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Dynamics of Proglycogen and Macroglycogen in Hepatocytes of Normal and Cirrhotic Rat Liver at Various Stages of Glycogenesis

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Abstract—The content and structure of glycogen in hepatocytes of normal and cirrhotic rat liver were examined at different time intervals after glucose administration to starving animals. We used an original cytofluorimetric method for detection and quantification of proglycogen (PG) and macroglycogen (MG) of isolated hepatocytes. The method is based on the use of reagents of the Schiff type with different spectral characteristics. The content of MG in hepatocytes of control rats was increased by 52% ($p < 0.01$) as early as after 10 min. The MG content in the cirrhotic liver cells was increased by 43% ($p < 0.05$) only 20 min after glucose administration to the starving animals. The correlation coefficient between MG content and the total glycogen content at various stages of glycogenesis in rats of both groups was from 0.90 to 0.99 ($p < 0.001$). Increase in the PG content in hepatocytes of control rats was observed in intervals of 10–30 and 45–75 min. The PG content in cirrhosis was increased only in 60 min after the beginning of glycogenesis, but in 120 min it was 1.5 times higher than the control values ($p < 0.001$). The correlation coefficients between PG and the total glycogen content in the cells were on average 0.86 ($p < 0.001$) and 0.77 ($p < 0.001$) in the control and experimental groups, respectively. Thus, the change in the total glycogen content in hepatocytes of normal and cirrhotic liver are associated mainly with changes in the MG level. The contribution of PG was most significant in normal liver at the beginning of glycogenesis (10–30 min); in cirrhotic liver, at later stages (75–120 min).

Keywords: proglycogen, macroglycogen, hepatocytes, glycogen synthesis, liver cirrhosis, glucose

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Glycogen belongs to the most important biopolymers incorporated into the human and animal cells. This glycoprotein, which is the main glucose source, provides energy to many metabolic processes in the body. Glycogen metabolism is distinguished by extraordinary complexity; its regulation is carried out through very complicated mechanisms involving different enzymes, hormones, and inhibitors and activators of enzymes, as well as metal ions (Ferrer et al., 2003; Greenberg et al., 2006; Jurczak et al., 2008). Violations in any link of these complex processes result not only in abnormal increase or decrease in glycogen content in the cells, but also in changes of its structure (Rozenfel'd, Popova, 1989; Mayatepek et al., 2010). For example, in glycogen storage disease type III, glycogen is characterized by shortened terminal branches of the molecule, while, in glycogen storage disease type IV, the accumulated glycogen has a few branch points, as well as very long and sparse side branches (Mayatepek et al., 2010). Mutations in the genes encoding laforin and malin are the cause of Lafora disease, which is accompanied by the accumulation of large hyperphosphorylated polyglucosane molecules in neurons, heart, liver, and skeletal muscles (Ganesh et al., 2001; Tagliabracci et al., 2007; Tagliabracci et al., 2011).

It is known that liver cirrhosis, which is one of the most widespread and dangerous human diseases, not only leads to the disruption of the morphological structure of the liver, but also causes various changes in the metabolism of glucose and glycogen. It was found that this disease is characterized not only by the accumulation of glycogen in the liver, but also by the change in its structure, as evidenced by the change in the ratio of easily accessible and inaccessible glycogen fractions in favor of the predominance of the latter (Kudryavtseva et al., 1992; Kudryavtseva et al., 2001).

Unfortunately, the currently available data on the glycogen structure in hepatocytes in liver cirrhosis are very few and characterize the cell population as a whole. Therefore, the purpose of this work was to study the structure of glycogen in isolated hepatocytes from normal and cirrhotic rat liver at different time intervals after glucose administration to the starving animals.

[1] *Abbreviations*: MG—macroglycogen, PG—proglycogen.

MATERIALS AND METHODS

Animals. Investigations were carried out in 54 white outbred male rats. Animal weight was 130–140 g at the beginning and 250–300 g at the end of experiment. The rats were kept on a standard diet. At the beginning of experiment the animals were divided into two groups, experimental and control.

To obtain experimental cirrhosis, animals of the experimental groups were subjected to the chronic inhalation exposure of carbon tetrachloride (CCl_4) vapors (7 mL per 100 L of volume) in a sealed chamber for 20 min 3 times a week for 6 months. The control group of animals was not subjected to the CCl_4 exposure.

