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CHARACTERISTICS OF GLYCOGENESIS IN HEPATOCYTES DURING CHRONIC LIVER DISEASES

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Dedicated to the memory
of Dr. Margarita V. Kudryavtseva

INTRODUCTION

Rationale of the study

Chronic hepatitides of various aetiology are widespread liver diseases of humans and animals. Chronic hepatitis (CH) gradually progresses to its final and most dangerous stage, liver cirrhosis (LC). Liver cirrhosis is one of the leading causes of mortality in many countries (Sun et al., 2019; Zhai et al., 2021a), a serious public health hazard and a significant economic problem (Mokdad et al., 2014; Scaglione et al., 2015; Sepanlou et al., 2020). The treatment of LC is a challenge, and no definitive solution has yet been found. Liver transplantation is currently considered the only way to radically cure this disease. However, the lack of suitable donors, the risk of rejection and the need for lifelong immunosuppression mean that liver transplantation cannot be considered a routine operation. Moreover, due to high costs, liver transplantation is unaffordable for many patients who badly need it (Jadlowiec et al., 2016).

The development of cirrhosis leads to profound changes in the liver architectonics, which have been described in detail in various works (Podymova, 1999; Malhi, Gores, 2008; Zhou et al., 2014; Torbenson, Washington, 2020). An overwhelming majority of LC studies has been conducted on experimental animals. Although no quantitative comparison of human LC with experimental LC models has been performed, many authors noted the similarity of this disease in humans and animals (Bresler et al., 1969; Verin, 1984; Michalopoulos, 1990; Chen, Hwang, 1994; Canturk, 1999; Kus, 2004; Planaguma, 2005).

Liver cirrhosis is accompanied not only by considerable changes in the microscopic structure of the liver but also by a significant impairment of its numerous functions, including glucostatic one. Glucostatic function, that is, the maintenance of a constant level of glucose in the blood, is one of the main functions of the liver. Its important mechanism is the ability of cells of the liver parenchyma (hepatocytes) to synthesise glycogen from glucose supplied from by blood or formed by gluconeogenesis from non-carbohydrate substances and to break it down according to the requirements of the organism.

Being a reserve substance, glycogen is found in almost all human and animal cells. It was discovered by Claude Bernard in 1857. Despite intensive studies of glycogen metabolism,

for which four Nobel Prizes have been awarded (Carl and Gerty Cori in 1947; Luis Federico Leloir in 1970; Earl Sutherland in 1971; Edmond Krebs and Edwin Fischer in 1992), the spatial structure of this branched polymer of glucose remains poorly understood. A fully-formed glycogen molecule, also called a β-particle (Takeuchi et al., 1978; Devos et al., 1983; Rybicka, 1996; Sullivan et al., 2014; Deng et al., 2016; Wang et al., 2022), is thought to be about 42 nm in diameter and to contain ~55,000 glucose residues connected by α-(1→4) and α-(1→6) glycosidic bonds. It consists of 12 concentric tiers of glucose residues, a self-glycosylating protein called glycogenin, which is located in the particle centre, and numerous proteins involved in the formation and degradation of the particle (Shearer, Graham, 2004; Roach et al., 2012). The four external tiers of the β-particle (macroglycogen, MG) contain ~95 % of all glucose residues, while the eight internal tiers form the so-called bone or proglycogen (PG) and contain about 5 % of all glucose residues (Melendez-Hevia et al., 1993; Rybicka, 1996; Melendez et al., 1999; Shearer, Graham, 2002). Proglycogen is believed to be a stable intermediate form of glycogen on the pathway to a complete β-particle (Lomako et al., 1991; 1993; Alonso et al., 1995).

Information about the mechanisms of formation of β-particles, their spatial structure and its changes in the norm and under pathological conditions is limited. Unfortunately, the spatial structure of this huge and complex molecule cannot be described with sufficient accuracy and completeness with the help of any known chemical or molecular biological methods (Brewer, Gentry, 2019; Brust et al., 2020). Almost all available methods are applicable to glycogen isolated from tissues, which poses a risk of disturbing its original structure. In addition, the methods currently in use can only provide information about the structure of glycogen molecules in a static state. Most of the data on the spatial structure of glycogen molecules are based either on theoretical concepts and mathematical calculations or on the results of determinations of the size of α- and β-particles obtained with the help of electron microscopy or ion-exclusion chromatography (Gunja-Smith et al., 1970; Goldsmith et al., 1982; Melendez-Hevia et al., 1993; Melendez et al., 1997; Sullivan et al., 2014; Rousset et al., 2022). Therefore, the development of a method for the study of the spatial structure of glycogen molecules and its dynamics directly in cells is urgently needed.

Glycogen is a readily available source of energy for various metabolic processes in many tissues and organs. Its content in the liver is a sensitive indicator not only of the state of the energy reserves in hepatocytes but also of the carbohydrate metabolism in the entire

organism. In the norm, the total glycogen content in liver cells fluctuates within rather narrow limits and is regulated by the ratio of its synthesis and breakdown (Devos et al., 1983; Rybicka, 1996; Sullivan et al., 2010). An excess or, on the contrary, a significant decrease of glycogen content in hepatocytes can cause severe liver diseases, which are accompanied by the disruption of the structure of glycogen molecules (Kudryavtseva, 1987; Rosenfeld, Popova, 1989; Ganesh et al., 2001; Tagliabracci et al., 2007; Mayatepek et al., 2010; Tagliabracci et al., 2011).

It is known that cirrhosis is often combined with other diseases (Olevskaya et al., 2018), with about 40 % of cirrhotic patients having comorbid status (Jepsen et al., 2008). One the most common comorbid conditions is Diabetes mellitus (DM) type 2 (Hickman et al., 2007; Garcia-Compean et al., 2009). The presence of DM in cirrhotic patients has been shown to markedly reduce their life expectancy (Tolman et al., 2007; Garcia-Compean et al., 2015; Kumar, 2018). However, the effect of comorbidity of DM and LC on the metabolism and glycogen content in hepatocytes is unstudied.

This means that an assessment of the metabolism, the content and the structure of glycogen in hepatocytes is important for determining the functional state of the liver, the prognosis of LC and the search for new approaches to its treatment.

Extent of prior research on the topic

Few data on the glycogen content in the cirrhotic liver are currently available. It has been shown that it is lower than in the normal liver (Owen et al., 1981; Krähenbühl et al., 1991; Krähenbühl et al., 1996, 2003). However, no definite conclusion about the glycogen level in the cirrhotic liver can be made based on the results of these studies because they were obtained with the use of indirect methods. The reliability of these methods strongly depends on the accuracy of measurements of the volume of the fibrous tissue and the parenchyma in the material under study.

Information about the glycogen content in the liver of patients with DM type 2 is also scarce and contradictory. Although it has been shown that the glycogen content in the diabetic liver is low (Whitton, Hems, 1975; Golden et al., 1979), the question remains open. The uncertainty is due to the fact that the information on the low glycogen content in the diabetic liver has mainly been obtained from animals with diabetes induced for a relatively short time (short-term diabetes), whereas in humans this disease develops over many months or even

years (long-term diabetes). Long-term development of diabetes can cause adaptive changes in the tissues as a response to hypoinsulinaemia and hyperglucagonaemia (Friedmann et al., 1963) and affect the glycogen content in liver cells.

It has been shown in numerous studies that glycogen consists of two fractions. One of them can be easily extracted with trichloroacetic acid (TCA). The extraction of the other fraction requires the treatment of tissues with strong alkali, which is explained by its binding to proteins (Prins, Jeanlos, 1948; Shearer, Graham, 2004; James et al., 2008). The presence of two glycogen fractions in cells was also confirmed *in situ* with the use of cytochemical methods (Kugler, Wilkinson, 1961; Kudryavtseva et al., 1974). It has been established that the content of TCA-soluble and TCA-insoluble fractions of glycogen in tissues varies under different physiological and pathological conditions (Kudryavtseva et al., 1992; Adamo, Graham, 1998; Graham et al., 2001; Battram et al., 2004; Shearer, Graham, 2004; Shearer et al., 2005; Wilson, 2009; Granlund et al., 2011). Our results indicate that the changes in the content of these fractions reflect not only their important role in glycogen metabolism but also considerable rearrangements in the structure of its molecules. Data on the content of glycogen fractions in cells can provide valuable information about the dynamics of the number of glucose residues, the degree of filling of the tiers, the number of proteins involved in the attachment and detachment of glucose residues, and other characteristics of glycogen molecules under normal and pathological conditions (Bezborodkina et al., 2018).

An acute shortage of donor livers for transplantation to patients with terminal cirrhosis has stimulated the search for alternative approaches to the treatment of this disease such as artificial biological and non-biological constructions, transplantation of mature hepatocytes and human stem cells as well as xenogeneic organs and cells (Burra et al., 2004; Buzhor et al., 2014; Heydari et al., 2020; Mirdamadi et al., 2020). Potentially, these methods seem promising for LC therapy. However, they are still at the experimental stage and cannot be widely used in clinical practice. Therefore, the research aimed at enhancing the regenerative potential of the pathological liver itself is being actively conducted.

There is much evidence that the liver with cirrhotic changes can revert back to an almost normal state (Sarkisov, Rubetskoy, 1965; Powell et al., 1970; Solopaev, 1980; Dufour et al., 1997; Kaplan et al., 1997; Lau et al., 1999; Benyon, Iredale, 2000; Wanless, 2001; Kweon et al., 2001; Hammel et al., 2001). Studies on LC reversibility usually focus on morphological changes in the liver rather than the recovery of its functions. At the same time,

it is the restoration of the functional capacity of the pathological liver that is the key indicator of the reversibility of LC. This means that the analysis of the content and structure of glycogen, an important source of energy for various metabolic processes in hepatocytes, is of particular importance for assessing the recovery potential of the liver.

Based on the above, the **aim of the present study was to determine the role of cellular and macromolecular mechanisms of glycogenesis regulation in hepatocytes of the normal and the pathological liver of rats and humans.** To achieve this aim, the following tasks were set:

1. To determine the levels of glycogen in hepatocytes and the activity of key enzymes of glycogenesis and glycogenolysis in the normal and the pathological liver of rats and humans at different stages of the food cycle.

2. To establish the effect of hepatocyte size and ploidy on their glycogen content in the normal and the cirrhotic liver of rats and humans.

3. To evaluate the effect of comorbidity of liver cirrhosis and diabetes mellitus type 2 on glycogen content of human hepatocytes.

4. To develop a cytofluorimetric method for the study of the spatial structure of glycogen molecules (β-particles) in individual hepatocytes.

5. To determine the structure of glycogen molecules in hepatocytes of the normal and the cirrhotic rat liver at different stages of the food cycle.

6. To determine the structure of glycogen molecules in hepatocytes of the normal and the pathological liver of rats and humans.

7. To evaluate the degree of recovery of structure and function of the cirrhotic liver of rats and humans after different treatments.

Scientific novelty

We established the role of polyploidy and hypertrophy of hepatocytes in the regulation of glycogenesis in the normal and the cirrhotic liver of rats and humans. We also showed the effect of LC and 2 DM type comorbidity on glycogen content in human hepatocytes. An original quantitative method for the study of the spatial structure of glycogen molecules in individual cells of liver parenchyma was developed. The contribution of the number and size of glycogen molecules to its content in the hepatocytes in the normal and the cirrhotic liver of rats and humans was determined. The dynamics of the spatial structure of glycogen molecules

in the hepatocytes of the normal and the cirrhotic rat liver during glycogenesis was studied. The spatial structure of glycogen molecules in hepatocytes of the normal and the cirrhotic liver of rats and humans was described, and then assessed in rat and human hepatocytes during liver cirrhosis rehabilitation.

Theoretical and practical significance

The results of this study demonstrate that despite profound structural reorganisations in the cirrhotic liver, the glycogen content in the cells of its parenchyma is proportional to the gene dose, as in the normal liver. The ability of hepatocytes to store glycogen in the normal liver depends on the size and mass (weight) of the cells, while in the cirrhotic liver no such dependence is observed. On the one hand, our data indicate that normal regulation of transcription and translation processes is retained in the pathologically altered liver. On the other hand, they demonstrate a significant disturbance of the post-translation mechanisms during cirrhosis. This disturbance results in a significant increase in glycogen concentration and in changes in its structure and metabolism in hepatocytes of the cirrhotic liver. The increase of glycogen content in cells during progression of chronic hepatitis is an indicator of the severity of liver damage and is associated primarily with a decreased activity of glycogen phosphorylase *a* and glucose-6-phosphatase. Our data suggest that glycogen content of hepatocytes is increased in DM type 2. However, the comorbidity of LC and DM does not result in either additive or synergistic effects.

We developed an original method for the study of the spatial structure of glycogen molecules in individual hepatocytes. The method makes it possible to determine the number of glucose residues and the distance between them on the external tiers of glycogen molecules. Using this method, we found that glycogen molecules in hepatocytes of the cirrhotic liver are smaller than in the normal liver. However, their number in hepatocytes of the cirrhotic rat and human liver was several times higher than in the norm.

Treatment of LC after the removal of the damaging agent markedly reduces the glycogen content in hepatocytes and normalises the spatial structure of its molecules.

Methods and materials of the research

To achieve the tasks of the study, we employed a broad array of modern methods of cell biology such as: confocal laser microscopy, cytochemical methods, absorption and

fluorescence cytophotometry, micro-interferometry, electron microscopy, and image analysis. The material used in the study was represented by puncture biopsies of the liver of patients with chronic hepatitis, liver cirrhosis and diabetes mellitus type 2. White mongrel rats, Wistar rats, C57BL/6N mice and transgenic homozygous OE-NPY$^{D\beta H}$ mice were used as experimental models. Statistical data were processed using the standard Sigma Plot for Windows 11.0 software package (Systat Software Inc., Chicago, IL, USA).

Structure and volume of the thesis

The thesis is expounded on 265 pages of typewritten text and consists of introduction, literature review, description of materials and methods of the research, results and their discussion, conclusion and list of references containing 741 sources. The work is illustrated with 92 figures and 19 tables.

Personal contribution of the author consists in the independent determination of the aim, tasks and research plan of the thesis; collection of material. The author independently carried out the vast majority of the experiments (a few experiments were carried out jointly with colleagues and students). Interpretation, statistical processing and final evaluation of the obtained experimental data, collection and analysis of scientific literature, as well as the writing the text of the thesis were carried out by the author independently. In publications on the topic of the thesis N.N. Bezborodkina is either the first author or her contribution to the preparation of publications is prevalent.

Validity and approbation of the results

The validity of the results of this study is ensured by the abundance of experimental and clinical material, a careful experimental design and a detailed analysis of literature data. The results presented in the thesis were obtained with the use of modern, extensively tested and informative methods, certified equipment and high-quality reagents from reliable manufacturers and suppliers. They have been published in leading international and Russian peer-reviewed journals.

The data were analysed with the help of a complex of statistical methods. The results were processed with the use of suitable specialised programmes.

The results of the study were presented and discussed at more than 30 Russian and international scientific events, including: XIII International Congress of Pharmacologists (Munich, Germany, 1998); VI and VII Congresses of the European Society for Analytical Cell Pathology (Heidelberg, Germany, 1999; Caen, France, 2001); II Pharmacological Congress (Budapest, Hungary, 1999); XI International Congress of Histochemistry and Cytochemistry (York, England, 2000); XIII and XIV Heidelberg Symposium on Cytometry (Heidelberg, Germany, 2000, 2001); All-Russian Conference with international participation "Problems of morphology: theoretical and clinical aspects" (Sochi, 2002); Conference "Cell and tissue engineering of plants and animals" (Moscow, 2002); 21st Annual Conference of the German Society for Cytometry (DGfZ) (Bonn, Germany, 2011); II All-Russian Scientific Conference of Young Scientists "Problems of biomedical science of the third millennium" (St. Petersburg, 2012); XXVII and XXVIII Congresses of the International Society for the Advancement of Cytometry (ISAC) (Leipzig, Germany, 2012; San Diego, California, USA, 2013); 23rd Conference of the Asia-Pacific Association for the Study of the Liver (APASL) (Singapore, Singapore, 2013); III and IV Conferences of Young Scientists of the Institute of Cytology, Russian Academy of Sciences (St. Petersburg, 2012, 2014); XVII All-Russian Symposium "Structure and Functions of the Cell Nucleus" (St. Petersburg, 2014); VI International Scientific and Practical Conference "Current Problems of Biology, Nanotechnology and Medicine" (Rostov-on-Don, 2015); 30th Annual Congress on Clinical Cytometry (Denver, Colorado, USA, 2015); International Congress on Microscopy (Kottayam, Kerala, India, 2015); 38th and 41st Congresses of the Federation of European Biochemical Societies (FEBS) (St. Petersburg, 2013; Virtual, 2021); Conference dedicated to the 95th Anniversary of Academician K. Zufarov (Tashkent, Uzbekistan, 2021).

The list of publications on the topic of the thesis numbers 16 articles in peer-reviewed scientific journals included in the international reference databases and citation systems defined by the State Commission for Academic Degrees and Titles of the Russian Federation, 3 monographs in Russian, a chapter in an international collective monograph.

List of Publications

The main results on the topic of the thesis are presented in the following articles published in peer-reviewed journals included in the List of the State Commission for

Academic Degrees and Titles of the Russian Federation (VAK) and equivalent foreign journals:

1. Okovityi S.V., Arkad'eva A.V., Bezborodkina N.N., Sakuta G.A., Yaroslavtsev M.Yu., Shulenin S.N., Kudryavtsev B.N. New protective effect of simvastatin in rats with experimental steatohepatitis / S.V. Okovityi, A.V. Arkad'eva, N.N. Bezborodkina, G.A. Sakuta, M.Yu. Yaroslavtsev, S.N. Shulenin, B.N. Kudryavtsev // Experimental and clinical pharmacology. — 2007. — Vol. 70(3). — P. 43–45. (In Russian).
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Main scientific results

1. The development of liver cirrhosis in humans is accompanied by more significant impairment of liver functions and a greater loss of hepatocytes (>50%) than in rats (~28%). Polyploidisation and hypertrophy of cells during cirrhosis in rats almost completely compensate for the loss of hepatocytes, while in humans these processes only partially compensate for the loss of cells (Bezborodkina et al., 2021 — pp. 5–7 (personal contribution of the author 90 %)).

2. Glycogenesis in the hepatocyte population of the cirrhotic liver is characterised by a lower rate (by 22%) than in the normal liver. The glycogen content in the normal liver cells directly depends on their size, whereas during cirrhosis there is no such relationship. Nevertheless, during glycogenesis, the glycogen content in the hepatocytes in the normal and the cirrhotic liver remains proportional to the level of cell ploidy (Bezborodkina et al., 2013 — pp. 1084, 1089 (personal contribution of the author 90 %); Bezborodkina et al., 2016 — pp. 357, 361, 363 (personal contribution of the author 90 %)).

3. An original microfluorometric method was developed that makes it possible to determine the content of the labile (macroglycogen) and the stable (proglycogen) fraction of glycogen in one and the same cell and to obtain information on the structure of glycogen in individual cells, the order of its synthesis and degradation in the liver. The method is based on the use of PAS reaction with Schiff's reagents of different colours and FRET (Förster Resonance Energy Transfer) technique (Bezborodkina et al., 2009 — pp. 1027–1030, 1035 (personal contribution of the author 90 %); Bezborodkina et al., 2011 — pp. 420, 422–423 (personal contribution of the author 80 %); Chestnova et al., 2015 — pp. 135, 138–139 (personal contribution of the author 80 %)).

4. Glycogen content in hepatocytes of transgenic homozygous OE-NPYDβH mice with prediabetic phenotype corresponds to the normal values, being 28.53±0.97 and 30.87±0.98 standard units, respectively. However, the structure of glycogen molecules is significantly different from that of mice of wild type (WT) phenotype: 0.62±0.02 standard units and 1.63±0.02 standard units, respectively, $p<0.001$. Glycogen synthesis and accumulation in hepatocytes of OE-NPYDβH mice occurs predominantly via an indirect pathway, by gluconeogenesis, while in the liver of WT mice the direct pathway is used (Ailanen et al., 2018, pp. 6, 7 (personal contribution of the author 70 %)).

5. An increase in the content of glycogen in hepatocytes is accompanied by changes in the spatial structure of its molecules, which are expressed in a greater number of chains and tiers filled with glucose residues, as well as in an increase in the number of proteins bound to glucose residues on the surface of the molecules. At the same time, the outer layer of glycogen molecules in hepatocytes is never completely filled with glucose residues and always contains residues bound to proteins (Bezborodkina et al., 2021 — p. 6 (personal contribution of the author 80 %)).

