34. M. J. Luzardo-Baptista, *Ann. Histochim.* **17** (1972) 141.
35. A. Lutkić, V. Puretić, and Lj. Svilokos, unpublished results.
36. B. Friedmann, E. H. Goodman, Jr., and S. Weinhouse, *Endocrinology* **81** (1967) 486.
37. R. Laskov and E. Margoliash, *Bull. Res. Counc. of Israel* **11 A 4** (1963) 351.
38. M. R. Stetten and D. Stetten, Jr., *J. Biol. Chem.* **232** (1958) 489.
39. F. Bertrand and L. Laszt, *Biochem. Z.* **327** (1956) 354.

IZVOD

Struktura glikogena u zavisnosti od njegove razine u jetri štakora

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Mjerili smo srednju dužinu lanca i veličinu ugradnje radioaktivne glukoze u jetrnji glikogen štakora tokom nagomilavanja glikogena u jetri. Štakori su bili obrađeni tako da imaju različite razine glikogena u jetri. Sve mjerene veličine povećavaju se s porastom koncentracije do oko 2.5%, a zatim se postupno smanjuju daljnjim porastom koncentracije glikogena. Povećavanje koncentracije jetrnjeg glikogena iznad razine od 2.5% ostvaruje se aglomeracijom već formiranih čestica. Struktura glikogena zavisna je jedino od razine glikogena u jetri, a nije pod utjecajem onih agensa koji mijenjaju koncentraciju jetrnjeg glikogena.

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