One week after the end of CCl_4 exposure to rats of the experimental group, animals from both groups were subjected to starvation for 48 h (water ad libitum) and then 30% glucose solution was administered per os at the rate of 4 g per 1 kg of body weight. Immediately after termination of starvation and 10, 20, 30, 45, 60, 75, 90, and 120 min after glucose administration, rats were decapitated (three animals for each period). Material (liver pieces) obtained from each animal was used for cytofluorimetric and histological studies.

Histological samples. Pieces of rat liver were fixed in 10% neutral formalin and embedded in paraffin according to the standard procedure. Then histological sections of 6–7 μm in thickness were prepared.

Sections were stained with Mayer's hematoxylin–eosin. Furthermore, to detect connective tissue, preparations were stained with picrosirius (0.01% solution of Sirius Red in a saturated aqueous solution of picric acid) for 1 h. After staining, preparations were rinsed in 0.01 N HCl for 2 min, dehydrated in alcohols of increasing concentration, and embedded in Canadian balsam.

Mapping of smear preparations of isolated hepatocytes. Smears of isolated hepatocytes were prepared according to the previously described procedure (Kudryavtseva et al., 1983). In order to find the location of each cell several times, a coordinate grid (a square size of about 1 mm^2) was applied to a glass slide with fixed cells using a diamond cutter. Next, we obtained images of selected sites of preparations using an Axioskop microscope (Carl Zeiss, Germany) and a DFC360 FX digital black-and-white high-sensitivity CCD camera (1392 × 1400). Then, on the obtained images of cells, we chose undamaged, separately located hepatocytes; each of them was given a specific number according to its location on a glass slide (Fig. 1).

During the work, to obtain images of separate areas of preparations, we used the lens 20 × 0.45. From 100 to 150 cells were labeled on each preparation.

Identification of PG and MG in isolated hepatocytes. Preparations were stained with a fluorescent variant of the PAS reaction. The mapped preparations of smears of isolated hepatocytes were placed into sodium periodate solution in diluted HNO_3 (200 mg of sodium periodate, 25 mL of 0.23% HNO_3) for 1.5 h. The oxidized preparations were then washed in running tap water for 5 min and then in one change of distilled water. After this, the preparations were first placed in EtBr–SO_2 (100 mL 10^{-5} M ethidium bromide, 0.2 mL thionyl chloride) for 40 min to detect MG (Fig. 2a) and, after rinsing in distilled water, they were placed in Au–SO_2 (300 mg auramine, 100 mL water, 0.2 mL thionyl chloride) for 50 min to detect PG (Fig. 2b). After the staining period, preparations were removed from the staining solution and washed with three changes of distilled water and three changes of sulfurous water (5 g potassium metabisulfite, 950 mL of water, 50 mL of HCl) for 3 min in each. After this, preparations were washed in running tap water for 20 min, rinsed in distilled water, and dehydrated in alcohols of increasing concentrations (70, 96, and 100 vol % for 5 min in each change of alcohols).

Cytofluorimetry. Images of the Au–SO_2- and EtBr–SO_2-stained cells were obtained using an Axioskop microscope (Carl Zeiss, Germany), a Plan-NEOFLUAR 20 × 0.50 lens, and a DFC360 FX digital black-and-white high-sensitivity CCD camera (1392 × 1400). To excite Au–SO_2 and EtBr–SO_2 fluorescence, we used interference filters at 450–490 and 546 nm; to record fluorescence, interference filters at 515–565 and 590 nm, respectively. The intensity of fluorescence of the cells stained with Au–SO_2 and EtBr–SO_2 was measured using ImageJ software. The total glycogen content in each cell was taken to be equal to a sum of PG and MG. The fluorescence of 100–150 cells was measured for each preparation.

Statistical processing of results, i.e., a comparison of the mean values by Student's test and correlation analysis with the calculation of correlation coefficients (r), were performed using SigmaPlot v 11.0 software.