6. Glycogen macromolecules (β-particles) are continuously synthesised and degraded in hepatocytes. Hepatocytes of starved rats contain 1.6 x 108 glycogen particles. Refeeding rats with glucose results in a rapid increase in the number of particles in the cytoplasm of hepatocytes. However, the main contribution to the increase in cellular glycogen content is the increase in the number of glucose residues in its molecules. Refeeding of rats with glucose for two hours leads to an approximately twofold increase in the size of glycogen molecules (Bezborodkina et al., 2022 — p. 7 (personal contribution of the author 90 %)).

The main theses for defence:

1. Mononucleate and binucleate hepatocytes of different ploidy in the normal and the cirrhotic liver do not differ in the rate of glycogen accumulation. In the normal liver, the degree of glycogen accumulation by hepatocytes depends on the cell size, while in the cirrhotic liver there is no such dependence.

2. Glycogen content in hepatocytes of the cirrhotic rat and human liver is significantly higher than in hepatocytes of the normal liver. The main causes of hepatocyte glycogenosis during cirrhosis are an impaired glycogenolysis and an increased gluconeogenesis.

3. An original microfluorometric method for the study of glycogen content and spatial structure of its molecules (β-particles) in individual hepatocytes was developed. It was shown that in hepatocytes of the normal rat liver the change in the content of total glycogen at the early stages of glycogenesis is caused by the synthesis of new molecules (β-particles), while at the later stages it is caused by the addition of glucose residues to the particles already present in the cells. In hepatocytes of the cirrhotic rat liver, the change in glycogen content during glycogenesis is due to the filling of the external tiers of β-particles. The increase in glycogen content in human hepatocytes during the development of liver cirrhosis is mainly due to the *de novo* formation of β-particles.

4. As cirrhosis progresses, the distance between chains of glucose residues on the external tiers of glycogen molecules increases in rat hepatocytes and decreases in human hepatocytes.

5. The distance between the external tiers of glycogen molecules decreases as they are being filled with glucose residues, which supports the hypothesis that the size of glycogen molecules is self-regulating.

6. Glycogen molecules in rat and human hepatocytes in the normal and the cirrhotic liver are smaller than in the normal liver, while their number is several times greater than in the normal liver.

7. The use of various treatments of cirrhosis does not lead to a complete restoration of the original lobular structure of the liver even after a long period of observation. However, treatment of cirrhosis after the removal of the damaging agent markedly reduces the glycogen content in hepatocytes and normalises the spatial structure of its molecules. Cirrhosis treatments leads to an increase in the number of glucose residues on the external tiers of glycogen molecules.

CHAPTER 1. LITERATURE REVIEW

1.1. Liver and its role in the mammalian body

The liver is the largest gland of mammals; its relative weight varies from 1.5% to 6% in different mammalian species (Prothero, 1982). The absolute weight of this organ varies greatly, for example, in the blue whale (*Balaenoptera musculus*) it reaches more than 1 tonne, in an adult human it is 1.2–1.8 kg, in rats and mice it is 8–10 g and 2–3 g, respectively, and in one of the smallest mammals, the pygmy shrew (*Sorex minutus*), it is only 0.18–0.31 g (Ivanter et al., 1985; Schmidt-Nielsen, 1987; Rogers, Dintzis, 2018).

The cell population of the liver consists of parenchymal and non-parenchymal elements. The latter are represented by sinusoidal (endotheliocytes and Kupffer cells) and perisinusoidal (Ito cells and Pit cells) cells. The main and the most numerous parenchymal cells of the mammalian liver are hepatocytes. They account for 60–80% of the total number of cells and 85–90% of the liver weight (Bioulac-Sage et al., 1999; Xu, Zhang, 2018). According to different estimates, the average number of hepatocytes in human liver is $100–250\times10^9$ cells (Bioulac-Sage et al., 1999; Arias et al., 2020), while in rats 1 g of liver contains from 1.96×10^8 (Knox, 1976) to 2.62×10^8 hepatocytes (Baidyuk, 2013).

The liver occupies a strategic position in the body. It performs up to 500 different metabolic functions, participating in the metabolism of proteins, lipids, vitamins, in bile secretion, detoxification, and pigment and mineral metabolism. It plays a central role in carbohydrate metabolism, maintaining a constant blood glucose concentration and regulating the accumulation and distribution of glucose in peripheral tissues. Some other organs can also store glucose in the form of glycogen, but only the liver can break it down and provide free glucose to the circulatory system (Kuntz, Kuntz, 2008; Chiang, 2014) (Fig. 1.1).

Metabolic pathways in the liver are under strict neurohumoral control (Reinke, Asher, 2016). The liver is innervated by the branches of the vagus nerve and the hepatic (sympathetic) plexus, while the hormones of the epiphysis, the adenohypophysis, the adrenal, the pancreatic and the thyroid gland are involved in the regulation of metabolic processes (Charni-Natan et al., 2019). The source of energy for the extremely energy-intensive tissue-specific functions of the liver is the aerobic oxidation of carbohydrates, fats and proteins derived from food or stored in various tissues (Rui, 2014). The main reserve substances in animals are glycogen and

triacylglycerides (fats). The complete oxidation of the latter produces significantly more energy in the form of ATP than oxidation of glycogen. However, glycogen has two advantages. It can be quickly mobilised for the metabolic needs of the organism and, what is even more important, can be the source of energy even under anoxic conditions.

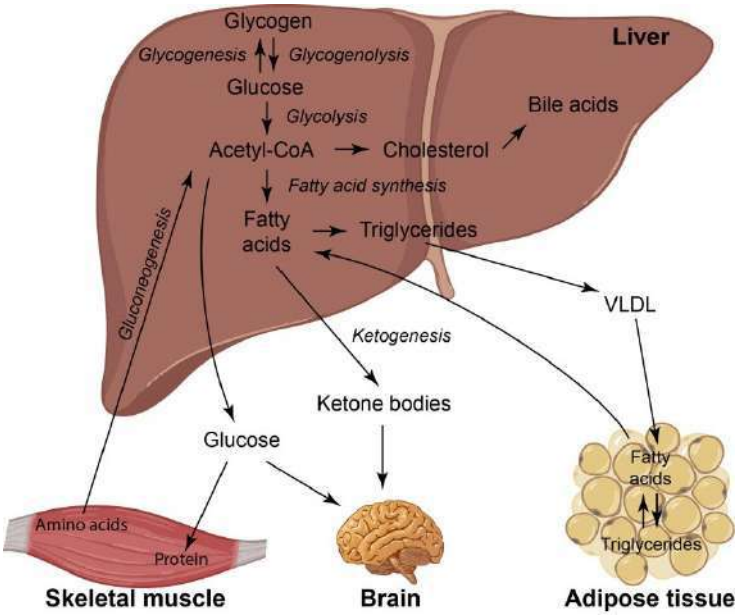


Figure 1.1 — Main metabolic functions of the liver.

1.2. Glycogen and its role in various tissues and cells of mammals

Glycogen is an easily accessible and universal source of energy for metabolic processes in living beings with various levels of organisation, from primitive heterotrophic prokaryotes (archaea and bacteria) to higher eukaryotes such as mammals (Ball et al., 2011). Glycogen or, as it was formerly called, animal starch, was first obtained and described by the French physiologist Claude Bernard. He found that when ethanol and an excess of glacial acetic acid were applied to the homogenate of fresh liver tissue glycogen was precipitated as a white substance, while its complete hydrolysis resulted in the formation of glucose (Bernard, 1857). Later it has been shown that glycogen is found in almost all the tissues but is present in the highest concentration in the liver, where it makes up 5–7 % of the total weight. The total glycogen content in the liver is approximately 0.4 g in the rat and 70–100 g in humans (Wasserman, 2009; Zois, Harris, 2016). Glycogen is also present in significant amounts in skeletal muscles. Although its concentration in them is much lower than in the liver, the total

amount is higher (Zois, Harris, 2016). For example, the total amount of glycogen in skeletal muscles in humans reaches 400–500 g (Wasserman, 2009; Jensen et al., 2011).

Glucose is stored in cells as a polymer because of the low osmolarity of this form. The glycogen reserve in the hepatocyte roughly corresponds to a glucose concentration of 0.4 M, but the actual concentration of insoluble glycogen in the cell is only 0.01 μM. Should the cytosol contain 0.4 M of glucose in its monomeric form, which has a high osmolarity, water movement into the cell might have caused cell lysis. Furthermore, at an intracellular glucose concentration of 0.4 M and a glucose concentration of 5 mM in mammalian blood, the change of free energy corresponding to the transport of glucose into the cell against an ~80-fold concentration gradient would have been prohibitive (Nelson, Cox, 2012).

Owing to its ability to be easily broken down to glucose and rapidly stored in cells, glycogen plays an extremely important role in human and animal life.

Firstly, during the late stages of mammalian embryogenesis, a lot of glycogen is accumulated in the organs of the foetus, especially in the liver, and is then used within the first hours after birth for the energy needs of the newborn (Doljanski, 1960; Kalhan, 2017). For human and animal embryos glucose is an energy source of paramount importance (Kalhan, Parimi, 2000; Dowling, McAuliffe, 2013). Its plasma concentration is maintained at a constant level by transport across the placenta from the mother's body (Mota-Rojas et al., 2011). Birth and the following several days are a critical stage in the life of mammals, and the accumulation of a large amount of glycogen in the liver before birth is one of the most important adaptive mechanisms (Hers, 1981; Arshavsky, 1982; Gruppuso, Brautigan, 1989).

Secondly, glycogen plays an important role in muscle contraction. Muscles of various functional purposes constitute up to 40–45% of the body weight of mammals (Frontera, Ochala, 2015), while the glycogen content in them reaches 0.5–2% of the muscle wet weight (Rosenfeld, Popova, 1989).

Thirdly, it is vitally necessary for the body to maintain blood glucose concentrations within a narrow physiological range required for the normal functioning of the nervous tissue, blood cells and testes. In fed state and in the postabsorptive period, the brain operates almost exclusively on glucose, the rate of its utilisation being estimated at 117–142 g/day (Nuttall et al., 2008). Another reason why the level of glucose in the blood should be strictly regulated is its toxicity. Modifications of proteins caused by their glycosylation (Degenhardt et al., 1998), glucose-induced oxidative cell damage (Huebschmann et al., 2006), and other glucose-

mediated harmful effects are particularly clearly manifested in people with severe forms of diabetes (Cleary et al., 2006).

1.3. Regulation of the glucostatic function of the liver and glycogen metabolism

Five phases of glucose homeostasis can be identified depending on the nutritional status of the organism (Ruderman et al., 1976) (Table 1.1). In absorptive **phase I** the level of glucose and insulin in the blood increases, while that of glucagon decreases. As a result, glucose formed in the intestine during carbohydrate digestion is absorbed: 55% by the liver, 25% by insulin-independent tissues (brain, nerves, blood cells, renal medulla and epithelium of testes) and 15% by insulin-dependent tissues (adipose tissue, skeletal muscles) (Tepperman, Tepperman, 1989). In postabsorptive **phase II,** glucose, insulin, and glucagon levels return to the baseline. The liver starts to produce glucose, which is to be used mainly by the brain, kidneys, red blood cells, adipose tissue, and skeletal muscle. A typical postabsorptive state in humans is the state of the body after a night's sleep. If no food is ingested for a day or more, the state of starvation (**phases III–V**) sets in. At all stages of starvation, due to depletion of glycogen stores, the only source of glucose is gluconeogenesis in the liver and kidneys (Ercan et al., 1994) (Table 2.1)

Table 1.1 — Phases of glucose homeostasis.

	Phase I	**Phase II**	**Phase III**	**Phase IV**	**Phase V**
Source of glucose	Exogenous carbohydrates	**Glycogenesis in the liver** Glycogen	**Gluconeogenesis in the liver** Glycogen	Gluconeogenesis in liver and kidneys	Gluconeogenesis in liver and kidneys
Tissues/ organs using glucose	ALL (liver takes up about 50% of glucose after a meal)	ALL (to a lesser extent: liver, muscle and adipose tissue)	ALL (to a lesser extent: liver, muscle, adipose tissue)	Brain, red blood cells, kidneys, muscle (small amounts)	Red blood cells, kidneys, brain (to a lesser extent)
Main source of energy for the brain	Glucose	Glucose	Glucose	**Glucose** Ketone bodies	**Ketone Bodies** GLUCOSE
Timing of the start	3-4 h after a meal	6-12 h after a meal	Starvation for 1 day	Starvation for up to 24 days	Starvation for more than 1 month

Thus, blood glucose level at different stages of the food cycle represents a balance between the intensity of glucose consumption by the peripheral tissues and the ratio of the rates of glycogen synthesis and breakdown in the liver.

Glycogen can be synthesised directly (glycogenesis) from blood glucose entering the cells and indirectly (gluconeogenesis) from non-carbohydrates. Degradation of glycogen (glycogenolysis) in the organism occurs by hydrolysis or phosphorolysis. Glycogen can be used in the course of aerobic or anaerobic glycolysis for the needs of the tissue itself or for glucose production as well as, via the pentose phosphate pathway, for the formation of NADPH-H and pentose phosphates necessary for the synthesis of fatty and nucleic acids (Fig. 1.2).

In most mammalian organs and tissues only some units of reactions associated with glycogen metabolism take place. For example, in skeletal muscles (Przybylski et al., 2006), the heart (Chandramouli et al., 2015), the brain (Rojas, Schwartz, 2014) and red blood cells (Moses et al., 1972) glycogen metabolism is directed towards glycolysis, whereas in the kidney cortex (Segura, Ruilope, 2013) and the small intestine (Mithieux, Gautier-Stein, 2014) the direction is towards glycogen synthesis via gluconeogenesis. The only mammalian organ with the complete set of glycogen metabolism is the liver. At the same time, the liver usually does not utilise the glucose formed during glycogenesis for its own needs but produces it for consumption by other organs.

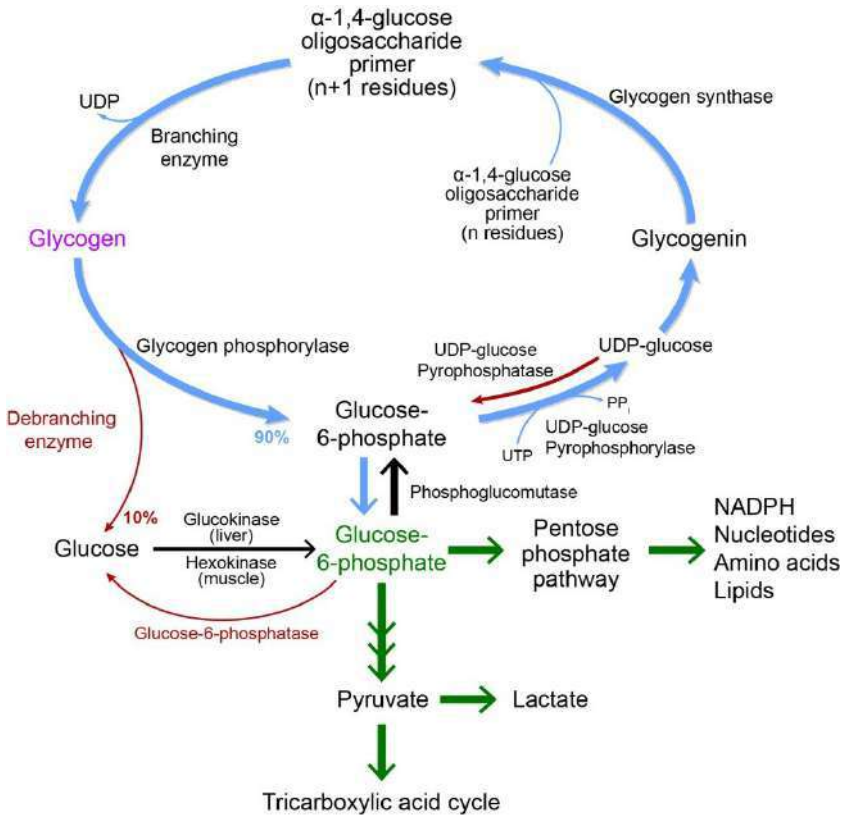


Figure 1.2 — General scheme of glycogen metabolism (Zois, Harris, 2016).

The basic scheme of reactions of glycogenesis and glycogenolysis has been described in detail (Bender, Mayes, 2015; Zois, Harris, 2016). This scheme is very similar in different tissues differing only in some slight details.

Glycogen synthesis begins with the attachment of UDP-glucose to glycogenin, a protein primer; the acceptor of the transferred glucose residue is glycogenin itself (Lomako et al., 2004; Chaikuad et al., 2011). In humans this protein is encoded by two genes, GYG1 and GYG2. The former is expressed in all the tissues, while expression of the latter is restricted to the liver, the heart and the pancreas (Henrissat, Davies, 2000). In contrast, rodents have only one glycogenin-encoding gene, GYG1 (Zhai et al., 2001).

Further formation of a complete glycogen molecule occurs with the participation of glycogen synthase (GS), branching enzyme (BE) and other enzymes after the self-glycosylation of glycogenin is completed. It has been suggested that glycogen is never completely broken down in cells and that its synthesis can begin immediately, on pre-existing glycogenin (Calder, Geddes, 1992). Glucose residues are transferred by GS to the non-reducing end of the oligosaccharide formed on the glycogenin and are linked together by α-1

→ 4 glycosidic bonds (Figure 1.3). As a result, a polysaccharide chain is formed, while the glycogenin remains inside the glycogen molecule.

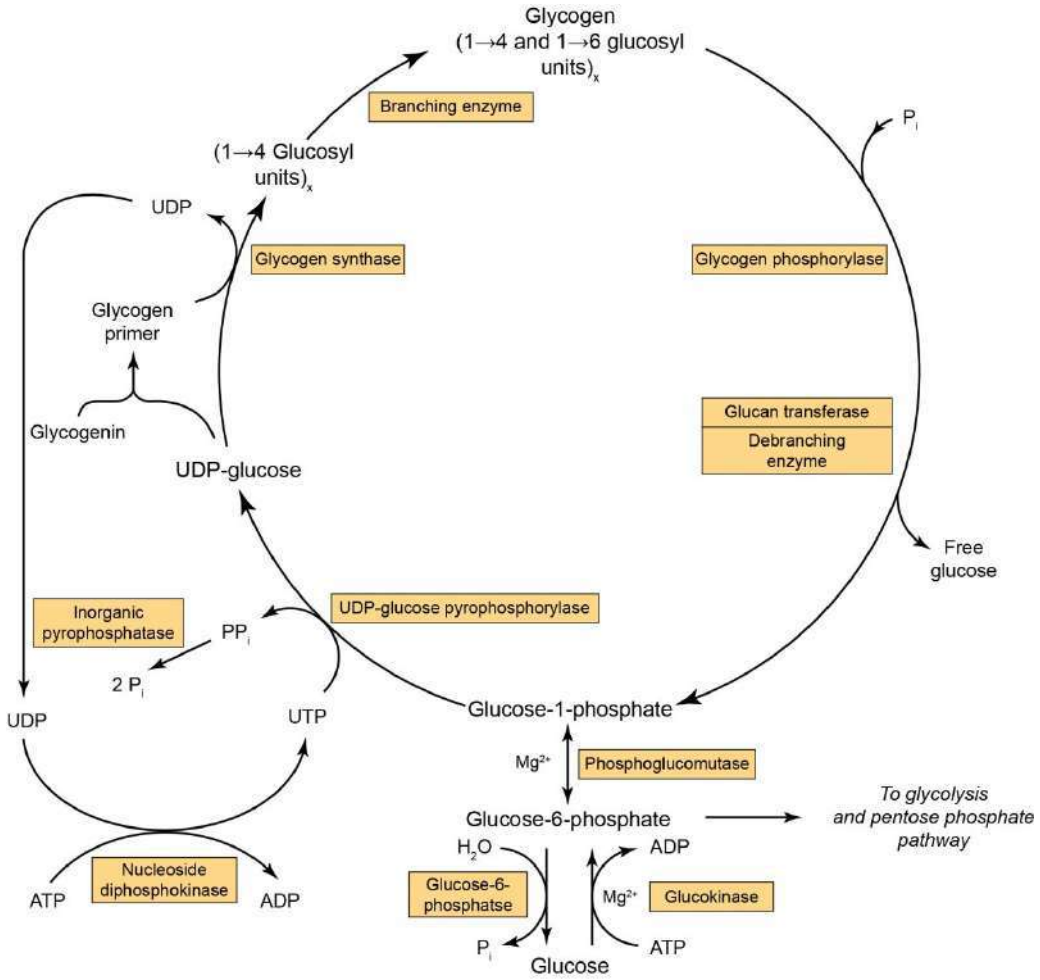


Figure 1.3 — Pathways of glycogenesis and glycogenolysis in the liver (by Bender, Mayes, 2015).

Mammals possess two GS-encoding genes: GYS1 is expressed in skeletal muscles and in most cells capable of glycogen synthesis, while GYS2 is expressed only in the liver (Qian et al., 2014). GS can be in an inactive phosphorylated state (b-form or I-form) and in an active non-phosphorylated state (a-form or D-form). Glucose-6-phosphate (G6P) can overcome the inactivation of the enzyme associated with phosphorylation and fully restore its activity. Owing to this property, it is possible to determine GS activity in the presence and in the absence of G6P and to estimate the degree of phosphorylation of this enzyme (Aiston et al., 2003). An additional mechanism of GS activity regulation is associated with changes in its intracellular localisation. It has been shown that incubation of hepatocytes isolated from the

liver of starving rats with glucose not only activates GS but also promotes its transition from a diffuse distribution in the cytoplasm to concentration in the cell periphery (Jurczak et al., 2008). This fact is in good agreement with the data that glycogen synthesis starts near the cell membrane of the hepatocytes; as glycogen accumulates, it moves from the cell periphery to the inner parts of the cell (Fernandez-Novell et al., 2002).

GS catalyses the transfer of glucoside residues only to a linear section of the chain. Once the number of linearly arranged glucose residues reaches 11, the chain begins to branch with the participation of the branching enzyme (BE). It forms α-1,6-glucoside bonds and results in the formation of numerous terminal monomers. Owing to this branching structure, glycogen molecules are compact and the rate of glycogen synthesis and degradation is significantly increased (Fig. 1.4, *a*).

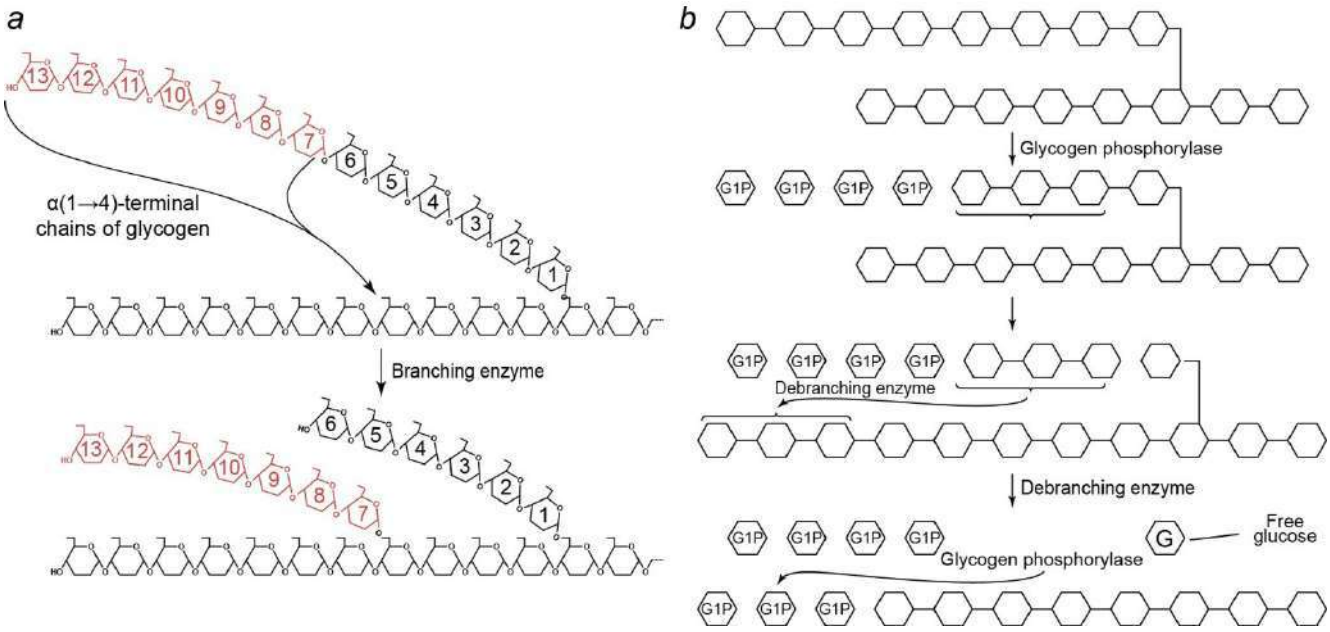


Figure 1.4 — (*a*) Mechanism of action of the branching enzyme. Branches are formed by the transfer of approximately seven terminal glucose residues from the linear section of a chain to the C6-OH group of the glucose residue of another chain. A new branching point is formed at a distance of at least 4 residues from any other existing branching point. (*b*) Mechanism of action of the debranching enzyme (Takusagawa F., 2010).

As already mentioned, glycogen degradation in cells can proceed along the phosphorolytic and along the hydrolytic pathway. The latter is effected by glucosidases. The best known glucosidase is α-amylase of the pancreas and the salivary glands, which cleaves α-1,4-glycosidic bonds. Glycogen can also be hydrolysed in lysosomes. This pathway has attracted scientific attention because of Pompe disease, associated with a mutation in the gene

of the lysosomal α-glycosidase (Hirschhorn, Reuser, 2000) and manifested in the accumulations of huge amounts of glycogen in lysosomes and vesicular structures (Fukuda et al., 2006).

Phosphorolysis of glycogen begins with the formation of glucose-1-phosphate. This reaction is catalysed by glycogen phosphorylase (GP), the key enzyme of glycogenolysis (Fig. 1.3). Its molecular structure, though complex, is now understood fairly well. It is known that the biological activity is characteristics of the dimer of GP. The transition of GP from the inactive to the active state occurs due to the addition of AMP and is accompanied by a change in the quaternary structure of the enzyme (Rath et al., 2000; Pautsch et al., 2008). During inactivation, GP, as well as GS, moves from the soluble fraction to the so-called "particle fraction" (Green et al., 2004).

GP can only cleave linear chains (α-1,4-bonds) of glycogen. As soon as approximately 4 glucose residues remain before the α-1,6 branching site, its action stops and the debranching enzyme (DBE) starts to work. The latter enzyme, which possesses transferase and 1,6-glucosidase activity, straightens the chain of glucose residues at the branching sites (Fig. 1.4, *b*). The catalytic units of DBE can act independently one from the other, which suggests the presence of multiple active centres, including those for glycogen binding (Zhai et al., 2016). Although DBE was discovered almost 50 years ago, its three-dimensional structure and the molecular basis of its function are poorly understood.

Eventually, through the combined action of DBE and GP, glycogen is cleaved to G1F, which is transformed into G6P under the impact of phosphoglucomutase. It is G6P that is involved in many metabolic pathways or converted into free glucose entering the blood.

To maintain a constant physiological blood glucose concentration, liver glycogen is used mainly between meals. It is believed that about 70–75% of glucose is formed by glycogenolysis in the postabsorptive period in humans (Kruszynska, 1999). During prolonged starvation, glycogen stores in the liver are depleted down to a few per cent of the initial level (Rothman et al., 1991). Under these conditions, glucose is supplied to tissues almost entirely by gluconeogenesis, which proceeds in the liver and, to a lesser extent, in the renal cortex, adrenal glands, and the small intestine (Mithieux et al., 2004; Watford, 2005). The sources of glucose synthesised *de novo* by the liver through gluconeogenesis are lactate, pyruvate, glycerol and some amino acids.

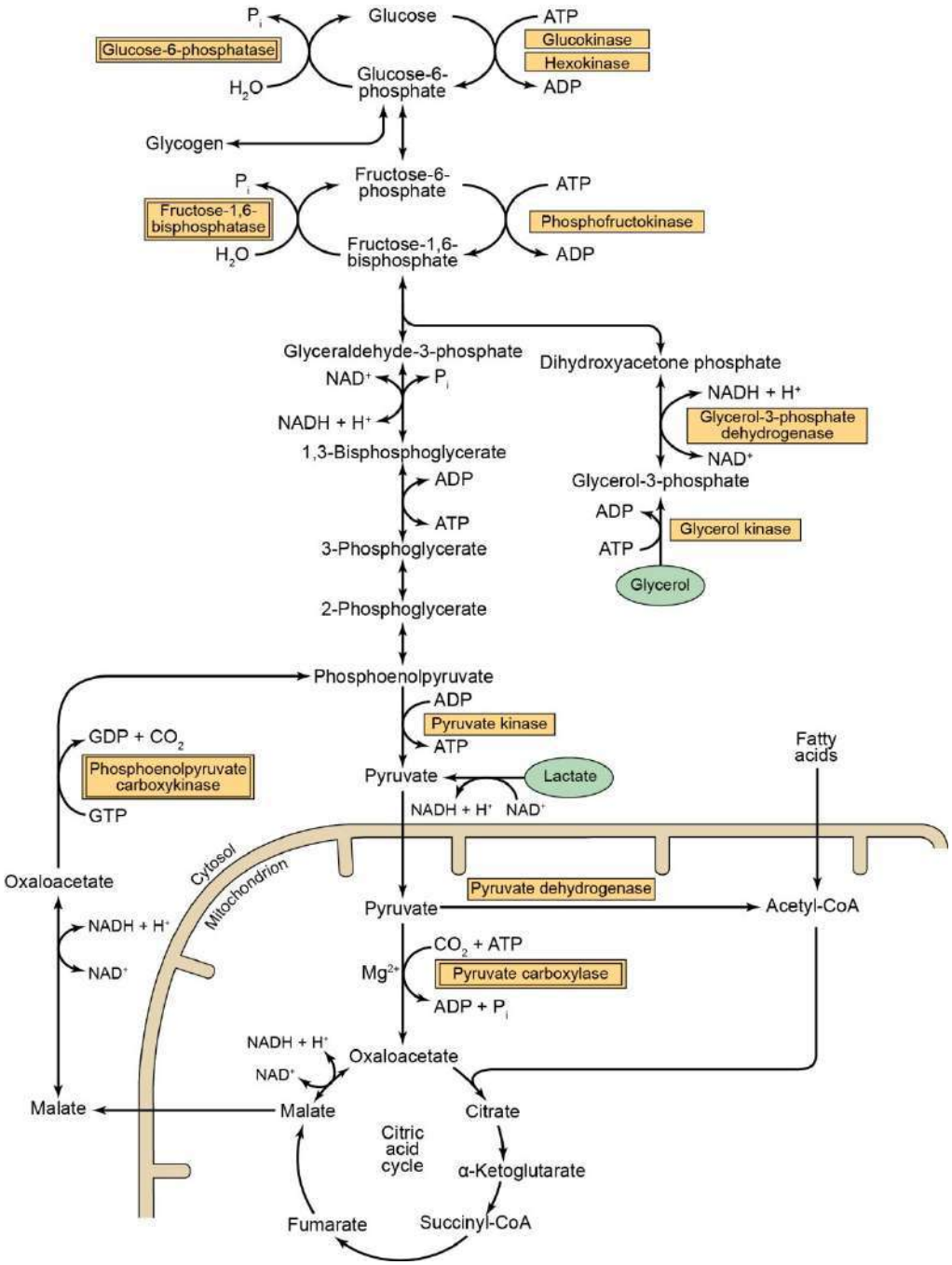


Figure 1.5 — A scheme of gluconeogenesis and glycolysis in the liver. The key gluconeogenic enzymes are enclosed in double boxes. ATP required for gluconeogenesis is derived from oxidation of fatty acids (by Bender, Mayes, 2015).

Almost all reactions of gluconeogenesis are reversals of glycolysis reactions and are catalysed by the corresponding glycolytic enzymes. However, since some of these reactions are irreversible, gluconeogenesis also involves some ‘bypass’ reactions of its own. One of the main steps of gluconeogenesis is the terminal reaction catalysed by glucose-6-phosphatase (G6Pase) (Fig. 1.5).

This enzyme occupies a strategic position in the regulation of blood glucose level. It is known that hepatic G6Phase has a molecular mass of 60–70 kDa (Reczek, Villee, 1982), consists of 5 basic polypeptides (Burchell, 1982) and is present (in mammals) in the microsomal fraction (De Duve et al., 1951). It has been shown that G6Phase is also found in kidneys, the intestine, the pancreas, adrenal glands, testes and the spleen, but its activity in these organs is low as compared to the liver (Burchell, Hume, 1995; Rajas et al., 1999; Croset et al., 2001).

In cells, G6Pase is localised predominantly on the inner side of the RER membranes, while its substrate is located in the cytosol (Waddell, Burchell, 1991). It was shown with the use monoclonal antibodies that G6P is transported across the membrane by a specific translocase T1, while glucose and Pi released during the substrate hydrolysis are transported to the cytosol by translocase T2 and translocase T3, respectively (Fig. 1.6) (Rodwell et al., 2015).

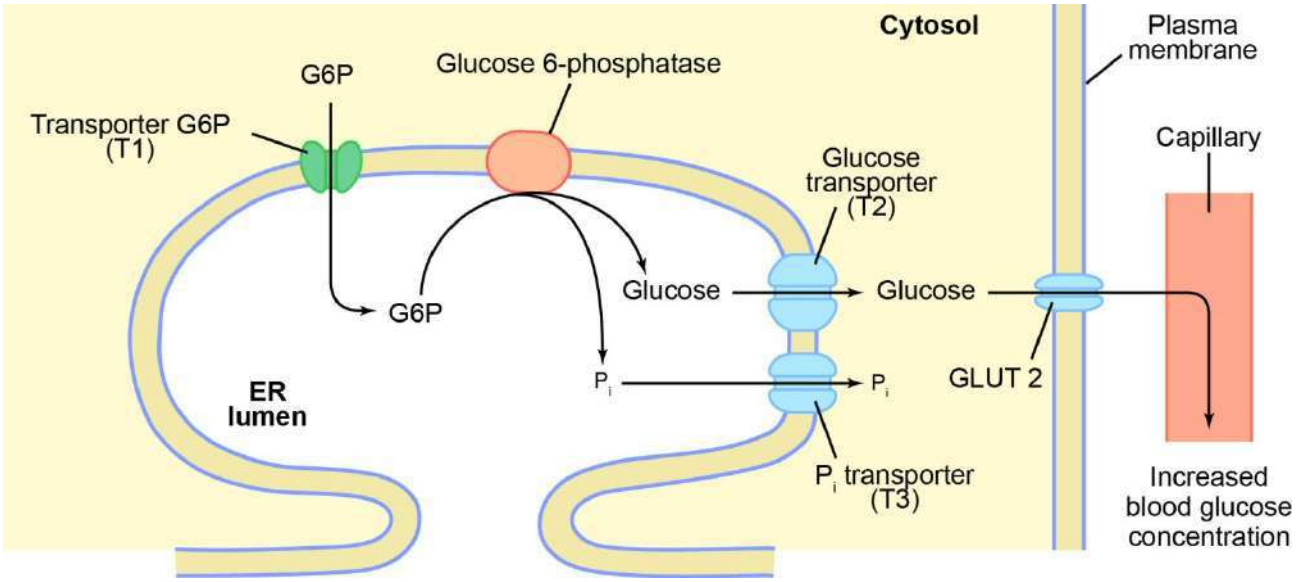


Figure 1.6 — Hydrolysis of G6P. The catalytic site of glucose-6-phosphatase faces the lumen of the rough endoplasmic reticulum (RER). GLUT 2 — glucose transporter (by Rodwell et al., 2015).

G6P is formed at the first stage of glycolysis, one of the most important as well as the most ancient metabolic processes in living organisms, under the action of hexokinase (Fig. 1.5). Many mammals, including humans, have four isoforms of hexokinase (Lowes et al., 1998; Coerver et al., 1998; Ehsani-Zonouz et al., 2001), but only hexokinase IV or glucokinase (GK) has a low affinity for glucose (Km about 20 mmol/l), is not inhibited by G6F, and functions efficiently at glucose concentrations above 1 mg/ml (Cornish-Bowden, Cardenas, 1991). Its share in the total hexokinase activity differs in different mammalian species: e.g., it

is 84–91 % in the liver of rats, mice, and hamsters (Ureta et al., 1971; Reyes, Cardenas, 1984) and only 40% in the human liver (Lowes et al., 1998).

In hepatocytes, GK forms a complex with the regulatory protein GKRP, which acts as a competitive inhibitor of glucose (Arden et al., 2005) and is the most important post-transcriptional regulator (Roncero et al., 2009). The loss of GCKR reduces both the amount of GK and its activity (Grimsby et al., 2000).

It has been shown that the absence of GK in the human liver leads to the development of mild diabetes (Malecki, Klupa, 2005). However, physiologically low GK activity in the liver does not lead to diabetes in ruminants, cats and birds. This is probably due to a different diet and also to the fact that these groups of animals obtain the energy in such a way that excess glucose can be removed from the circulation without an obligatory participation of GK (Ureta, 1982; Aschenbach et al., 2010; Polakof et al., 2011; Verbrugghe et al., 2012).

Most data on glucostatic function, metabolism and glycogen content have been obtained without taking into account the characteristics of the tissue structure of the liver and the structure of its cell population. As a rule, the studies were conducted on tissue homogenates, or separate cells and organelles, or isolated enzymes, or else by determining the concentration of metabolites in the inflowing and outflowing blood, etc. Meanwhile, the liver is an organ with a highly organised metabolism, a distinct histological structure and a cell population in which each cell type has a strict localisation and performs a specific function.

1.4. Influence of tissue and cell factors on metabolism and content of glycogen in the liver

All metabolic pathways associated with glycogen accumulation and degradation are confined to hepatocytes. As a cell type, they arise from progenitor cells (hepatoblasts) in the early embryonic period: in humans approximately on the 18^{th} day and in rats, on 10^{th}–11^{th} day of prenatal development (Zhao, Duncan, 2005; Shin, Monga, 2013) (Fig. 1.7).

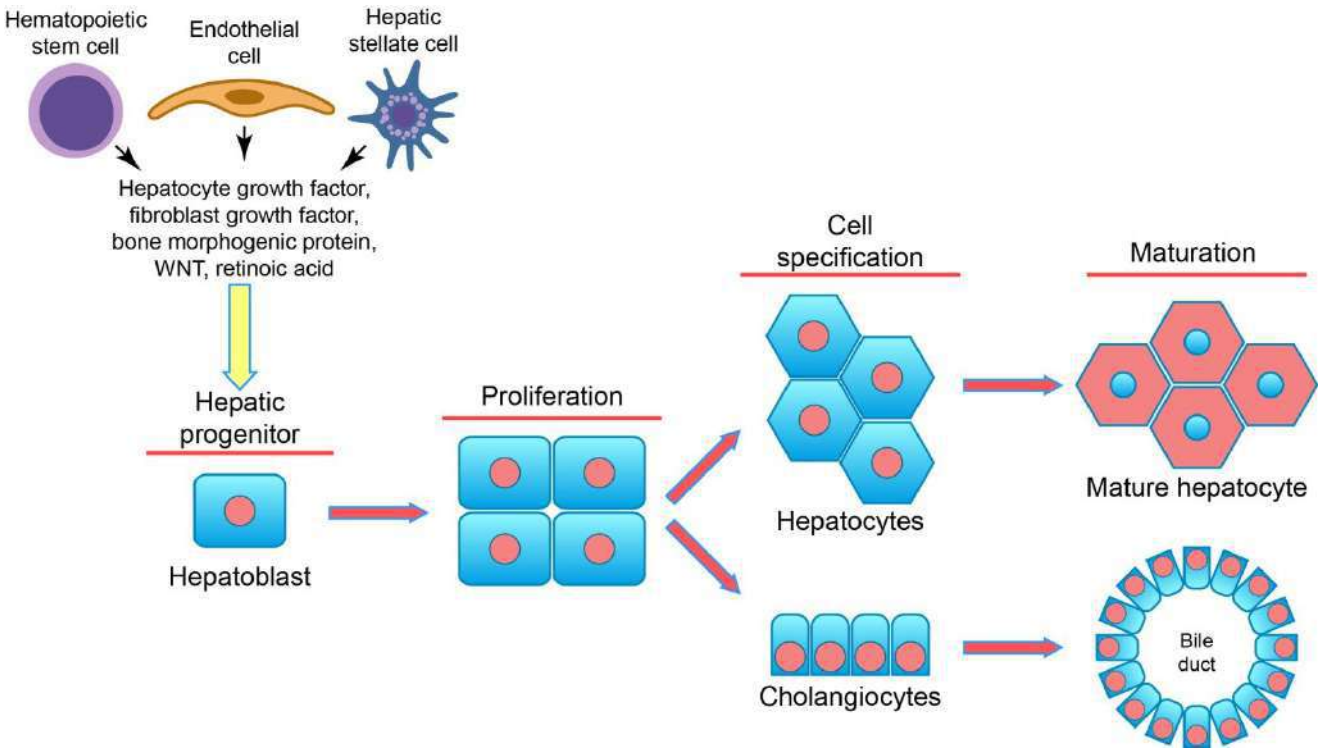


Figure 1.7 — A scheme of liver morphogenesis. Hepatoblasts, which are bipotential stem cells, differentiate into hepatocytes or cholangiocytes (biliary epithelial cells). In the course of morphogenesis, these cells mature, acquire additional characteristics such as polarity and become capable of performing the key functions of the liver.