RESULTS AND DISCUSSION

Chronic intoxication of animals with CCl_4 reproduces many of the characteristic symptoms of human cirrhosis (Kus et al., 2004; Planaguma et al., 2005). The development of cirrhosis is known to be accompanied by a significant rearrangement of the liver structure, which leads to a loss of lobular organization of the organ, change in vascular bed, and increasing ratio of nonparenchymal cells to parenchymal ones due to enhanced proliferation of the first and death of the last (Sherlock, Dooley, 2002). The histologic analysis of sections of rat liver showed that the parenchyma structure in control animals is typical for the normal organ. It is characterized by hepatocyte trabeculae that are arranged radially around the central vessels, a small amount of connective tissue, and distinct boundaries of portal vessels and bile ducts (Figs. 3a, 3c).

We observed diffuse leukocyte infiltrates in liver sections from rats that were exposed to CCl_4 poisoning

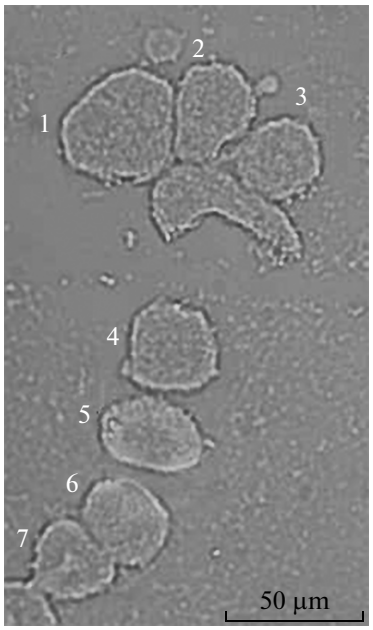

Fig. 1. Mapped preparation from isolated hepatocytes.

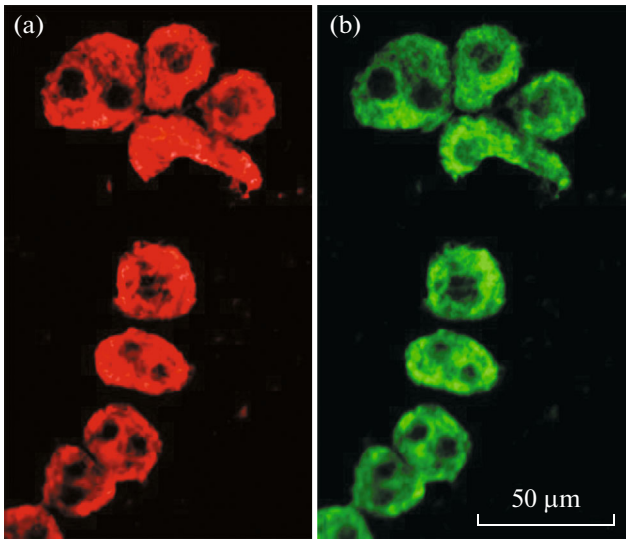

Fig. 2. Identification of (a) MG and (b) PG in the same hepatocytes with EtBr–SO_2 and Au–SO_2, respectively.