The embryonic liver grows fast due to the high proliferative activity of hepatocytes (Zavarzin, 1967), but their mitotic activity rapidly decreases after birth. In adult rats, the number of hepatocytes in the DNA synthesis phase make up as little as a fraction of one percent (Stöcker et al., 1972). As the proliferative activity of hepatocytes declines, they undergo polyploidization, which is particularly intense during the first 3–4 weeks after birth, when the young rats switch from suckling to independent feeding on carbohydrate-rich food (James et al., 1979; Brodsky, Uryvaeva, 1981; Bogdanova et al., 1990). It is believed that polyploidization is carried out by alternation of acytokinetic mitoses and bimitoses; as a result, the liver of adult rats contains mononuclear and binuclear hepatocytes of different ploidy classes: 2c, 2c×2, 4c, 4c×2 and 8c (Brodsky, Uryvaeva, 1977; Donne et al., 2020) (Fig. 1.8).

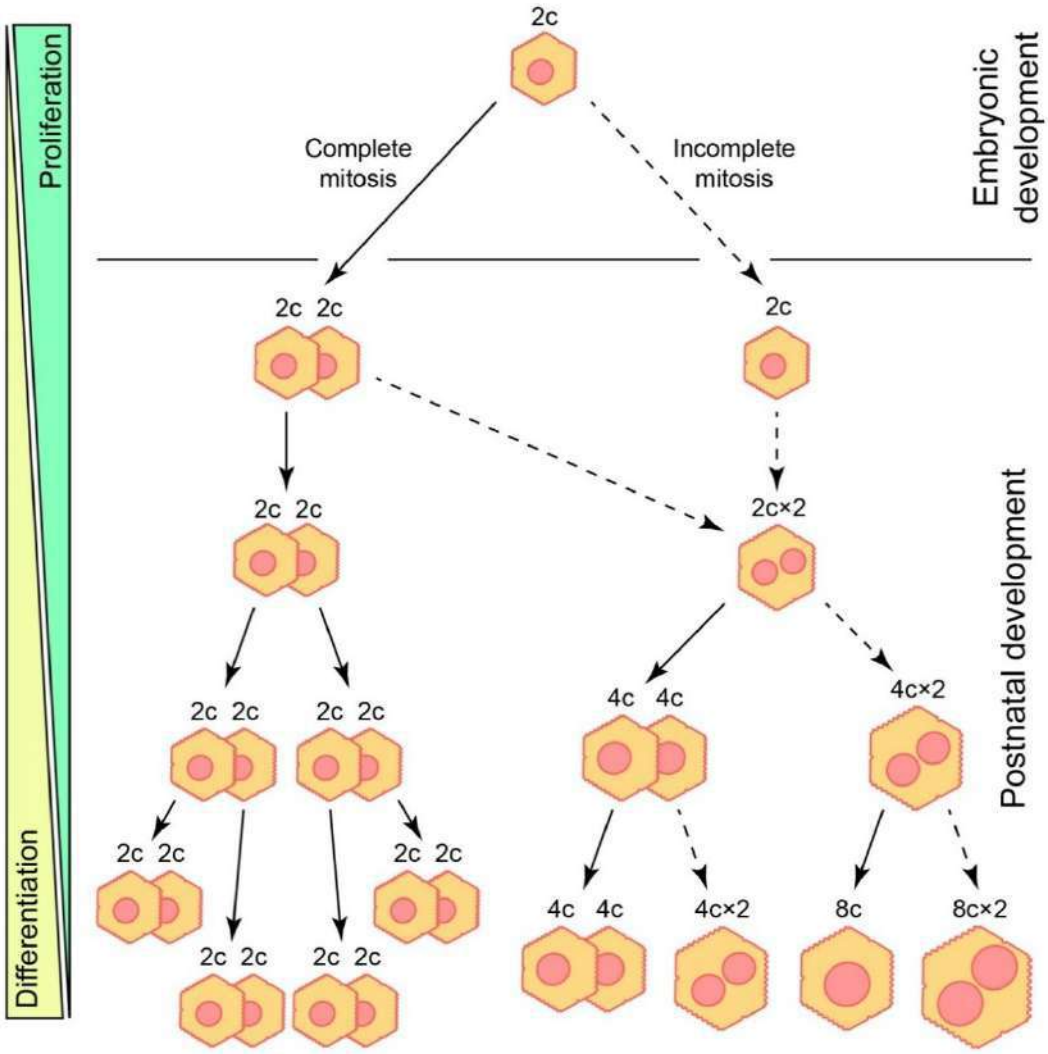


Figure 1.8 — Formation of hepatocytes of different ploidy in rat liver (Okovity et al., 2022).

In the liver of adult rats, mononuclear tetraploid hepatocytes are the most numerous, accounting for ~80% of the population of parenchymal cells (Bogdanova et al., 1990; Sakuta, Kudryavtsev, 2005). Hepatocytes of higher ploidy are rare, arising during aging or in case of liver pathologies. In contrast, in adult humans 80–90% of hepatocytes of the normal liver are represented by mononuclear diploid cells. It is only after the age of fifty that the number of polyploid hepatocytes in humans becomes noticeable, however, even at the age of 90, almost 60% of the hepatocytes are mononuclear diploid cells (Kudryavtsev et al., 1982; Watanabe et al., 1984; Kudryavtsev et al., 1993).

In addition to cell factors, glucose and glycogen metabolism in hepatocytes depends on the characteristic structure of the liver parenchyma, which is due to its unique blood supply (Jungermann, 1987). The liver simultaneously receives arterial and venous blood from, respectively, the hepatic artery and the portal vein (Fig. 1.9).

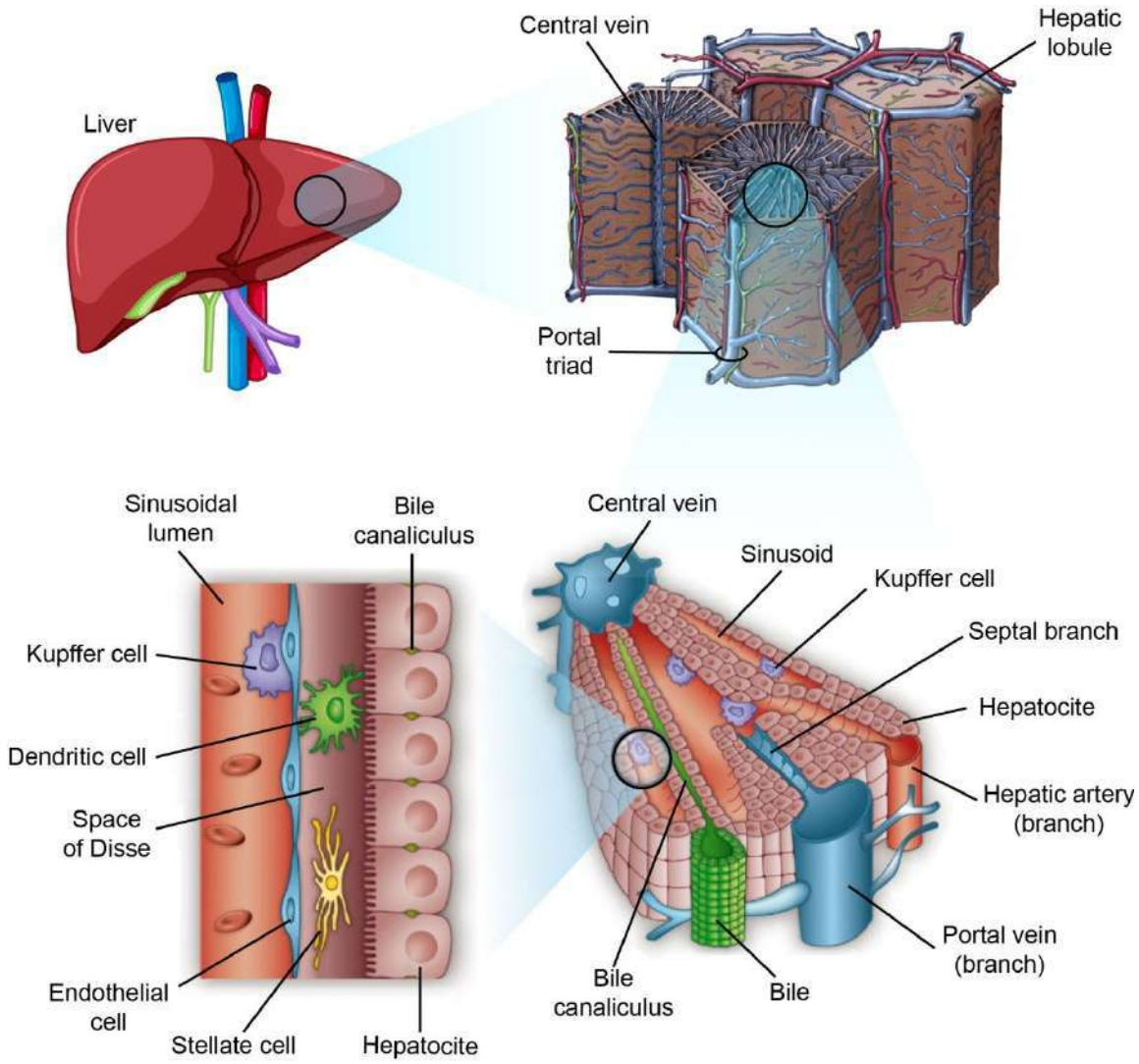


Figure 1.9 — Microscopic structure of the liver (a scheme) (Okovity et al., 2022).

The hepatic lobule is traditionally considered to be the basic morphofunctional unit of the liver. There are several views on its structure reflecting different aspects of its organisation and function (Sasse et al., 1992; Romert et al., 1993; Teutsch, 2005). According to the classical view, a lobule is shaped as a hexagonal prisms. Portal tracts with the branches of the portal vein, the hepatic artery, the bile ducts, the lymphatic vessels and the nerve fibres are located at the corners of their bases. In the centre of the lobule the central vein is located, through which blood flows to the vessels carrying blood to the inferior vena cava and, further, to the right ventricle of the heart (Fig. 1.9, 1.10, *a*). Trabeculae consisting of about 20 hepatocytes are located around the central vein (Fig. 1.10). In many animal species, the radial direction of the trabeculae in the lobules is often obscured by the anastomoses between them.

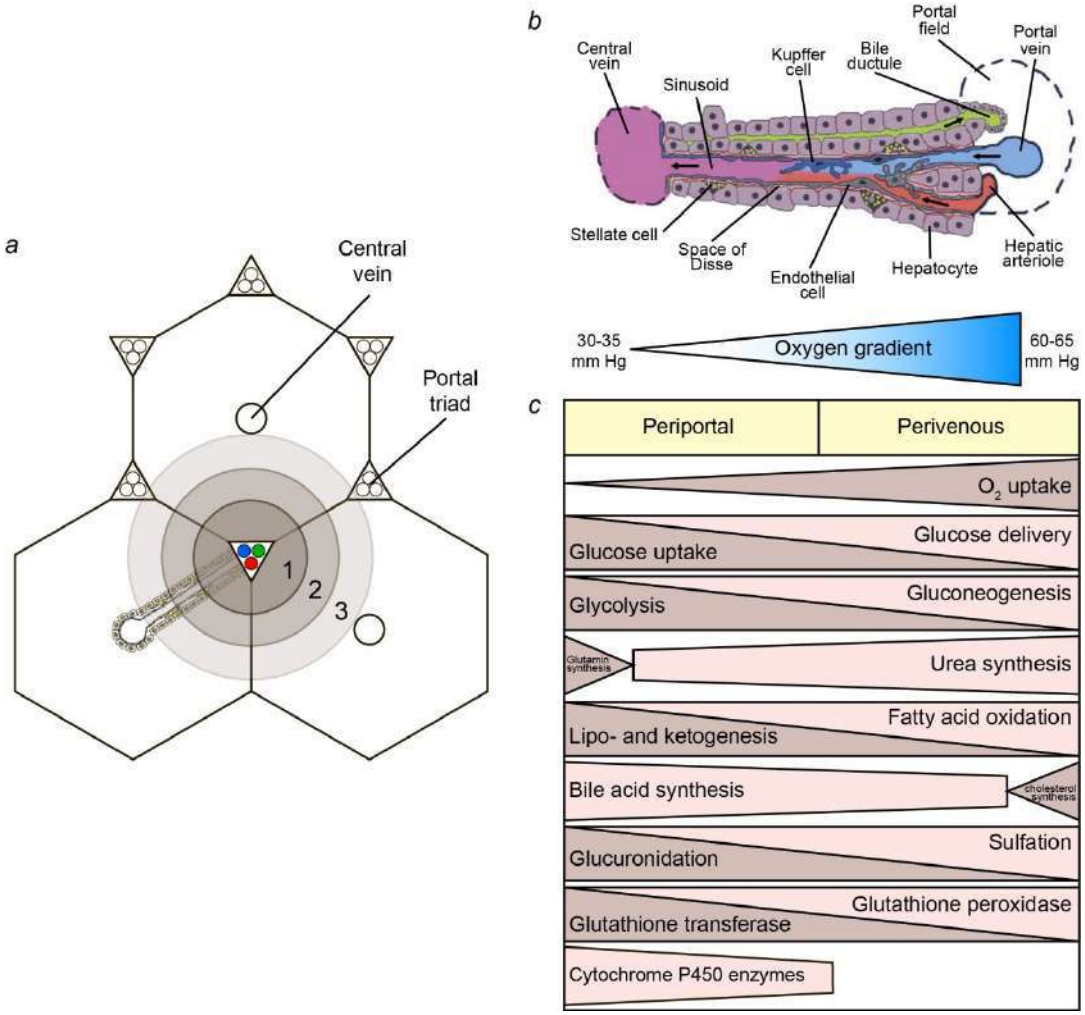


Figure 1.10 — Liver microstructure, oxygen gradient and metabolic zonation. *a* - Classic liver lobule. Branches of the portal vein (blue dot), hepatic artery (red dot), bile duct (green dot). The acinus and its three zones. 1 - periportal zone; 2 - intermediate zone; 3 - perivenous zone. *b* - Liver sinusoid and oxygen gradient. *c* - Functional activity of the hepatic acinus depending on oxygen zonation (after Kietzmann, 2017; Lee-Montiel et al., 2017).

The term "acinus" is often used to describe the structure of the liver. The acinus is formed by segments of two adjacent classical lobules (Fig. 1.10, *a*). The blood supply of the acinus, unlike that of the lobules, is from the centre to the periphery (Rappaport, 1981). Three zones are distinguished in the acinus: the periportal zone, supplied with blood with a high oxygen content, the intermediate zone and the perivenous zone. The latter is adjacent to the central vein and receives blood which has a low oxygen concentration but is enriched with the end products of cell metabolism (Kietzmann, 2017) (Fig. 1.10, *a*). Zones 1, 2 and 3 of the hepatic acinus correspond to the portal, the intermediate and the central zone of the classical lobule.

Oxygen pressure in the periportal zone exceeds that in the central zone 3.1–3.5-fold (Kietzmann, 2017; Lee-Montiel et al., 2017) (Fig. 1.10, *b*). Periportal hepatocytes, being more

“aerobic”, have a larger mitochondrial volume (20% v/v) and the area of the cristae (33 000 μm^2) as compared to the cells in the pericentral zone, whose mitochondrial volume is 12% v/v and the total area of the cristae, 23 000 μm^2 (Arias et al., 2020). As a consequence, periportal hepatocytes have a higher respiration rate and oxidative phosphorylation (Sell, 2001).

The concentration gradient of oxygen, substrates and hormones between different zones of the liver lobule obviously influences glucose and glycogen metabolism in the hepatocytes located in these zones (Fig. 1.10, *c*). Periportal cells have a higher capacity for glucose synthesis by gluconeogenesis. Accordingly, they have higher activities of G6Pase (Teutsch et al., 1999), fructose-1,6-biphosphatase (Schmidt et al., 1978) and phosphoenolpyruvate carboxykinase (Wimmer et al., 1990). An intensive glycolysis in perivenous hepatocytes is manifested, in particular, in a high level and activity of GK (Toyoda et al., 1995).

It is believed that periportal glycogen is synthesised mainly from gluconeogenic precursors, while perivenous glycogen is synthesised directly from glucose (Jungermann, Katz, 1989; Jungermann, Kietzmann, 1997). It remains unclear in which zone of the lobule glycogen synthesis begins after food intake and at what rate it proceeds in different zones. It has been shown, however, that periportal hepatocytes accumulate glycogen faster after starved rats have been fed on a mixture of glucose and fructose (Kudryavtseva et al., 1992). It is also known that in the postabsorptive period the glycogen content in hepatocytes of the portal zone of the normal human and rat liver is higher than in the cells of the central zone (Kudryavtseva et al., 1996; Kudryavtseva et al., 2001b).

Hepatocytes differ greatly in glycogen content. These differences seem to be associated mostly with the different activity of the enzymes and different concentration of the substrates of glycogen metabolism in individual cells. However, the heterogeneity of the hepatocytes in respect of ploidy level, size and proliferative activity can also make a significant contribution and influence their functional activity. An *in situ* study showed that during DNA synthesis in rat hepatocytes the level of glycogen in the cells of different ploidy decreases by about 26% as compared to the cells in G_{0+1}-phase of the cell cycle. As hepatocytes pass through the S-phase of the cell cycle, their glycogen content gradually decreases (Shalakhmetova et al., 1981). It has also been established that the glycogen content in mononuclear and binuclear hepatocytes of different ploidy during postnatal ontogenesis (Kudryavtseva et al., 1979; Shalakhmetova et al., 1981) and in regenerating rat liver (Maytesyan, 1983) corresponds to their degree of ploidy. However, the dependence of glycogen content in hepatocytes on their ploidy level has

been investigated only for one mammalian species, the rat. No such data are available on other species, including humans. In addition, it is unclear whether this pattern depends on the nutritional status of the organism and whether it persists at different phases of glucose homeostasis.

The role of the size of hepatocytes on their function is much less clear than that of their proliferative activity and ploidy. Cell size is a fundamental parameter that has a significant impact on the general plan of cell structure and function (Jorgensen, Tyers, 2004). However, most studies dealing with the influence of the size of the cells on their functional activity focus on ion-dependent changes of the function.

It has been shown that swelling and dehydration of hepatocytes elicit changes in oxygen consumption, glucose production, gluconeogenesis and glycolysis rates, and the activity of key enzymes of carbohydrate metabolism in these cells (Hallgren et al., 2003; Krumschnabel et al., 2003). In general, cell hydration stimulates anabolic processes and proliferation, while dehydration leads to increased catabolism and increases sensitivity to apoptosis (Schliess, Häussinger, 2003). Rapid changes in the size of hepatocytes caused by swelling or dehydration have a small amplitude (about 35%) and are unaccompanied by changes in the number of organelles and various structures in the cells (Espelt et al., 2008). In contrast, the variability of hepatocyte weight (size) in mammalian liver is quite large. For instance, the dry weight and protein content in individual hepatocytes of equal ploidy can differ several times over (Zavadskaya et al., 1989; Bogdanova et al., 1990). It has been suggested that the amount of glycogen that can be accumulated in hepatocytes strongly depends on their size: the larger the cell, the greater the amount of glycogen can be deposited in its cytoplasm (Newsholm, Start, 1975). However, there are still no data to support or refute this hypothesis.

1.5. Chronic pathologies associated with impairment of metabolism, content and structure of glycogen in the liver

The key role of the liver in the mammalian carbohydrate metabolism, of which glycogen metabolism is an important part, suggests that diseases of this organ associated with impaired glycogen metabolism can have serious consequences for the organism. Numerous liver diseases in humans and animals associated with defects in glycogen metabolism are currently known. They can be conventionally divided into inborn and acquired ones.

1.5.1. Inborn diseases associated with impaired glycogen metabolism

Glycogen storage diseases (GSD) or glycogenoses are a group of genetic diseases. They are characterised by a deficiency of one of the enzymes associated with the synthesis or degradation of glycogen (Figure 1.11).

The principle of classification of GSD is based on the deficiency of an enzyme of glycogen metabolism and the type of tissue where it is absent. A total of twelve different GSD are currently known (Chandramouli et al., 2015; Curtino, Aon, 2019). These diseases are mainly manifested in episodes of hypoglycaemia and hepatomegaly, but their characteristic features are very variable.