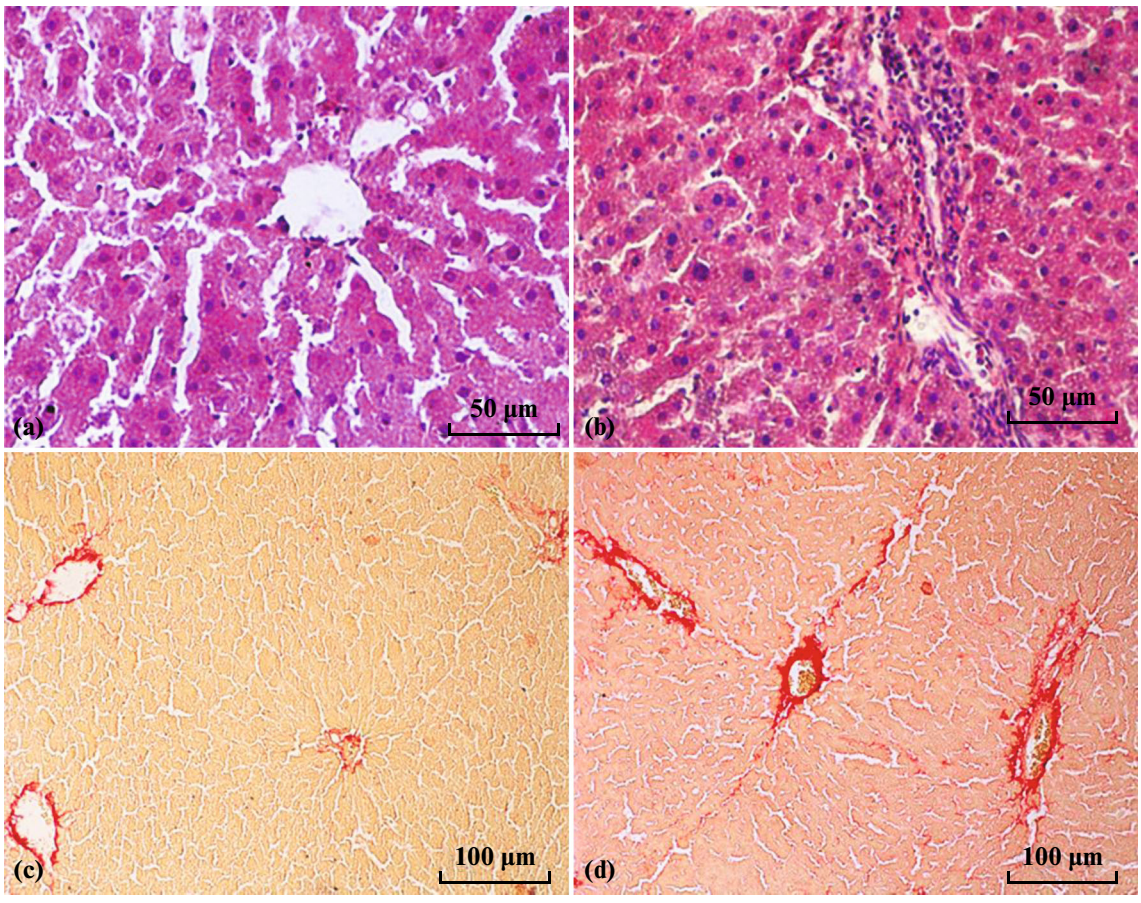

Fig. 3. Histological sections of (a, c) normal and (b, d) cirrhotic rat liver. Staining with (a, b) hematoxylin–eosin and (c, d) picrosirius. Thick arrows indicate diffuse leukocyte infiltrates; thin arrows, thickened walls of central veins and connective tissue fibers along the sinusoids. Plan-NEOFLUAR lens (a, b) 40 × 0.75 and (c, d) 20 × 0.50.

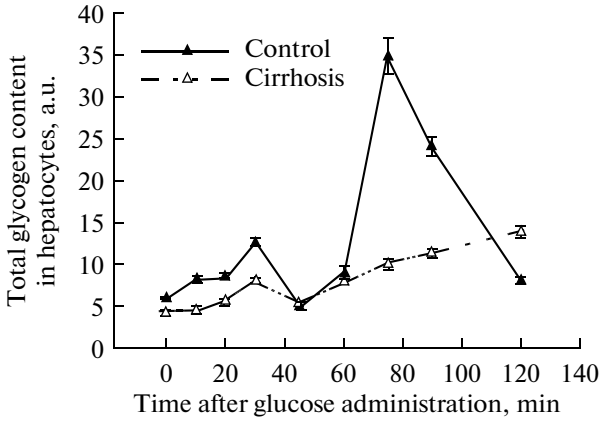

Fig. 4. The total glycogen content in hepatocytes of normal and cirrhotic rat liver at various time intervals after glucose administration to starving animals.

within 6 months, which indicates the activation of inflammatory processes (Fig. 3b). In addition to inflammation processes in the liver tissue of rats of the experimental group, we observed signs of fibrotization of the organ. Parenchyma was germinated by connective tissue strands that violate the lobular structure of the organ. The thickened walls of central veins and connective tissue fibers along sinusoids also were detected by staining with picrosirius (Fig. 3d).

Fibrotization of sinusoids and central veins of the liver prevents hepatocytes from coming into contact with the blood entering the organ, making their functioning difficult. Since the liver plays a key role in maintaining a constant level of glucose in the blood, the deterioration of glucose uptake and its deposition as glycogen in hepatocytes may be crucial in the development of glucose intolerance and liver cirrhosis. The currently available data on the synthesis of glycogen in the cirrhotically altered liver are few and contradictory. On the one hand, it has been found that glycogen synthesis in the liver of cirrhosis patients does not differ from that in the liver of healthy people (Schneiter et al., 1999). On the other hand, it has been shown that the glycogen content in cirrhotically altered and rat human liver after ingestion is significantly lower than in the normal liver (Krahenbuhl et al., 1991; Giardina et al., 1994; Petersen et al., 1999).