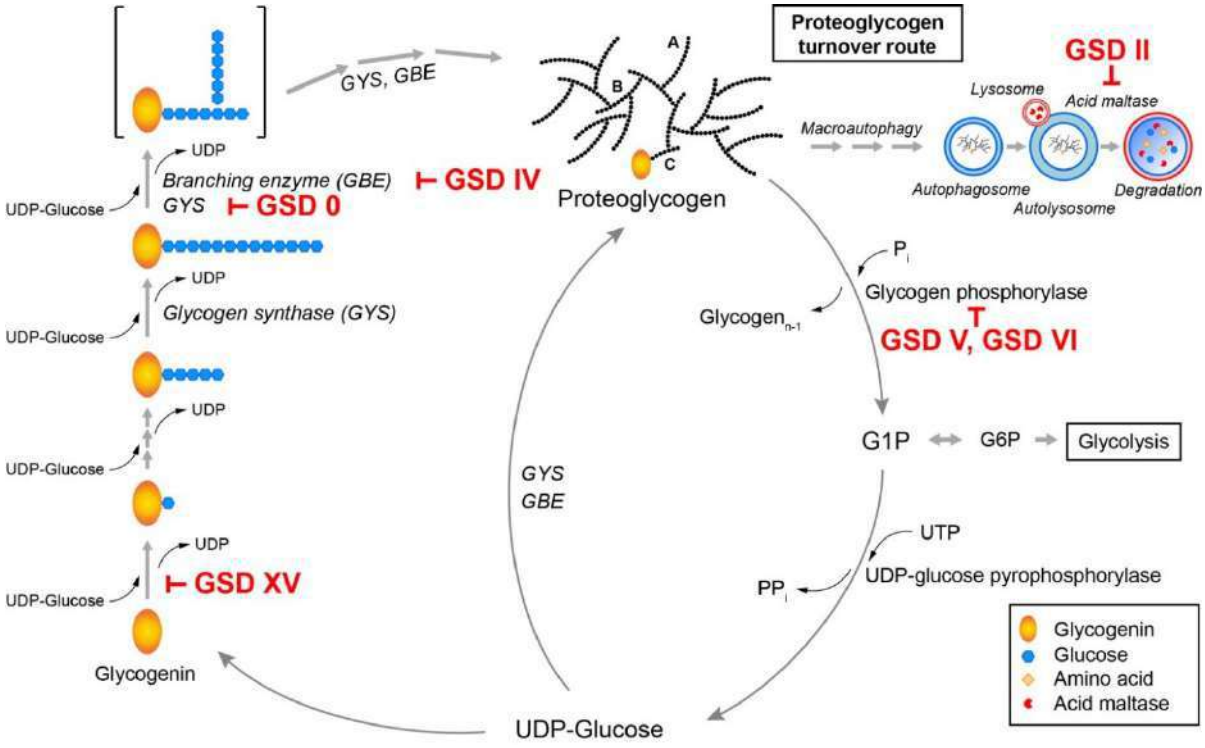


Figure 1.11 — Glycogen biogenesis and inborn defects of its metabolism causing glycogenoses in humans (Curtino, Aon, 2019).

GSD of types I, m, K and n make up about 80% of all GSD of the liver (Ozen, 2007; Curtino, Aon, 2019). The best known of these are von Gierke's disease (type I) and Cori's disease (type m). The former is caused by the deficiency of G6Pase. The liver of patients with GSD type 1 is characterised by swollen hepatocytes; their cytoplasm stains weakly for glycogen but their nuclei containing large amounts thereof. Cori's disease results in the loss of amylo-1,6-glycosidase and causes excessive accumulation of poorly branched glycogen, which

adversely affects hepatocyte function. GSD type m accounts for about 24% of all cases of GSD (Ozen, 2007).

1.5.2. Non-hereditary disorders of glycogen metabolism

In comparison with the low (0.002–0.005%) prevalence of hereditary hepatic disorders, the prevalence of acquired liver diseases, which are associated with severe abnormalities in glucose and glycogen metabolism, is very high. They are a significant health hazard and, in many countries, a threat to economy. For instance, in China liver diseases affect more than 300 million inhabitants (Wang et al., 2019). Among the diseases with a high prevalence rate, non-alcoholic fatty liver disease (NAFLD) should be mentioned. About 20–25% of NAFLD cases are classified as non-alcoholic steatohepatitis, which often leads to the development of fibrosis, liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (Prikhodko et al., 2022).

However, the most severe liver diseases are chronic hepatitis (CH) of various aetiology and diabetes mellitus (DM). They have a global distribution and pose a great hazard to human health.

Chronic hepatitides, whose terminal stage is LC, are widespread diseases of humans and animals. They are mainly caused by excessive alcohol consumption and abuse of medications, as well as by infection, via blood, by various viruses from the family *Hepadnaviridae* (B, C, D, E etc.) (Mauss et al., 2015). There are approximately 2.3 billion carriers of these viruses worldwide (Jefferies et al., 2018). Alcoholic and "drug" hepatitis are thought to be the cause of LC in more than a half of CH patients, while hepatitides of viral aetiology account for 10–24% of LC cases (Sherlock, Dooley, 2002). LC is a severe, irreversible and often lethal disease. Its annual mortality rate is ~1.16 million (Asrani et al., 2014). It is believed that compensated LC often goes undetected for a long time, with up to 1% of the population possibly having "histological" cirrhosis (Schuppan, Afdhal, 2008).

Liver cirrhosis is associated with hepatocyte death, nodular transformation of the parenchyma and the formation of fibrous septa connecting the system of portal vessels with the system of hepatic veins. Its main diagnostic features are considered to be fibrosis of most the parenchyma, pronounced regeneration of hepatic tissue, unusually small portal tracts and an excessive number of central veins in the lobules as well as the impairment of the lobular structure of the organ (Schuppan, Adfhal, 2008; Okovity et al., 2010). It should be noted that

though the diseases of the liver may have a widely different aetiology, the main stages of its pathogenesis are similar (Table 1.2) (Okovity et al., 2022).

Table 1.2 — Main pathogenetic mechanisms of acute and chronic liver diseases.

Diseases		**Main pathogenetic mechanism**
Viral hepatitides	Acute and chronic HBV	Hepatocyte cytolysis is associated with T-killer activity (in the presence of HBsorAg and HBAg)
	Acute and chronic HCV	Direct cytotoxic effect of the virus; immune cytolysis
	Acute and chronic HDV	
Autoimmune hepatitis	Primary biliary cirrhosis	Autoantibody-dependent cytotoxicity: immune-mediated necrosis of hepatocytes or cholangiocytes
Drug and alcohol-induced liver impairments	Fatty liver dystrophy without necrosis Acute hepatitis, steatohepatitis	Blockade of enzymes involved in the synthesis of lipoproteins, phospholipids, etc. Increased lipid peroxidation of hepatocyte membranes with accumulation of highly active forms and compounds of oxygen; blockade of enzymes involved in the detoxification function of the liver (cytochrome P-450 and other microsomal enzymes).
Wilson-Konovalov disease, haemochromatosis		Increased lipid peroxidation, activation of fibrogenesis
Liver cirrhosis		Corresponds to the etiological factor; activation of fibrogenesis; autoantibody-dependent cytotoxicity; disruption of microsomal oxidation system

Continuous cell death during the development of the pathological process in the liver stimulates a regenerative response. Its main cellular mechanisms are proliferation, polyploidization and hypertrophy of hepatocytes (Fig. 1.12) (Goss, 1966; Bogdanova et al., 1990; Sakuta, Kudryavtsev, 2005).

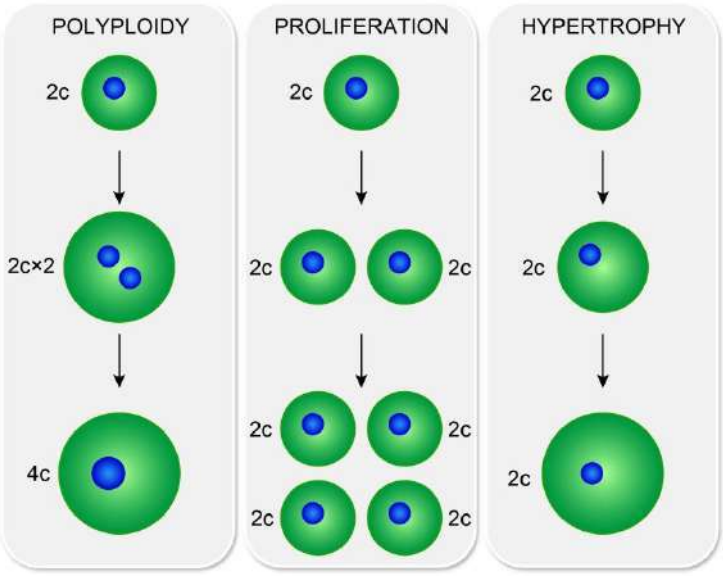


Figure 1.12 — Cellular mechanisms of liver regeneration (Okovity et al., 2022).

The leading role in the reparative growth of the liver affected by damaging agents of various aetiology is played by the processes associated with DNA synthesis: proliferation and polyploidization of hepatocytes. These processes lead to an increase in the number of cells and/or the number of genomes in the cells. The proliferative response of the hepatic parenchyma during chronic exposure to hepatotoxins is characterised by a lower probability of hepatocytes completing the cell cycle. Incomplete cell divisions in the parenchyma result in an increased ratio of the cells with polyploid nuclei. As a consequence, the average ploidy of hepatocytes increases significantly (Zavadskaya, 1989; Kudryavtsev et al., 1993). Under conditions of a chronic poisoning of rats by the hepatotropic poison CCl_4 hepatic, about 50% of the reparative growth of the liver is due to the proliferation of hepatocytes, 30%, to polyploidization of cells and 20%, to hypertrophy of their cytoplasm (Sakuta, Kudryavtsev, 1996).

In humans, too, liver damage is accompanied by an increased proliferative activity of hepatocytes and their increased ploidy level. An increased ploidy of hepatocytes has been shown in case of CH and LC of various aetiology, fatty hepatosis, extrahepatic cholestasis and other human diseases of the liver (Koike et al., 1982; Kartashova, 1985; Fang et al., 1994, Melchiorri et al., 1994). However, polyploidization of hepatocytes of pathological human liver has its own characteristic features: in contrast to the liver of rats and mice, the ratio of 2c×2 hepatocytes in the cirrhotic human liver increases with the progressing severity of the liver

impairment. At the same time, the ploidy of hepatocytes never increases as much as it does in rodents (Kudryavtsev et al., 1982; 1993).

Excessive growth of the fibrous tissue and the impairment of microcirculation and the lobular structure of the liver parenchyma reach the peak at the final stage of CH and LC. These processes lead to a decreased supply of hepatocytes with oxygen and substances necessary for their activity, causing disorders of numerous tissue-specific functions of the liver, including glucose and glycogen metabolism (Sherlock, Dooley, 2002).

The data on glycogen content in the liver of LC patients are contradictory (Owen et al., 1981; Kudryavtseva, 1987; Krähenbühl et al., 2003; Kudryavtseva et al., 1992). On the one hand, it has been shown that glycogen content in hepatocytes of patients with CH and LC after overnight fasting is several times higher than in normal hepatocytes and increases further with the increasing severity of the liver impairment (Kudryavtseva, 1987; Kudryavtseva et al., 1992). It has also been shown that the distribution of glycogen in the portal and the central zone of cirrhotic liver lobules differs from the norm and depends, in humans, on viral or alcoholic aetiology of the disease (Kudryavtseva et al., 2001b). On the other hand, glycogen content in the cirrhotic liver was found to be lower than in the normal one (Krähenbühl et al., 1991, 2003).

An increased level of glycogen in hepatocytes may be associated with changes in the activity of GS and GP, the key enzymes of glycogenesis and glycogenolysis. Some studies have shown a decrease in GS activity in experimental LC (Giardina et al., 1994; Krähenbühl et al., 1996). However, most data indicate that GS activity in the cirrhotic rat and human liver does not differ from the norm or is slightly lower (Krähenbühl et al., 1991; Kudryavtseva et al., 1992; Krähenbühl et al., 2003). Data on the rate of glycogenolysis in the cirrhotic liver are also scarce and contradictory. In some studies glycogen degradation was found to be faster in the cirrhotic liver than in the normal one (Krähenbühl et al., 1991), while in some other studies it found to be, on the contrary, slower (Petersen et al., 1999). These contradictions may be due to the fact that the material used in these studies was obtained from the liver of organisms with a different nutritional status.

Chronic liver impairment leads to significant changes not only in the glycogen metabolism in this organ but also in the carbohydrate metabolism in general. Several features of carbohydrate metabolism in cirrhotic patients such as tissue glucose intolerance (Mion et al., 1996), decreased GK activity in the liver (Aiston et al., 2001; Wu et al., 2005) and cell

resistance to insulin (Holstein et al., 2002; Garcia-Compean et al., 2009) are also characteristic of another common and dangerous disease, diabetes mellitus (DM). This disease is a major medical, economic and social problem in many countries. According to WHO, in 2021 the incidence of DM was more than 537 million people, which is about 10% of the world population aged 20–79 years, and it may reach 643 million by 2030 (Ogurtsova et al., 2017; IDF Diabetes Atlas 2021). Currently, DM ranks fourth in the list of lethal diseases, with diabetes type 2 being the most common (90–95% of cases). While genetic factors play a certain role, especially in case of DM type 1, the lifestyle, in particular, the diet and the level of physical activity, is much more important for the development of this disease (Xu et al., 2018; Zhang et al., 2022).

Hyperglycaemia and an impaired hormonal status, which are characteristic of DM patients, are accompanied by disruptions of glycogen metabolism in the liver. However, there are few data on glycogen content in hepatocytes of DM patients, and the available information is contradictory. Using ^{14}C-labelled precursors, it has been shown that glycogen synthesis is impaired in rats with experimental DM (Friedmann et al., 1970; Whitton, Hems, 1975). An increased contribution of the indirect pathway of glycogen synthesis (C_3-precursors → G6P → glycogen) as compared to the direct pathway (glucose → G6P → glycogen) was found in the liver of rats with DM (Giaccari, Rosetti, 1992). At the same time, studies on mice with hereditary and streptozotocin-induced diabetes did not reveal any differences in the glycogen content in the "diabetic" liver as compared to the control (Stearns, Benzo, 1977; Roesler et al., 1990).

Glycogen concentration was lower in the liver of patients with DM type 2 before meals than in the control (227±6 and 275±10 mmol/l of liver respectively, $p < 0.001$), while the total glycogen synthesis rate was lower after meals (Krssak et al., 2004). Similar data were obtained by Magnusson et al. (1992), who found that 4 h after a meal, the glycogen concentration was 131±20 mmol/L of liver in patients with DM and 282±60 mmol/L of liver in the control ($p < 0.05$).

The data on the activity of the key enzymes of glycogenesis and glycogenolysis in DM are also contradictory. On the one hand, it has been found that DM is accompanied by an increase in GS activity (Akatsuka et al., 1983; Niewoehner, Nuttall, 1986) and a decrease in GP activity (Gannon, Nuttall, 1990; Magnusson et al., 1992; Hundal et al., 2000). On the other hand, in the liver of db/db mice with hereditary DM, GS levels were not different from the

norm, while the total GP activity and the activity of GP*a* were increased*;* at the same time, some changes in the kinetic properties of these enzymes in mice with DM were found (Roesler et al., 1990). In addition, a lower GS activity in the liver of starved rats with DM as compared to the control rats were recorded (Golden et al., 1979).

1.6. Characterisation of hepatocyte population and glycogen metabolism during cirrhotic liver regeneration

The liver has a unique ability to regenerate after damage (Liozner, 1960; Taki-Eldin et al., 2012; Gilgenkrantz, Collin de l'Hortet, 2018). In experiments on rodents, complete liver weight recovery occurred within approximately one week after removal of 2/3 of the normal liver (Nadalin et al., 2004; Kele et al., 2012; Forbes, Newsome, 2016). However, the extent of parenchyma loss is much greater in the cirrhotic liver. In the course of the development of experimental cirrhosis, the weight of dead hepatocytes approximately corresponds to five times the weight of the liver in the beginning of the experiment (Peters, 1962; Brodsky, Uryvaeva, 1981; Macdonald et al., 1986).

Despite a high regenerative potential of the liver, its continuous exposure to various damaging agents leads to a situation when the rate of hepatocyte death becomes higher than the rate of the population recruitment. As a result, the remaining hepatocytes cannot carry out numerous specialised functions of the liver, and the disease eventually ends in the liver failure, which is often lethal.

In modern regenerative medicine, two main approaches are used to treat chronic liver damage: cell therapy and tissue or organ engineering. Clinical trials have shown that transplantation of hepatocytes obtained from various sources through the portal vein is a safe and effective procedure, however, it results only in short-term partial correction of metabolic disorders (Fox et al., 1998). The second approach is associated with the development of artificial liver tissues and their engraftment to patients. Implantable constructs of the liver tissue or the entire organ are among therapeutic approaches used in tissue engineering (Kholodenko et al., 2016; Camp et al., 2017; Stevens et al., 2017; Gilgenkrantz, Collin de l'Hortet, 2018). Though these methods seem promising, their practical outcome for the therapy of cirrhosis is still unclear.

It is broadly believed that there is currently no complete cure for LC and that the available treatments are ineffective in prevention of the liver failure. Liver transplantation is thought to be the only lifesaving option. The first human liver transplantation was performed in 1963 by the American surgeon Thomas Starzl. Since then, the number of such operations has been steadily increasing. For instance, 584 liver transplantations were performed in Russia in 2019. The success is considerable (~85% of patients survive for 1 year and about 70% live for 5 years or more), but there is a significant gap between supply and demand. In Russia, the average waiting time for transplantation is ~3.6 years, which means that at least 15% of patients die while waiting for surgery (Chistiakov, 2012; Hannoun, 2016). Besides the shortage of donor organs, liver transplantation is aggravated by other serious problems such as high costs, postoperative graft rejection and the need for long-term immune suppression (Duan et al., 2013). Therefore, it is imperative to continue the search for effective options of LC treatment besides transplantation. Whatever these approaches may be, it should be taken into account that the reversal of cirrhotic changes in the liver is only possible if the causes of the changes are also addressed. In addition, these approaches should be based on the fundamental knowledge of the cellular mechanisms of regeneration in the liver, since the course of liver diseases and their outcome largely depend on these mechanisms.

Although many scientists believe that after a certain point cirrhotic changes in the liver lead inevitably to the final lethal stage, several authors suggested that LC may in fact be reversible (Solopaev, 1963; Sarkisov, 1970). This optimistic viewpoint is based on the assumption that if the cause of the disease is eliminated, cirrhotic changes in the liver become reversible or at least may be normalised. This assumption is based on three premises. Firstly, it is the extremely high capacity of the mammalian liver to regenerate by compensatory hypertrophy. Even after several repeated partial resections of the liver, with the total weight of the removed tissue exceeding the initial one by 3–3.5 times, this organ can restore its preoperative weight quite quickly and completely (Monoszon, 1968; Bobyleva, Ladygina, 1975). Secondly, it is the enormous compensatory potential of the liver, which confirmed by experimental and clinical observations (Davydovsky, 1969; Sarkisov et al., 1983; Callea et al., 1991). Thirdly, it is the possibility of "reversing" the pathological changes, evidenced by experimental and clinical data that the liver can restore its morphological and functional parameters after cessation of the damaging effect. Excessive growth of connective tissue becomes reversible under certain conditions (Solopaev, 1963; Sarkisov, 1970). Dystrophic and

necrotic changes in the cirrhotic liver were also shown to be reversible, disappearing after the elimination of the pathogenic factor by means of intensive proliferation of hepatocytes (Kolpashchikova, Alymov, 1970; Sarkisov, 1974). In the case of experimental LC, a certain normalisation of the organ structure and function was observed after the elimination of hepatotoxic influence (Tarao et al., 1993; Mera et al., 1994; Szende et al., 1994).

The analysis of hepatocyte population kinetics 6 months after the cessation of the damaging effect showed that the average level of hepatocyte ploidy decreased, though remained 25% higher than in the control, while the protein content in rats that had had cirrhosis was comparable to the norm. Calculations show that post-cirrhotic regeneration of the liver is occurs almost exclusively by means of hepatocyte proliferation. At the same time, the structure and composition of the parenchyma cell population is not restored completely (Sakuta, Kudryavtsev, 1996). It is also noteworthy that some functional indices of carbohydrate metabolism of the liver, such as the level of glycogen in cells and the activity of GP and GS, are restored faster and more completely after cessation of exposure of rats to CCl_4 (Sakuta, Kudryavtsev, 1996).

Experimental data and clinical observations have shown that the reversibility of structural and pathological changes in the cirrhotic liver largely depends on the degree of the damage; in case of an advanced pathological process, normalisation of these changes is apparently impossible (Okazaki, Maruyama, 1985; Sarkisov et al., 1995). On the other hand, a poor reversibility of the cirrhotic changes may be due to inefficient regeneration of the pathological organ. Therefore, it can be assumed that the enhancement of the regeneration may promote a more complete restoration of the structure and function of the liver.

Reparative regeneration of the pathological liver is primarily aimed at the normalisation of stromal-parenchyma relationships and can proceed both by increasing the volume of the functioning parenchyma and by resorption of excessive collagen. Insufficiency of reparative regeneration may be a prerequisite for the pathological process in the organ becoming chronic (DuBois, 1990; LaBrecque, 1994). Therefore, it is important not only to find ways to stimulate regenerative activity but also to reveal objective indicators of the degree of restoration of the structure and function of pathologically altered liver after therapeutic impact.