The study of the total glycogen content dynamics in hepatocytes showed that, after the administration of glucose to starving rats, there was a rapid accumulation of glycogen in hepatocytes in rats of the control group (Fig. 4). As a result, as early as in 10 min, the glycogen content in hepatocytes of the normal liver was 1.4 times higher than in starving animals ($p < 0.001$). The maximum glycogen content in rat hepatocytes was observed at the 75th minute of the experiment, and it exceeded the initial level by 5.9 times ($p < 0.001$). In contrast to the normal liver, the glycogen accumulation in hepatocytes in cirrhosis was delayed and started 20 min after glucose administration. However, 120 min after the beginning of the experiment the total glycogen content in hepatocytes of rats of the experimental group was 1.7-fold higher than in rats of the control group ($p < 0.001$).

If the total glycogen content during glycogenesis was continuously increased in the cirrhotic liver, then the process of glycogen accumulation in the normal liver had a rhythmic wavelike nature; periods of rapid increasing the glycogen content alternated with periods of its reduction (Fig. 4). It is difficult to speak at present of the reasons for rhythmic fluctuations in the glycogen content during its synthesis in the liver. Currently, near-hourly (20–90 min) rhythms of protein synthesis, activity of various enzymes, hormones, calcium concentration, and concentrations of cAMP and other cellular components are known (Brodsky, 1975; Brodsky, Nechaeva, 1988; Lloyd, Rossi, 1992; Brodsky, 2006). It has been found that near-hourly rhythms have an endogenous nature; they are caused by intercellular contacts, and are a reflection of synchronization of the functionally interrelated cells (Brodsky, Nechaeva, 1988). It can be assumed that the disappearance of the rhythm of glycogen accumulation in rat hepatocytes in the experimental group is associated with significant damage to lobular structure of the liver parenchyma, which, in turn, leads to disruption of intercellular relations between hepatocytes.

It is known that the total glycogen content in the liver cells and the number of its α- and β-particles can vary considerably depending on the nutritional status of the body (Devos et al., 1983; Sullivan et al., 2010). It was shown that a fully formed glycogen molecule (β-particle) consists of 12 concentric tiers. Four external tiers of macroglycogen contain 94–97% of all glucose residues, and eight internal ones that form a so-called skeleton or proglycogen contain from 3 to 6% of the glucose residues in the glycogen molecule (Rybicka, 1996; Melendez et al., 1999; Shearer, Graham, 2002). Proglycogen has a molecular weight of about 400 kDa and is distinguished from macroglycogen by insolubility in dilute trifluoroacetic acid due to its strong association with proteins. It is believed that proglycogen is a stable intermediate form of glycogen during the formation of a complete β-particle (Judd et al., 1992; Alonso et al., 1995). It is assumed that the accumulation of glycogen may occur either by increasing the number of external tiers of its molecule (increase in the MG content) or by the formation of new initiation points of its synthesis (increase in the PG content).