Unfortunately, most studies of the regenerative potential and the degree of reversibility of pathological changes in the damaged liver focus on the dynamics of structural changes in the parenchyma. The characteristics of tissue-specific functional activity of the organ have

hardly been studied, except for some indirect clinical indicators. At the same time, since it is hepatocytes that are responsible for the main functions of the liver, only studies made directly on these cells can provide a sound assessment of the completeness of the recovery of this organ after damage.

To sum up, the available data indicate that the structure and function of the liver does not completely revert back to normal even a long time after the elimination of the pathogenic impact, though the tissue structure and the cellular composition of the parenchyma can improve significantly. Therefore, further search for methods and conditions ensuring a successful therapy of the cirrhotic liver and a deep understanding of the mechanisms of maintenance of tissue-specific functions in the pathologically changed organ are extremely important research directions. In particular, this concerns the most important function of the liver: the synthesis and degradation of glycogen in the cells of its parenchyma.

1.7. Structure of glycogen molecules. α- and β-particles and their role in glycogen metabolism

Glycogen content in hepatocytes is determined not only by the rates of its synthesis and degradation but also by the number and size of its molecules (β-particles) in the cells. At the same time, the mechanisms of formation and degradation of glycogen molecules, the dynamics of their composition and number in normal and pathological conditions as well as the role of structural changes in glycogen molecules in the regulation of glycogenesis and glycogenolysis remain largely unknown.

In various types of mammalian cells, glycogen is usually diffusely distributed in the cytoplasm, often localised near SER and near mitochondria (Nielsen et al., 2010) and sometimes may apparently be present in the nuclei (Bennett et al., 2008; Levene, Goldin, 2010). Its amount in the cytoplasm of hepatocytes varies from 0.5 to 19%, depending on the satiety of the organism (Reith et al., 1973). In hepatocytes, glycogen is represented by two types of granules: β-particles (molecules) and α-particles consisting of several dozens of β-particles (Devos et al., 1983; Sullivan et al., 2014; Deng et al., 2016) (Fig. 1.13).

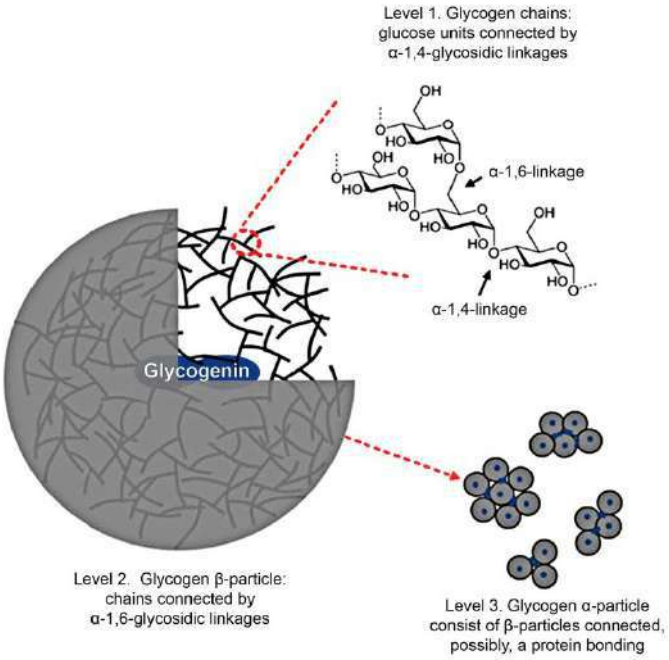


Figure 1.13 — Schematic representation of the three levels of glycogen structure (Li, Hu, 2020).

Glycogen is also present in skeletal muscles and some other tissues in the form of individual β-particles lying freely in the cytoplasm; their molecular weight is ~10^7 Da (Wanson, Drochmans, 1968). As for α-particles, which are a complex of 20–40 covalently bound β-particles appearing on electron micrograms as "rosettes" of various sizes, they are characteristic only of hepatocytes (Fig. 1.14). The diameter of α-particles can reach 200–300 nm (Devos et al., 1983; Sullivan et al., 2010).

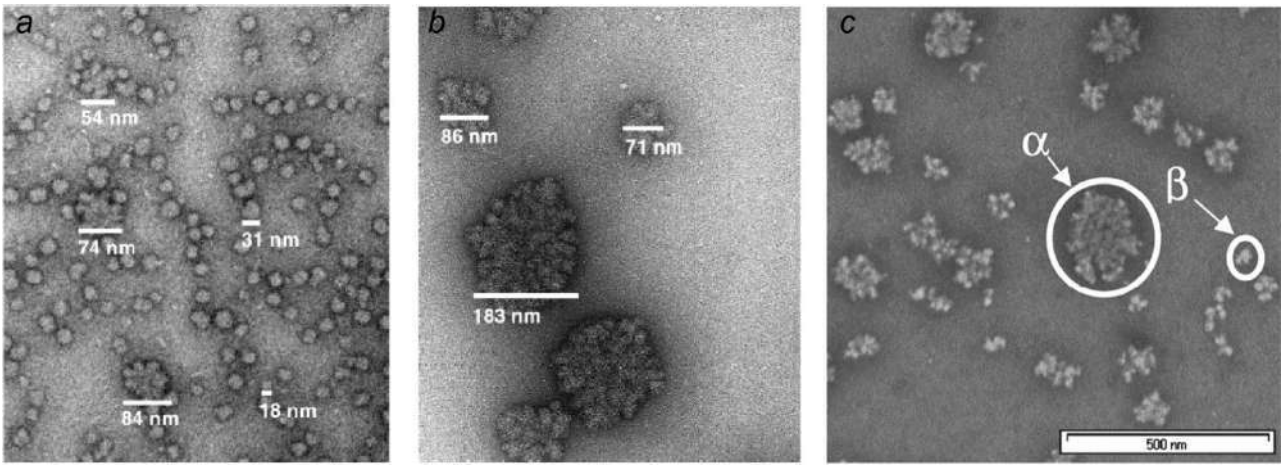


Figure 1.14 - Electronograms of glycogen particles isolated from the marine gastropod mollusc *Crepidula fornicata* (*a*), rat liver (*b)* and mouse liver (*c*). Molluscan glycogen mostly consists of β-particles with a diameter of about 20 nm, while rat liver glycogen mostly consists of α-particles (average diameter ~100 nm) *a, b* - Sullivan et al., 2010; *c* - Li, Hu, 2020.

The presence or absence of α-particles in the tissue may determine the order of glycogen degradation. It is thought that in the liver, where there are many α-particles, glycogen degradation follows a certain order, whereas in adipose tissue, where glycogen is mostly present in the form of β-particles, this process is random (Devos, Hers, 1980). It is assumed that two populations of glycogen particles exist in hepatocytes. The first population is represented by predominantly growing particles associated with GS. They are present in the cell when glycogen are low, and their number is limited. The second population consists of particles that have reached their maximum possible size; in principle, their number is unlimited (Devos et al., 1983).

The nature of the bonds holding β-particles together during the formation of α-particles is unclear. It was shown that they are not hydrogen or ionic bonds (Orrell, Bueding, 1964). Nakamura believed that β-particles were linked together by α-1,4-glycan chains (Nakamura, 1977). Treatment of glycogen with 2-mercaptoethanol or protease did not change the size and shape of the particles, which means that proteins do not seem to be responsible for holding β-particles together, either (Hata et al., 1984). A study of the dependence of the average size of glycogen β-particles treated with dimethyl sulfoxide on the concentration of LiBr showed that the formation of α-particles did not involve hydrogen bonds or protein-protein interactions (Sullivan et al., 2010). It has recently been suggested that α-particles are formed and kept stable with the help of non-glycosylated glycogenin (Fig. 1.15) (Tan et al., 2018).

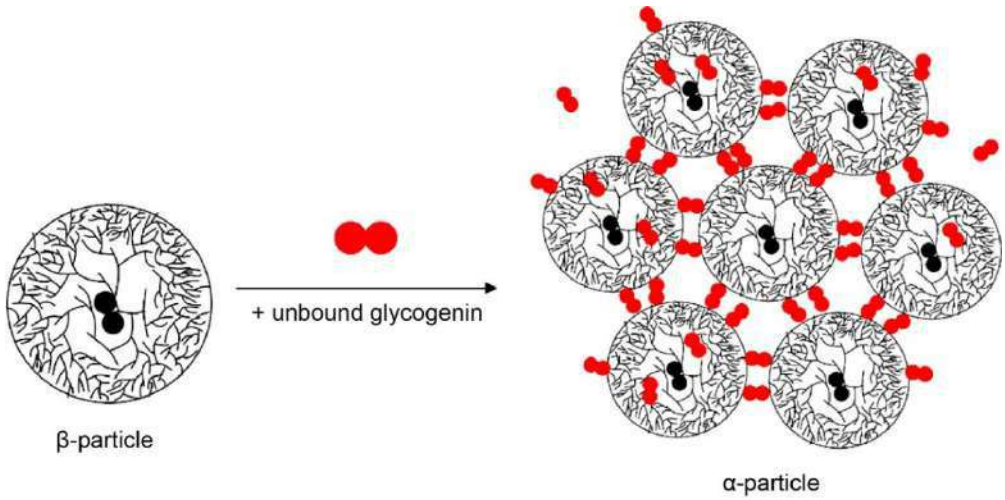


Figure 1.15 — Hypothetical model of assembly of α-particles. Glycogenin (primer) is present in the core of all β-particles (black colour). Non-glycosylated glycogenin dimers (red) non-covalently bind the terminal α-(1→4) glucose on the surface of the β-particle, thus connecting the neighbouring β-particles and making α-particles stable (Tan et al., 2018).

The total glycogen content in the liver cells, i.e. the number of α- and β-particles, fluctuates within certain limits and depends on the physiological state of the organism (Devos et al., 1983; Rybicka, 1996; Sullivan et al., 2010). The data on the size distribution of glycogen particles in the liver of mice during its synthesis, degradation and during starvation obtained by exclusion chromatography were used to suggest the "recycling" model of glycogen metabolism (Sullivan et al., 2014).

Food intake increases the level of blood glucose, and it becomes necessary to synthesise a large amount of glycogen. According to the authors of the model, the formation at this time (early and late synthesis stages) of β-particles, which are smaller than α-particles, increases the surface/volume ratio, increasing the affinity of GP*a* to glycogen and facilitating the incorporation of glucose into glycogen (Fig. 1.16). During glycogen synthesis, the α-particles remaining from the previous diurnal cycle dissociate into individual β-particles. If this were not the case, those α-particles that did not degrade during one diurnal cycle would continue to grow during the next synthesis phase, and their average size would become progressively larger with each day. However, this is not observed (Sullivan et al., 2014).

In the postabsorptive period, after glycogen synthesis is complete, glucose is slowly slow released into the blood. This process is controlled by means of the aggregation of β-particles into larger α-particles (Fig. 1.16). Glycogen degradation (early and late breakdown stages) mostly affects relatively small β-particles, while larger α-particles are affected to a lesser extent (Sullivan et al., 2014). This conclusion is supported by the fact that GP is more active in glycogen with smaller molecular weight (Stetten, Stetten, 1958).

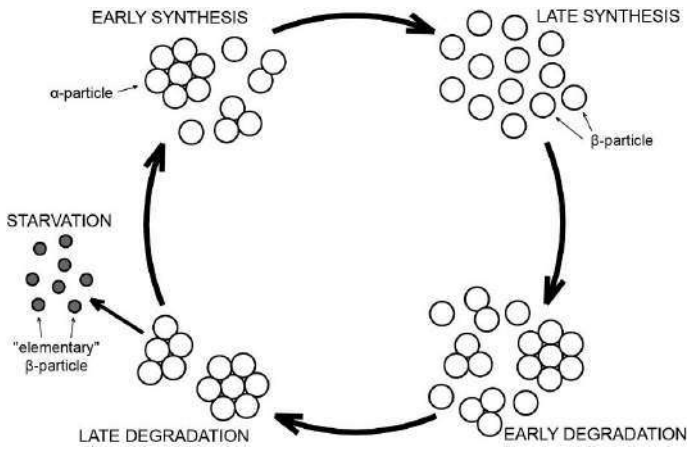


Figure 1.16 — Changes in glycogen structure in the liver of mice within 24 hours and during starvation (Bezborodkina et al., 2018).

The data on the incorporation of labelled glucose into glycogen also indicate that the metabolic rate of larger particles is lower than that of small particles. Degradation of α-particles at the stage of "early synthesis" is accompanied by the appearance of β-particles. This fact suggests that the surface area of glycogen molecules plays an important role in controlling their degradation (Geddes, Stratton, 1977).

It is interesting that in a fasting organism glycogen particles are small and have a low dispersion in size (Fig. 1.17). This circumstance possibly plays an energy-saving role. The *de novo* formation of glycogen molecules after food intake would require much greater energy expenditure and more time than the preservation of small "elementary" glycogen molecules, to which a large number of new glucose residues can be rapidly attached (Sullivan et al., 2014).

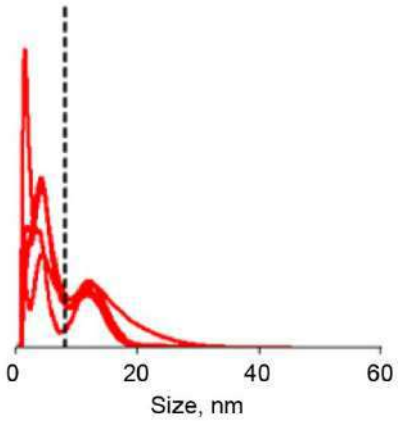


Figure 1.17 — Size distribution of glycogen particles in the liver of mice after 16 h of fasting. The dotted line separates glycogen peaks from contamination peaks (Sullivan et al., 2014).

The characteristics of α-particles may differ not only in the normal state but also in case of various diseases. For example, the ability to form α-particles in hepatocytes is lower in diabetic mice than in the control animals. At the same time, α-particles of diabetic mice are more resistant to degradation (Gilbert, Sullivan, 2014). It was also found that the glycogen of healthy mice has a higher proportion of large particles with a greater number of tightly bound β-particles than the glycogen of diabetic mice (Sullivan et al., 2011).

More than 160 years have passed since the discovery of glycogen in 1857. During this time, conisiderable progress has been made in the study of its metabolism (Roach, 2002; Jensen, Richter, 2012; Zois, Harris, 2016; Petersen et al., 2017). In particular, the structure of the polysaccharide part of glycogen molecules has been described in detail. The description was based on the results of chemical analyses determining the chain length of glucose residues,

the chain branching pattern and the molecular weight distribution of glycogen isolated from tissues of different organisms (Gunja-Smith et al., 1970; Goldsmith et al. 1982; Geddes, 1985; Melendez-Hevia et al., 1993; Melendez et al., 1997) as well as the electron microscopic data on the size of β-particles (Wanson, Drochmans, 1968).

The molecular structure of glycogen is based on D-glucose residues linked by α-(1→4) glycosidic bonds in a chain branching as a result of the formation of α-(1→6) bonds. Analyses of glycogen isolated from various mammalian tissues have shown that the chain length of glucose residues varies little, making up, on average, ~13 residues (Melendez-Hevia et al., 1993; Melendez et al., 1997).

To date, there are two main structural models of glycogen: Meyer-Bernfeld model (Meyer, Bernfeld, 1940) and Whelan model (Whelan, 1971). According to Meyer and Bernfeld, the chains of glucose residues grow evenly, and the non-reducing ends of all the chains are located on the surface of the molecule. In Whelan model, too, the total number of A-chains (unbranched chains) is approximately the same as that of B-chains (branched chains), but only A-chains are located on the surface of the molecule. Whelan also allowed for the presence of “hidden” A-chains in the glycogen molecule. Later, it was confirmed that the number of unbranched and branched chains in the glycogen molecule was approximately the same, but no “hidden” A-chains inside the molecule were found (Goldsmith et al., 1982). According to Whelan, each B-chain is joined by two other chains and, as branching progresses, the number of the chains increases exponentially (Fig. 1.18) (Bezborodkina et al., 2018).

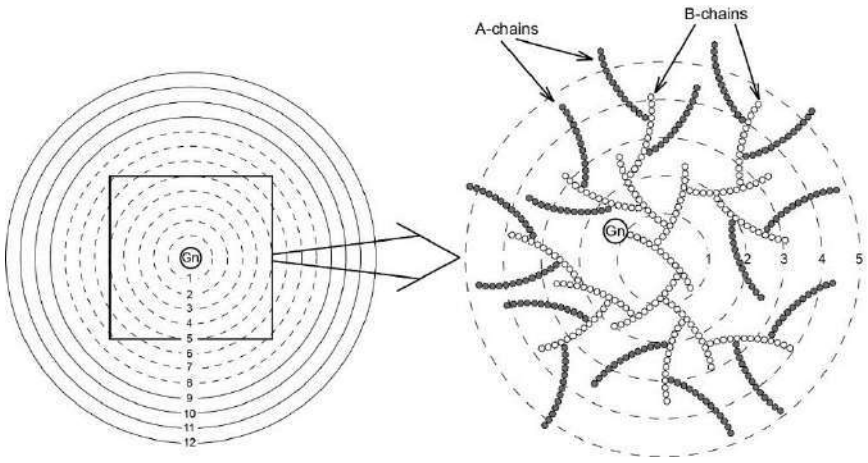


Figure 1.18 — Glycogen structure according to Whelan model. The complete glycogen molecule (β-particle) is spherical, with 12 concentric tiers of glucose residues. A self-glycosylating protein glycogenin (Gn) is in the centre of the particle (Bezborodkina et al., 2018).

All unbranched chains are located on the outer tier[*)] of the β-particle (Goldsmith et al., 1982; Roach et al., 2012). As a result, the number of chains on tier **n** is twice larger than the number of chains on tier **n – 1,** while the number of outer A-chains on the tier furthest from the centre of the particle is equal to the sum of all B-chains located inside the particle ($2^n = \Sigma 2^{n-1}$). In this case, one would expect GP to remove half of the glucose residues contained in the A-chains in the outer tier of the β-particle during each breakdown cycle. It turned out, however, that GP removes less than 50% of the glucose residues of the outer tier, leaving a stub of 4 glucose residues, which is apparently too small for enzyme action (Walker, Whelan, 1960). Nevertheless, the structure of the glycogen molecule allows GP to rapidly introduce into metabolic turnover about a third (50% × 9/13 = 34.6%) of all glucose residues in the molecule even without the participation of the debranching enzyme (Shearer, Graham, 2002; Graham et al., 2010).

Mathematical modelling of the β-particle structure makes it possible to predict its maximum size, beyond which its further growth is impossible. If a glycogen molecule contained not 12 but 13 tiers, the density of glucose residues on the surface of the molecule would be so high as to cause steric hindrance to the functioning of catalytic sites of GS and GP (Goldsmith et al., 1982; Melendez-Hevia et al., 1993). A similar idea was also suggested in one of the earlier studies (Madsen, Cori, 1958). It has been concluded that the size of the glycogen molecule is regulated by the characteristics of its own structure.

Thus, the 12-tier structure of the β-particle appears to be optimal for the following reasons: the maximum amount of glucose is stored in the least possible volume; a large number of non-reducing ends on the outer tier allows GP to break glycogen down at a high rate even without the participation of the branching enzyme, providing the cells with the required "fuel" as quickly as possible.

Theoretically, the structure of glycogen molecules can be described using only three parameters: the degree of branching (r), the number of tiers (t), and the number of glucose residues in each chain (g_c) (Table 1.3) (Bezborodkina et al., 2018).

[*)] A tier is a spherical space occupied by chains of glucose residues formed by sequential branching of B-chains and located equidistantly from the particle centre. The 1st tier corresponds to the space occupied by the primer chain formed by self-glycosylation of glycogenin; the 2nd tier corresponds to the space occupied by two chains of residues formed by branching of the primer chain; the 3rd tier comprises 4 chains formed by branching of two chains of glucose residues on the previous (2nd) tier, etc.

Table 1.3 — Distribution of glucose residues in β-particle of glycogen.

Tier	1	2	3	4	5	6	7	8	9	10	11	12
Number of residues glucose/tier	13	26	52	104	208	416	832	1664	3328	6656	13312	26624
Number of chains/tier	1	3	7	15	31	63	127	255	511	1023	2047	4095
Total number of glucose residues	13	39	91	195	403	819	1651	3315	6643	13299	26611	53235

The values of these parameters in calculations of the structure of glycogen molecules are: r = 2 (Melendez et al., 1997), t_{max} = 12 (Melendez et al., 1998) and g_c = 13 (Melendez-Hevia et al., 1993). These values are very similar in organisms at different evolutionary levels, from bacteria to humans (Melendez-Hevia et al., 1993; Melendez et al., 1997). This similarity indicates, on the one hand, that glycogen structure is an ancient phenomenon, which has been "polished" by evolution for hundreds of millions of years, and, on the other hand, that the theoretical ideas about the glycogen structure underlying these calculations are fairly reliable.

Based on the available data, we can conclude that a fully formed glycogen molecule (β-particle) has the following structural parameters:

- 42–44 nm in diameter (Wanson and Drochmans, 1968);

- molecular weight ~ 10^7 Da (Wanson, Drochmans, 1968; Geddes, 1986); total number of glucose residues ~ 55000 (10^7/180 – Mr glucose);

- ratio of the number of A-chains to the number of B-chains ~ 1:1 (Marshall, 1974);

- consists of 12 tiers of glucose residues (Melendez et al., 1997; Goldsmith et al., 1982; Melendez-Hevia et al., 1993) connected by α-1-4- and α-1-6- glycosidic bonds. The latter are uniformly distributed within the particle and account for 7–10% of all glycosidic bonds in the molecule (Shearer, Graham, 2002); the distance between tiers is ~ 1.9 nm (Goldsmith et al., 1982; Shearer, Graham, 2004);

- contains ~ 4095 chains of glucose residues (at the 12th tier, ~2048 A-chains), each consisting of 13 glucose residues (Gunja-Smith et al., 1970; Melendez-Hevia et al., 1993; Melendez et al., 1997). Each of the inner B-chains has two branching points located at least 4 residues apart (Goldsmith et al., 1982; Whelan, 1971).

Since glycogen does not have a crystalline structure, it cannot be studied by X-ray analysis. Nevertheless, it is known that polymeric glucose can, in principle, form helical structures. A heptamer of glucose can form a left-handed helix with 6.5 glucose residues per turn and a rise of 2.4 Å per residue (Goldsmith et al., 1982), while a cyclic polymer of 26 glucose residues can form a double helix (Gessler et al., 1999). Theoretically, the outer A-chains in the β-particle of glycogen should contain 13 glucose residues, but in reality this is unlikely because of the constant and rapid turnover of glycogen. For example, the average length of the outer chains in glycogen from human liver is 7.7 glucose residues (Mercier, Whelan, 1970). Therefore, it cannot be ruled out that helical structures may be present in large glycogen molecules with a strong chain branching.

It has been suggested that the β-particle can be consider as a fractal owing to repeatability, self-control and self-similarity of glycogen structure at each level of its formation (Melendez et al., 1999). The fractal structure makes possible a rapid synthesis and degradation of the molecule and provides a simple mechanism for the regulation of metabolic flows (Melendez-Hevia et al., 1994). An important consequence of the fractal structure of glycogen is the stability of its molecule. The spherical shape promotes the maximum potential energy at the surface, in the form of glucose residues, maximises the number of hydrogen bonds on the surface and, as a result, enhances still further the stability of the molecule (Melendez et al., 1999).

Traditionally, glycogen is referred to as a polysaccharide or glycan. However, in addition to glucose residues, glycogen contains numerous proteins involved in its metabolism, including proteins performing regulatory and scaffolding functions (Fig. 1.19).

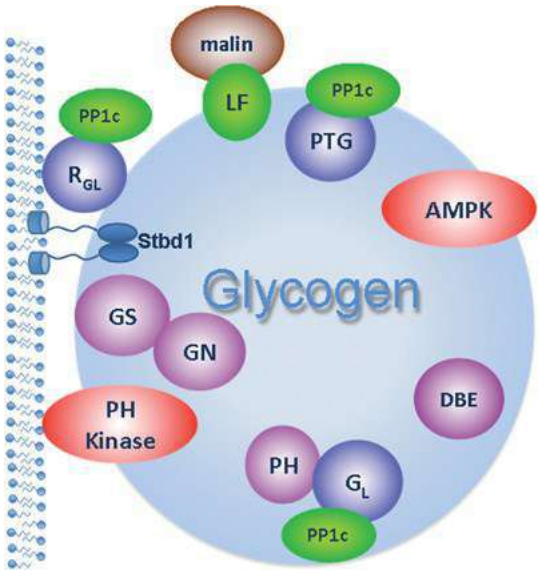


Figure 1.19 — Proteins of β-particle: enzymes of glycogen metabolism (purple) — glycogenin (GN), glycogen synthase (GS), glycogen phosphorylase (GP) and debranching enzyme (DBE); protein kinases (red) — phosphorylase kinase (PK) and AMP-activated protein kinase (AMPK); phosphatases (green) — catalytic subunit type 1 (PP1c) and laforin (LF); PP1-glycogen associated subunits (blue) — R_{GL}, G_L and PTG; Stbd1 protein associated with cell membrane. Phosphorylase kinase, Stbd1 and R_{GL} are associated with the membrane (Roach et al., 2012).

Under normal conditions, the pool of proteins comprising the β-particle can account for up to 80 % of its total weight (Shearer, Graham, 2004). Glycogen particles contain glycogenin and low molecular weight proteins participating in its glycosylation, GS and GP**,** DBE, various kinases and phosphatases, and actin (Roach, 2002; Shearer, Graham, 2004; Roach et al., 2012). Laforin (phosphatase) and malin (ubiquitin ligase E3) are also associated with glycogen molecules. They form a functional complex and play an important role in the pathogenesis of the neurodegenerative Lafora disease (Garyaly et al., 2014). A newly discovered Stbd1 protein (genetonin 1) is required for the attachment of glycogen to intracellular membranes (Jiang et al., 2011) and its transport into lysosomes (Sun et al., 2016). Proteomic analysis of glycogen from mouse and rat liver confirmed that most of the proteins mentioned above were present in β-particles (Stapleton et al., 2010). The absence of BE was a curious fact. The reason probably is that it interacts with the glycogen molecule without forming a strong bond and can be easily separated from it (Caudwell, Cohen, 1980).

Enzymes work at the surface of the β-particle, that is, at its outermost tier. It contains about a half of the glucose residues contained in the molecule, and about one third of them are available for the action of GP without the involvement of DBE. This feature of the glycogen molecule organisation allows a rapid provision of energy to the cells. Experiments with the introduction of ^{14}C-glucose have shown that the outermost tier is always more radioactive than

the inner tiers of the particle, indicating that the surface of this polysaccharide is the first to be involved in the turnover (Stetten, Stetten, 1954).

Unfortunately, there are almost no data on the number, interactions and dynamics of different proteins on the surface of the β-particle. It is assumed that up to 40–50 dimers of GP may be located on the surface of a fully formed particle (Madsen, Cori, 1958). They can break glycogen down very fast due to the presence of numerous non-reducing ends in its molecule (Goldsmith et al., 1982). Traditionally, one glycogen molecule is thought to contain one GS molecule (Roach et al., 2001), but there is evidence that the β-particle can be associated with several GS molecules (Prats et al., 2009). The number of GS molecules probably increases with the growth of the particle.

Glycogen is supposed to store the maximum amount of glucose in the smallest volume (Melendez-Hevia et al., 1993), but its polymeric structure imposes certain geometric constraints on the enzymes involved in glucose incorporation and release. Mathematical modelling has shown that the normal structure of glycogen particles during their growth and degradation can be maintained under the following conditions: (1) GS should operate preferentially on inner chains, attacking non-reducing ends; (2) the movement of GS from the point of attack should be much faster than that of GP. Otherwise, the growth of glycogen particles will lead to excessive and incorrect branching of glucose residues (Figure 1.20) (DiNuzzo, 2013).

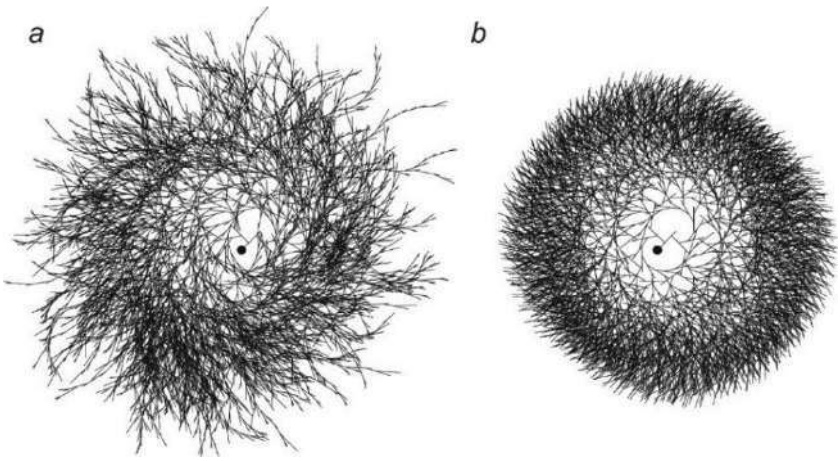


Figure 1.20 - Effect of glycogen synthase (GS) affinity to inner chains on the structural homogeneity of the glycogen molecule. (*a*) Without steric constraints on GS, the glycogen molecule becomes heterogeneous. (*b*) If GS operates predominantly on inner chains, the structure of the glycogen molecule becomes homogeneous. Both forms contain the same number of glucose residues (DiNuzzo, 2013).

The fact that different enzymes of glycogen synthesis and degradation have a different accessibility to glucose residues results in a faster turnover of the outer regions of the glycogen molecule as compared to the inner ones. The spatial constraints on GS and GP activity are thought to be optimal for the formation of large amounts of glucose within a short time and a rapid replenishment of glycogen stores. At the same time, as shown by isotope labelling, the attachment and detachment of glucose residues follow the last-in-first-out principle (Stetten, Stetten, 1954; Devos, Hers, 1979; Elsner et al., 2002).

It was established using chemical methods that two fractions of glycogen were present in tissues: lyoglycogen and desmoglycogen. The former is easily soluble in trichloroacetic acid (TCA), while the latter is insoluble (or poorly soluble) in TCA but can be extracted by treating the tissues with strong alkali[*)] (Willstatter, Rhodewald, 1934; Bloom et al., 1951; Weisberg, Rodbard, 1958). The presence of two glycogen fractions in tissues *in situ* was demonstrated in histo- and cytochemical work using PAS (Periodic Acid-Schiff) reaction (Kugler, Wilkinson, 1961; Kudryavtseva et al., 1974). Desmoglycogen, unlike lyoglycogen, was thought to be bound to proteins (Prins, Jeanlos, 1948). Later, however, it has been suggested that desmoglycogen is an artefact (Kits Van Heijningen, Kemp, 1955). A similar conclusion was reached by Rosenfeld and Popova (1989), who showed that glycogen could be almost completely isolated the liver by multiple repeated extractions with TCA, without resorting to the use of alkali. The authors suggested that it would be more correct to speak of “easily” and “poorly” extractable glycogen fraction (Rosenfeld, Popova, 1989). However, the aim of these works was not to study the structure of glycogen molecules but to extract the maximum amount of glycogen from tissues. Besides, the conclusion about “easily” and “poorly” extractable glycogen confirms rather than disproves the hypothesis about the existence of two glycogen fractions and indicates that glycogen may be bound or not bound to proteins.

An important milestone in the development of ideas about the spatial structure of glycogen molecules was the discovery of two forms of glycogen differing in the ratio of proteins and polysaccharides. One form was a “classical” macromolecular glycogen containing ~0.35% of glycogenin, while the other form (p400) was represented by glycogen in which

[*)] Note: in the works of different authors, glycogen fraction soluble in TCA has been referred to as lyoglycogen, easily accessible fraction and macroglycogen, while the fraction extractable only by KOH has been referred to as desmoglycogen, poorly accessible fraction or proglycogen. The indicators used to assess the content or concentration of fractions in tissues after treatment with TCA or KOH were also different: the amount of glucose extracted, the molecular weight of the extracted product, or the intensity of staining. However, despite the differences in terminology and methods of determining glycogen fractions in tissues, it has always been emphasised that different sensitivity of the fractions to TCA is based on differences in the interactions of glucose residues with proteins.

glycogenin content reached ~10% (37 kDa/400 kDa), owing to which this form was not precipitated by TCA (Lomako et al., 1991). Since the p400 fraction was converted into macromolecular glycogen *in vitro*, it was named proglycogen (PG). It has a low molecular weight (~4×10^5 Da) and is a stable intermediate form on the pathway of formation of macroglycogen (MG), whose molecular weight is ~10^7 Da (Lomako et al., 1991) (Figure 1.21).

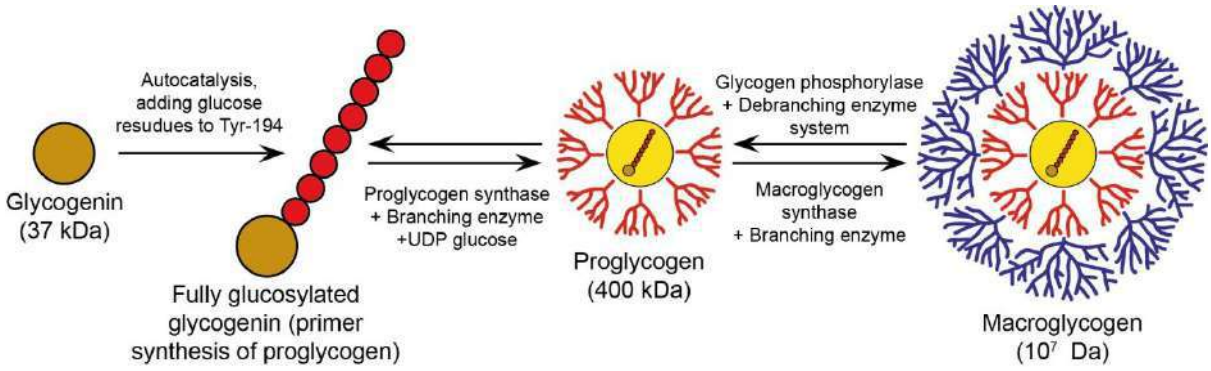


Figure 1.21 – Model of biogenesis of proglycogen and macroglycogen (Bezborodkina et al., 2018).

The strong difference in the molecular weight of MG and PG suggested that only the four outer tiers (9–12th) of its molecule, which represent MG, participate in glycogen metabolism, while the eight inner tiers (1–8th), i.e. PG, are uninvolved (Melendez et al., 1997). This difference in the metabolism of the outer and the inner tiers appeared a plausible mechanism, since the content of the "fuel" in the eight inner tiers makes up ~ 5% of the total amount of glucose residues in a complete glycogen molecule.

The ratio of PG and MG varies greatly depending on the tissue type. In skeletal muscles and heart, PG makes up most of the glycogen, while in the liver its content is small (Lomako et al., 1993). Therefore, the dynamics and physiological role of PG and MG in these tissues has received considerable attention. Theoretically, during the resynthesis of glycogen in cells its reserves can be replenished in two ways:

1) by forming new glycogen granules;

2) by increasing the size of the already available glycogen granules, i.e. by increasing the number of tiers in β-particles and the degree to which the tiers are filled with glucose residues (Fig. 1.22).

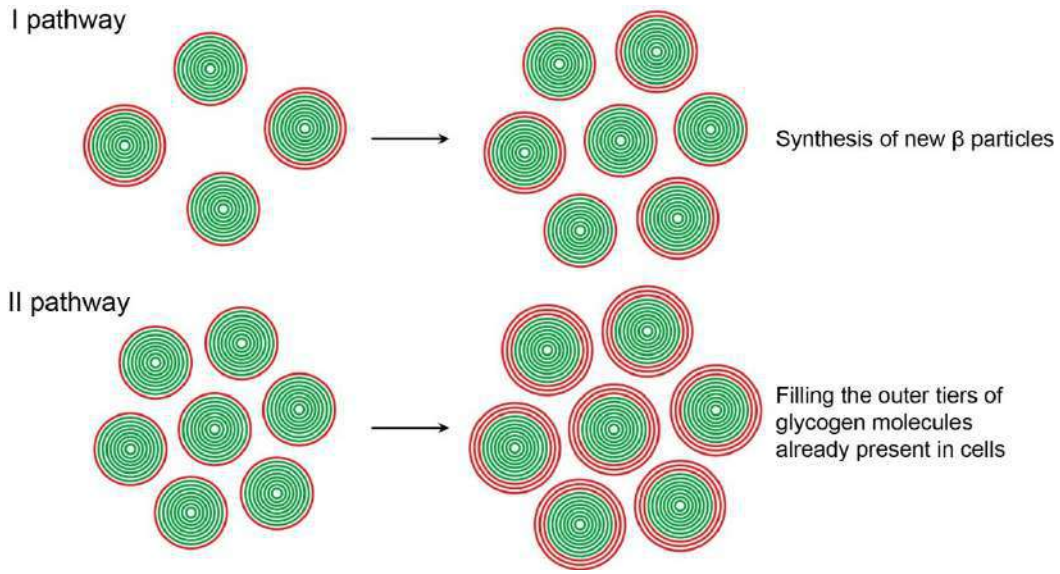


Figure 1.22 — Model of glycogen accumulation in hepatocytes (Bezborodkina et al., 2018).

The first pathway assumes that the number of β-particles in the tissue remains constant, they only become larger. In this case, the synthesis of new glycogenin molecules is unnecessary for increasing the glycogen content in the cells. This pathway should eventually lead to a decrease in the ratio of PG and an increase in the ratio of MG. The second pathway implies the formation of new glycogen granules. It should be accompanied by an increasing ratio of PG and a slow accumulation or an unchanged ratio of MG. This pathway obviously requires additional synthesis of glycogenin (Bezborodkina et al., 2018).

Since glycogenin is the basis of the entire polysaccharide structure of the glycogen molecule, it can be assumed that its content will determine the number of glycogen particles and the dynamics of its total content in cells. Indeed, several studies have shown that the level of glycogenin mRNA increases 2–3 times after prolonged muscle exertion and during the first hours of the recovery (Kraniou et al., 2000; Shearer et al., 2005). During a five-hour muscle recovery period, its amount increases by 70% (Arkinstall et al., 2004; Shearer et al., 2005). These data suggest that glycogenin degrades during exercise and is synthesised during rest. However, in another study, no changes in glycogenin content in the muscles during prolonged exercise and subsequent recovery were found (Marchand et al., 2007).

It has been shown that during the first 0–30 min of the recovery phase of human and rat skeletal muscle fibres, glycogen is mainly formed owing to PG, while MG synthesis starts only after several hours, when glycogen concentration reaches a rather high level (Adamo, Graham, 1998; Wilson, 2009; Granlund et al., 2011). It appears that the rate of glycogen degradation is

also the highest for PG, while MG catabolism begins to predominate only after the rate of glycogenolysis decreases (Graham et al., 2001). There is evidence that in the early stages of muscle recovery from exercise, glycogen synthesis proceeds through the formation of new molecules. When their number reaches a critical threshold, they begin to increase in size and the contribution of MG to the replenishment of glycogen stores becomes noticeable (Graham et al., 2001; Shearer et al., 2005).

However, the authors of another study arrived at an opposite conclusion (James et al., 2008). Investigating the dynamics of TCA-soluble and TCA-resistant glycogen in skeletal muscles of rats during starvation and at different stages after refeeding, they concluded that the greatest contribution to the change in the content of total glycogen was made by the TCA-soluble fraction, i.e. MG. The level of TCA-resistant glycogen (i.e. PG) remained stable or increased only slightly under these conditions (James et al., 2008).

To sum up, our current knowledge about the spatial structure of glycogen molecules is mainly based on the results of chemical analysis, the data on the size of α- and β-particles obtained by electron microscopy or exclusion chromatography and on theoretical ideas. A general idea about the structure of the β-particle was obtained with the help of mathematical description and modelling of the glycogen molecule, the determination of its approximate dimensions, the length of the chains and the branching patterns of glucose residues, the identification of proteins within the particle, the establishment of the order of attachment and detachment of glucose residues. However, while providing valuable information on the structure of glycogen molecules, all the methods mentioned above have fundamental drawbacks. Classical chemical methods and exclusion chromatography analyse glycogen isolated from tissues, and there is always a danger of artefacts due to disruption of the original structure of huge glycogen molecules (Devos et al., 1983; Roach, 2002). Electron microscopy makes it possible to study α- and β-particles of glycogen directly in cells and tissues and to obtain information on their geometrical parameters but not to “look inside” the particles. However, the main setback of the modern views about the spatial structure of the glycogen molecule is their static character. They cannot provide information about the dynamics of glycogen under various normal and pathological conditions. This means that the search for methods of studying the structure of glycogen molecules directly in tissues and/or individual cells is a promising research direction.

CHAPTER 2. MATERIALS AND METHODS OF RESEARCH

2.1. Objects of research

2.1.1. Experimental animals

Our studies were mainly made on 350 white breedless male rats weighing 120–180 g at the start and 250–300 g in the end of the experiment. In addition, 50 Wistar male rats weighing 180–200 g and 12 male mice (lines C57BL/6N and OE-NPY^{DbH}) weighing ~35 g were also used in the experiments.