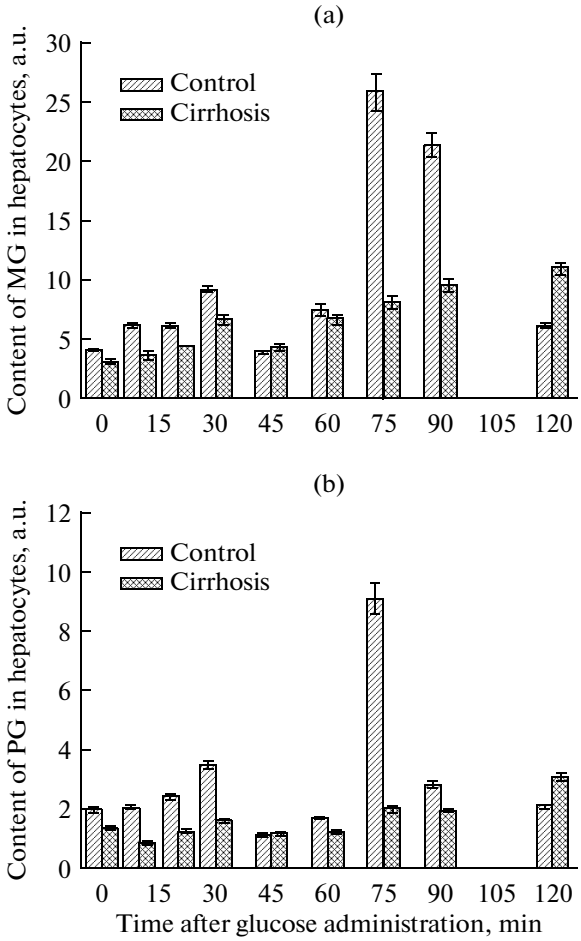

Fig. 5. Content of (a) MG and (b) PG in hepatocytes in the norm and cirrhosis at various time intervals after glucose administration to starving animals.

The results shown in Fig. 5a indicate that the MG content in hepatocytes of control rats increased by 52% ($p < 0.01$) as early as in 10 min. In a similar manner as the total glycogen content in hepatocytes, the periods of fast increase in the MG content were alternated with the periods of its reduction. Unlike the control, the MG content in hepatocytes in cirrhosis began to increase only 20 min after the beginning of glycogenesis (Fig. 5a). It should be noted that the MG proportion in some cells of rats of both groups could reach 88% with the accumulation of glycogen in hepatocytes.

The PG content in normal liver hepatocytes was changed wavelike throughout the entire experiment. The increase in PG content was observed in intervals of 0–30 and 45–75 min, and in the intervals of 30–45 and 75–90 min there was a drop in PG content (Fig. 5b). Unlike the control, the PG content in cirrhosis varied only slightly in the first 60 min of glycogenesis. Nevertheless, at the end of the experiment, the PG content in rat hepatocytes in the experimental group was 2.3 times higher than its initial concentration ($p < 0.001$). It should be noted that the PG content in cirrhotic rat liver hepatocytes in 120 min after the glucose administration was 1.5 times higher ($p < 0.01$) than in normal liver hepatocytes. An increase in the PG content for a certain period after glucose administration to starving rats may indicate the appearance of additional points of initiation of glycogen synthesis in the cell at these stages of glycogenesis.

Analysis of dependence of the total glycogen in hepatocytes on MG showed that the glycogen accumulation in the liver and fluctuations of its content in the cells in both the control and experimental rats at different stages of glycogenesis are related mainly to changes in the MG content. This is confirmed by the high correlation coefficient (r), which ranged from 0.906 to 0.998 in the control and from 0.915 to 0.997 ($p < 0.001$) in the experimental group (Fig. 6).

Despite the fact that MG makes the main contribution to the synthesis of glycogen, the correlation coefficients between the total content of glycogen and PG were also high at some stages of glycogenesis and

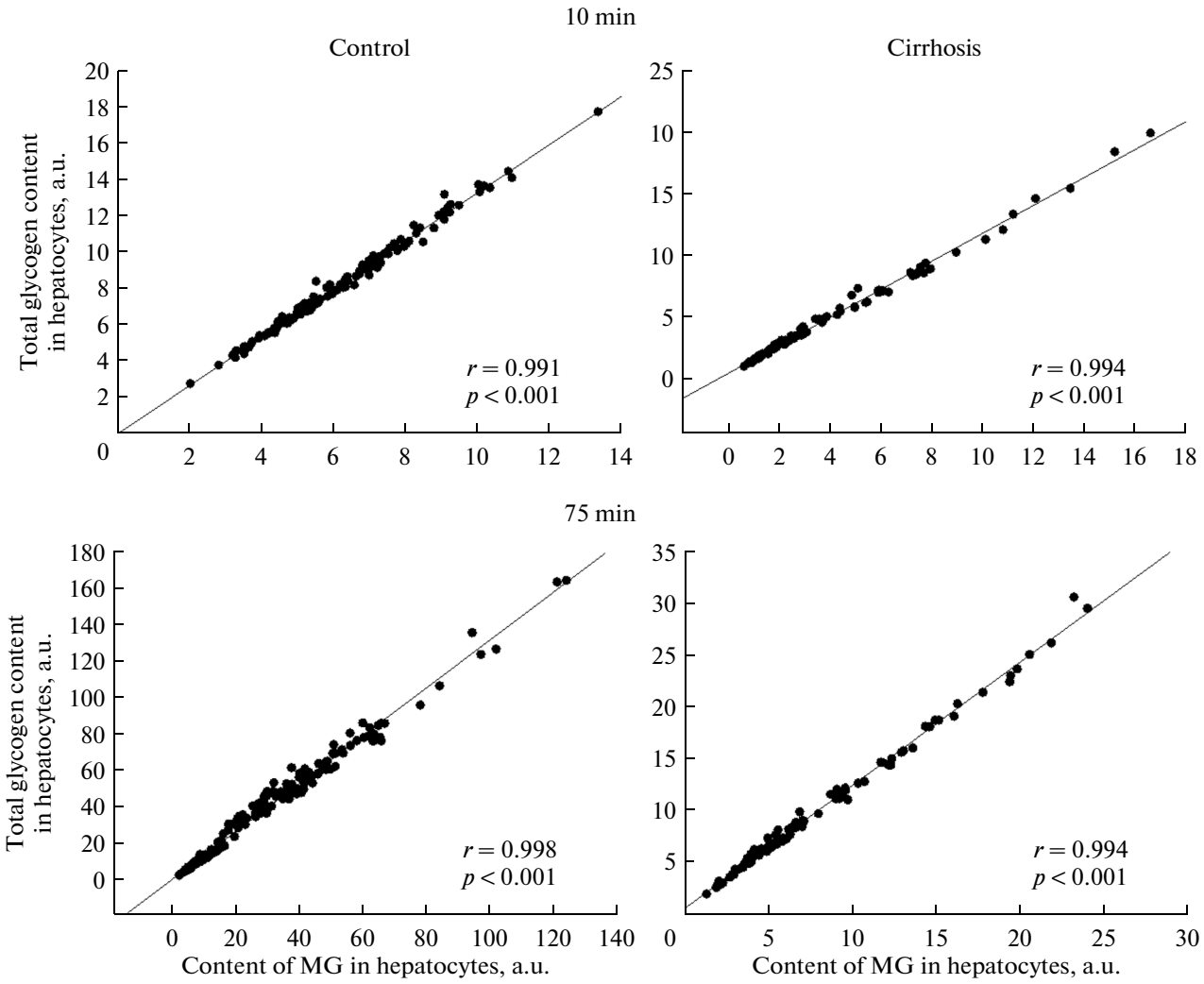

Fig. 6. Dependence of total glycogen content on MG content in hepatocytes of normal and cirrhotic rat liver at different glycogenesis stages.

averaged 0.863 ($p < 0.001$) in the norm and 0.772 ($p < 0.001$) in cirrhosis (Fig. 7)

Similar results were obtained by Wilson, who showed that the rate of MG resynthesis in the rat muscles exceeded the rate of PG resynthesis in the time interval of 0–1 h, but was lower in the time interval of 1–4 h during the recovery from exhausting physical exertion (Wilson, 2009). It is of interest to note that the glycogen accumulation in equine skeletal muscles after prolonged physical exercises occurs to a greater extent through MG (Bröjer, 2006). The MG fraction had a limited resynthesis rate in the first hour and the highest rate for the remaining 24 h of recovery after physical exertion. Increasing of the PG content was a slower process (Bröjer, 2006). However, other authors note that, in human and rat muscles, glycogen resynthesis after prolonged exercise occurs mainly due to PG, whereas the MG concentration increases markedly only after 1–2 h after completion of exercises (Shearer, Wilson, 2005; Wilson, 2009).

One of the explanations for the difference between the rates of PG and MG resynthesis may be related to the level of glycogen depletion. The extreme degrees of its depletion are likely to require the formation of new glycogen granules rather than adding glucose to the existing granules. This hypothesis is confirmed by data obtained by Shearer et al. (Shearer et al., 2005), which showed that changes in the glycogenin level were not observed during glycogenolysis until significant reduction of glycogen occurred.

Thus, based on our data, we can conclude that, after glucose administration to starving rats, total glycogen content in hepatocytes varies mainly due to MG both in the norm and in cirrhosis. In this case, it should be noted that PG also contributes significantly to glycogen accumulation in the cells: in the normal

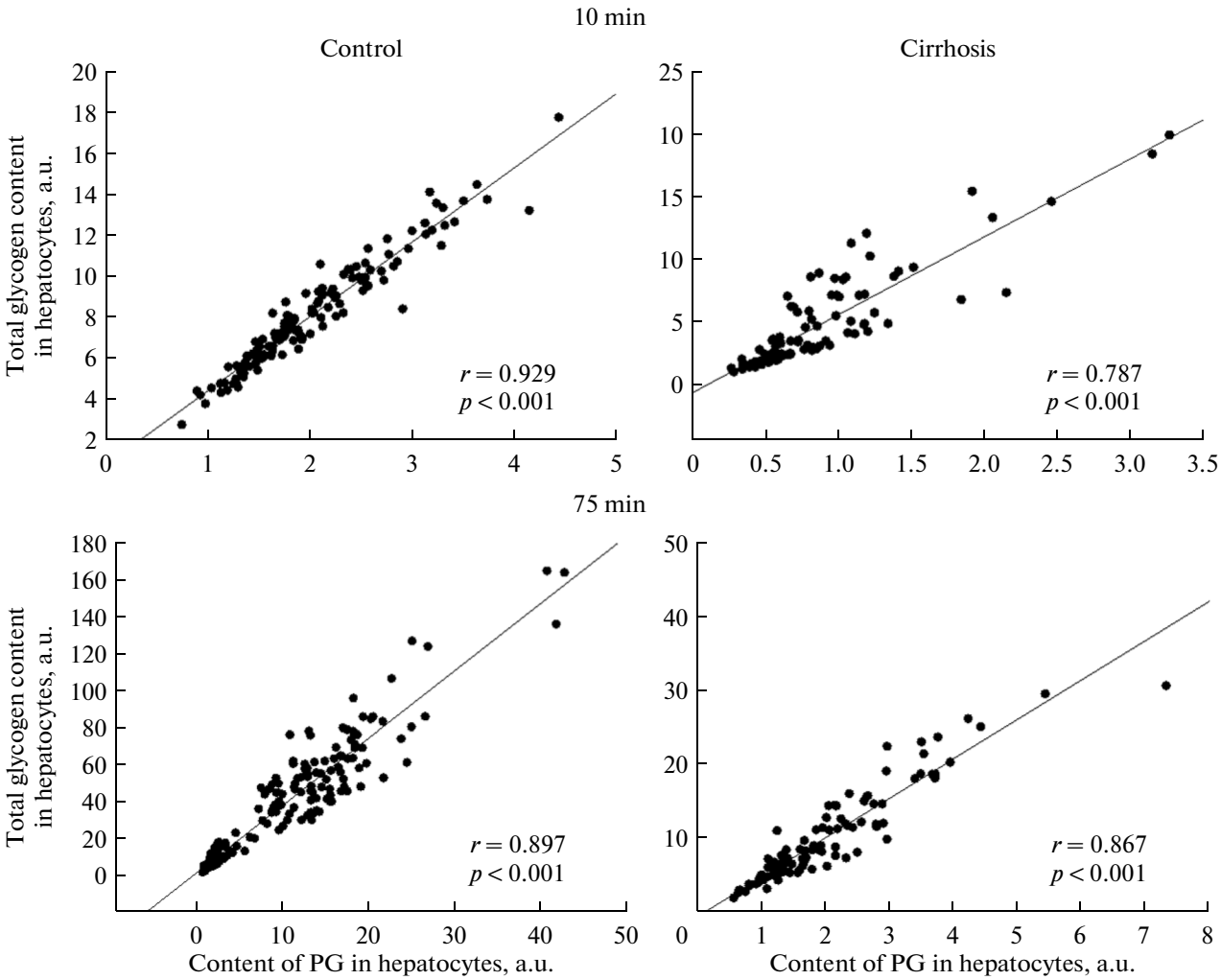

Fig. 7. Dependence of total glycogen content on PG content in hepatocytes of normal and cirrhotic rat liver at different glycogenesis stages.

liver at the beginning of glycogenesis (10–30 min) and in the pathologically altered organ at its later stages (75–120 min).

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