Rats were obtained from the "Rappolovo" animal breeding station (Leningrad Region, Russia), while mice were provided by the Institute of Biomedicine of the University of Turku (Turku, Finland). All animals were kept under standard light regime conditions: 12-h light/dark cycle. Experimental animals had *ad libitum* access to water and standard food for rats (Laboratorkorm, Moscow, Russia; GOST 2874-82) or mice (SDS, Essex, UK).

Obtaining experimental liver cirrhosis (LC). White breedless rats were divided into two groups in the beginning of the experiment. Rats of one group were exposed to chronic inhalation of carbon tetrachloride vapour (CCl_4) with a concentration of 0.05 g/l in a closed chamber for 20 min 3 times a week for 6 months (the experimental group). Rats of the other group were unexposed for the same time and served as the control (the control group). In order to exclude the effect of acute exposure to CCl_4 on the liver, all further experiments were performed one week after the cessation of the treatment (Kudryavtseva et al., 1999).

Obtaining material for the study of hepatocyte populations of the normal and the cirrhotic liver at different stages of the food cycle. To study prolonged fasting and absorptive period of the food cycle, 27 rats from the control group and 27 rats from the experimental group were starved for 48 h (water *ad libitum*) and then orally administered a 30% glucose solution at a rate of 4 g/kg mass. Immediately after the end of starvation and 10, 20, 30, 45, 60, 75, 90 and 120 min after glucose administration, the animals were decapitated. The material (blood and liver pieces) obtained from each animal was used for cytophotometric and biochemical studies.

To study the postabsorptive period of the food cycle, rats from the control and the experimental group were decapitated after overnight starvation. The obtained material (blood

and liver pieces) was used for histological, cytophotometric, electron-microscopic and biochemical studies.

Different methods LC treatment in rats:

1) To determine the level of spontaneous regeneration of the cirrhotic liver, rats were unexposed to any additional treatment for 6 months after the end of the hepatotropic exposure to CCl_4 (WM group);

2) During the 1st, the 2nd, the 4th and the 6th month, along with the exposure to CCl_4 three times a week, the rats were injected 2-ethylthiobenzimidazole hydrobromide (bemithyl) at a rate of 12 mg/kg body mass (BM group) (Kudryavtseva et al., 2002b; Kudryavtseva et al., 2003);

3) After the cessation of the toxic effect of CCl_4 the rats underwent partial hepatectomy according to Higgins and Anderson (1931) under ether anaesthesia with extirpation of 2/3 of the liver (PH group) (Kudryavtseva et al., 1998);

4) During 3 months after the last session of CCl_4 treatment the rats were daily given, in addition to the standard food, an aqueous solution of a mixture of fructose (150 g/l) and glucose (50 g/l) in the ratio 1:1 (CD group) (Kudryavtseva et al., 1999);

5) After cessation of the 6-month exposure to CCl_4 the rats were daily (once a day) given intramuscular injections: during 3 days, of human chorionic gonadotropin 170–200 units/animal together with 1% ATP solution at a concentration of 10mg/kg mass (Solopaeva et al., 1967), and during the next 20 days, collalysine (GIPKh, St. Petersburg) at the rate of 5 KU/animal (HCG group) (Kudryavtseva et al., 2001a);

6) Every day for 5 days after the cessation of the toxic exposure, the rats were intraperitoneally injected dipeptide Lys-Glu (preparation "Vilon", St. Petersburg Institute of Bioregulation and Gerontology, Russian Federation patent no. 2080120, 1997) at a concentration of 1.7 mg/kg mass (Vilon group) (Kudryavtseva et al., 2000);

Material from each animal for histological, cytophotometric and biochemical studies after the last session of CCl_4 poisoning was obtained after: 7 days (BM group); 2 weeks and 1 month (Vilon group); 1, 3 and 6 months (in PH, CD, HCG, WM groups).

Same-aged rats, not poisoned with CCl_4 and not receiving any treatment, were used as the control for all other groups. The material was taken from the control rats at the same time intervals as from the rats of the experimental group.

The general scheme of the experiment for white breedless rats is presented in Figure 2.1.

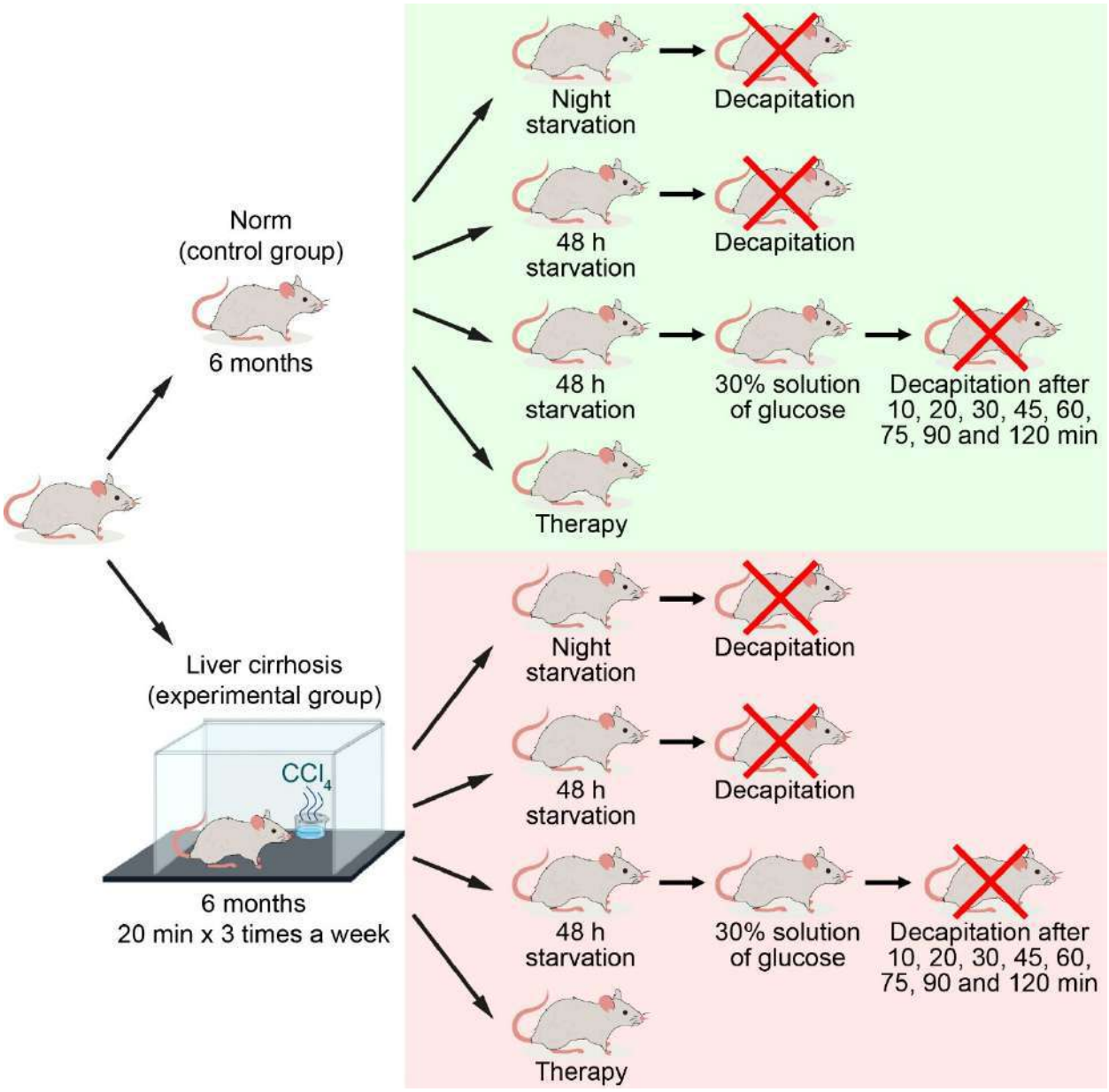


Figure 2.1 — Schematic of experimental procedures for white breedless rats.

Obtaining experimental toxic hepatitis in Wistar rats. For 4 weeks, the rats were daily injected intragastrically a 50% solution of CCl_4 in vaseline oil at a rate of 0.2 ml/kg and received 5% ethanol as drink (Beinert, Albracht, 1982; Ischenko, Michurina, 2005; Okovity et al., 2007). One week after the last CCl_4 injection the rats were killed using thiopental anaesthesia. Same-aged rats unexposed to the hepatotropic effects were used as a control. The livers of animals of the control and the experimental group were used to obtain the primary culture of hepatocytes (Baidyuk et al., 2009).

Methods of therapy of prediabetes type 2 in mice. Transgenic homozygous male OE-NPY^{DbH} mice (Ruohonen et al., 2008; Vähätalo et al., 2015) with the metabolic phenotype of hepatosteatosis and prediabetic state (Vähätalo et al., 2015; Ailanen et al., 2017) were used to investigate the therapeutic effects of metformin. Male C57BL/6N mice (wild type, WT) served as controls. From the age of 17 weeks, the mice received metformin (Enzo Life Sciences LTD, Exeter, UK) with drinking water at a rate of 300 mg/kg/day or water without metformin for 4 weeks. Drinking bottles were changed twice weekly and water consumption was measured to confirm drug intake (Ailanen et al., 2018). At an age of 20 weeks, the mice were subjected to the glucose-tolerance test by intraperitoneal injection of glucose at a concentration of 1 g/kg body mass (Vähätalo et al., 2015) and then were sedated using CO_2. Their liver was extracted and used for cytophotometric studies.

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (http://www.nap.edu/catalog/10498.html), the Directive of the Council of European Communities (https://op.europa.eu/en/publication-detail/-/publication/cc3a8ccb-5a30-4b6e-8da8-b13348caeb0c/), and ARRIVE (https://www.nc3rs.org.uk/) and ICLAS (https://iclas.org/) guidelines.

2.1.2. Clinical material

We used the material of liver puncture biopsies from 114 patients treated in the Clinic for Internal Medicine no. 2 of the St. Petersburg State Medical Academy named after I.I. Mechnikov. Biopsies were obtained on an empty stomach (after overnight fasting) at the same time (from 10:00 to 12:00) by Mangini method. Patients in the control group had no clinical, morphological, immunochemical and biochemical signs of the liver damage. Patients with chronic viral hepatitis (CVH) and liver cirrhosis (LC) received basic treatment including enterosorbents, B and K vitamins and detoxification solutions. Nine patients with CVH and nine patients with LC were additionally treated with 2-ethylthiobenzimidazole hydrobromide (bemithyl) in addition to the basic treatment. The drug (0.25 g) was administered twice a day after meals in three short courses of five days, with breaks of two days between the course (Kudryavtseva et al., 2002a). The distribution of patients across groups is presented in Table 2.1.

Table 2.1 — Characteristics of the patients who participated in the study.

no.	Diagnosis	Total number of patients (male/female)	Average age, years	Average duration of the disease, years
1	Conditional norm (control group)	29 (20/9)	44.0±3.6	-
2	Chronic hepatitis	35 (22/13)	42.1±4.9	5.7±1.2
3	Liver cirrhosis	33 (28/5)	49.9±5.1	6.8±1.3
4	Diabetes mellitus type 2	12 (12/0)	54.5±4.0	-
5	Diabetes mellitus type 2 and liver cirrhosis	5 (5/0)	49.6±7.5	-

Biochemical blood analysis was performed for all the patients. It included determination of the concentration of glucose and total bilirubin (TB) and the activity of alanine aminotransferase (AlAT), aspartate aminotransferase (AsAT) and alkaline phosphatase (ALP). The puncture biopsy material was used for histological, cytophotometric and biochemical analysis. The study was conducted in accordance with the 1989 Helsinki Declaration of the World Medical Association (https://jamanetwork.com/journals/jama/fullarticle/1760318). All patients consented to participation in the study, including the publication of its results.

2.2. Methods of making preparations

2.2.1. Preparations of primary culture of rat hepatocytes for detection of functioning mitochondria in living cells

Isolated rat hepatocytes were obtained according to the modified Seglen method (Seglen, 1976). For this purpose, the liver was perfused *in situ* through the portal vein for 5 min with a buffer (pH 7.4) heated to 37°C containing 8.3 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, 2 g/L glucose and 5.5 ml/L 1 M NaOH with the help of a micropump. The liver was then extracted and placed in a sterile Petri dish in the same buffer solution. Further manipulations were performed under sterile conditions in a laminar box (Baidyuk et al., 2009). The obtained monolayer of hepatocytes was incubated on coverslips with a layer of 0.1% gelatine for 1 day in Eagle medium with rhodamine 123 (10 μg/ml) (Sigma, USA) for 30 min in an atmosphere with 5% CO_2 at 37°C (Johnson et al., 1980). The incubation medium was

then drained, the coverslips were covered with pure Needle medium and incubated for 5 min under the same conditions. This procedure was repeated twice. To preserve the viability of hepatocytes during microscopic analysis, the coverslips were placed on a slide with a previously prepared sealed chamber filled with a heated culture medium. Fluorescence of hepatocytes was excited by 480 nm light, and their mitochondria were qualitatively analysed using a Leica TCS SL confocal microscope (Leica Microsystems Inc., Germany) (Baidyuk et al., 2009).

2.2.2. Preparation of smears of isolated hepatocytes

Smears of isolated hepatocytes made on standard slides were used for cytophotometry. For this purpose, pieces of rat liver (~2 mm^3) and human liver biopsies (~1 mm^3) were placed for 10 min in phosphate buffer I (pH 8.0) (475 mL 0.066 M Na_2HPO_4 x $2H_2O$, 25 mL 0.067 M KH_2PO_4, 500 mL 0.15 M sucrose), then transferred for 15 min into 0.067 M K, Na-phosphate buffer II (pH 7.4). After that, the liver pieces were taken with tweezers and gently shaken in a drop of buffer, and the resulting cell suspension was smeared on the surface of the slide using a quartz glass with a ground edge. Immediately afterwards, smears of isolated hepatocytes on slides were fixed at room temperature with 100% methanol, air-dried for 3–5 min and stored in a dark place (Kudryavtseva et al., 1983; Bezborodkina et al., 2021b).

To study the kinetics of hepatocytes staining with Schiff's reagents, smears of isolated hepatocytes from the liver of fed rats of the control group were prepared on coverslips according to the method described above.

2.2.3. Histological preparations

Pieces of rat liver (~4 mm^3) and human liver biopsies (~2 mm^3) were fixed in 10% neutral formalin for at least 48 h at 20–22 °C and embedded in paraffin blocks. Histological sections ~5 μm thick were cut using a Reichert microtome (Austria).

To detect connective tissue, the preparations were stained with van Gieson picrofuchsin (Biovitrum, Russia) or with picrosirius, a 0.01% solution of sirius red F3BA (Bio-Optica Milano SPA, Italy) in saturated aqueous solution of picric acid, for 1 hour. After staining, the preparations were rinsed in 30% acetic acid, dehydrated in an ascending ethanol series and

embedded in Canadian balsam. Rat liver sections were additionally stained with Mayer's hematoxylin-eosin (Chestnova et al., 2015; Bezborodkina et al., 2021b).

Images of sections stained with hematoxylin-eosin, hematoxylin-picrofuchsin and picrosirius were obtained using an Axiovert 200M microscope (Carl Zeiss, Germany) (10×/0.30, 20×/0.50 objectives) equipped with a Leica DFC420C digital camera (Leica Microsystems Inc., Germany).

2.2.4. Fixation and preparation of liver tissue for electron-microscopic study

Liver samples were fixed in 2.5% glutaraldehyde solution prepared in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 h. Subsequent (pre)fixation of the liver tissue was performed in a 1% OsO_4 solution on the same buffer at room temperature for 2 h. The pieces were then dehydrated in an ascending ethanol series: 30-40-50-60-70-80-90-96-100%. Simultaneously with dehydration of the samples, they were contrasted with uranyl acetate (Bezborodkina et al., 2008). For this purpose, uranyl acetate was added to the alcohols starting from 70% to achieve a concentration of 0.5–1.0% (Komarov, 1987). After dehydration in ethanol, the samples were embedded in araldite according to Glouert (Glouert, Glouert, 1958). Ultrathin sections prepared with an ultratome LKB III (Sweden) were placed on copper grids and additionally contrasted with lead nitrate.

Photographs were taken on GEM-100 CX electron microscope (Japan) at a magnification of 8000x or 20000x.

2.3. Cytophotometric methods

2.3.1. Determination of hepatocyte dry mass using an interference microscope

Dry mass (DM) of rat and human hepatocytes was measured on unstained methanol-fixed preparations embedded in glycerol according to a previously described method (Brodsky, 1966; Beneke, 1969). Measurements were made with the help of a MBIN-4 interference microscope (LOMO, Russia) in monochromatic light using an interference light filter λ_{max} = 550 nm and an objective 40×/0.65.

DM was calculated according to the formula (Brodsky, 1966; Beneke, 1969):

$$\mathbf{M} = \frac{\boldsymbol{\delta} \times \mathbf{S}}{\mathbf{100} \times \boldsymbol{\alpha}} \quad \textbf{(1)}$$

where: **M** - dry mass of the cell, pg; **δ** – path-length difference, cm; **S** - cell area, cm^2, **α** - specific refractive index increment, cm^3/g; its value for proteins in glycerol is 0.00095 cm^3/g (Pellegrino et al., 1963).

When measuring the DM of hepatocytes, the path-length difference for the cell and the medium was first determined (Fig. 2.2).

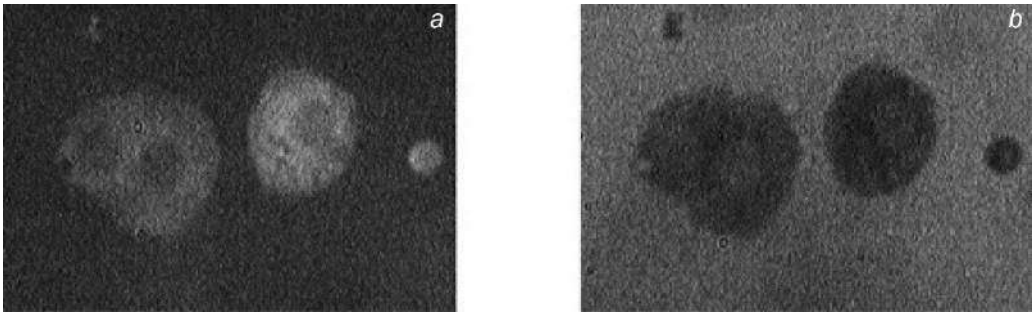


Figure 2.2 — Unstained hepatocytes, interference microscope: *a* — adjusted to dark field; *b* — adjusted to dark object. 40×/0.65 objective.

The optical path difference was determined according to the formula:

$$\boldsymbol{\delta} = \frac{\boldsymbol{\varphi_1} - \boldsymbol{\varphi_2}}{\mathbf{K}} \times \boldsymbol{\lambda} \quad \textbf{(2)}$$

where: **δ** – path-length difference, cm; **φ_1, φ_2** - counts on the Senarmont compensator scale, degrees; **λ** - wavelength of light, 550 nm; **K** = 180°.

Then, the cell area (in μm^2) was measured using the ImageJ programme (NIH, USA). Depending on the task, 100–450 cells were measured on each preparation (Bezborodkina et al., 2016).

2.3.2. Methods of quantitative cytochemistry

2.3.2.1. Identification and measurement of glycogen and its fractions in isolated rat hepatocytes

Glycogen content in hepatocytes was measured with the help of the fluorescent PAS reaction (Kudryavtseva et al., 1974). First, smears of isolated hepatocytes were placed in 0.8% KIO_4 solution prepared with 0.23% HNO_3 for 1.5 h. The preparations were then washed in running water for 15 min and in one change of distilled water. Fluorescent stain auramine OO

(Reanal, Hungary) was used to detect glycogen in hepatocytes using Schiff's reagent. Preparations were stained with Schiff's reagents (0.3% solution of auramine-SO_2 [Au-SO_2] + 0.2 ml of thionyl chloride ($SOCl_2$)/100 ml of stain solution) at room temperature for 90 min. After staining, the preparations were washed with three changes of distilled water and three changes of sulphurous water (5 g $K_2S_2O_5$, 950 ml water, 50 ml 1 N HCl) for 3 min each time to remove non-specifically bound stain. The preparations were then washed in running water for 20 min, rinsed with distilled water, dehydrated in ethanol series (5 min each time in two changes of 70°, 96° and 100° ethanol), air dried and stored in the dark. Before measurements, preparations were embedded in non-fluorescent vaseline oil (Kudryavtseva et al., 1999).

Images of cells stained with Au-SO_2 (Figure 2.3) were obtained using an Axioskop fluorescence microscope (Carl Zeiss, Germany) equipped with a DFC360FX digital camera (Leica Microsystems Inc., Germany).

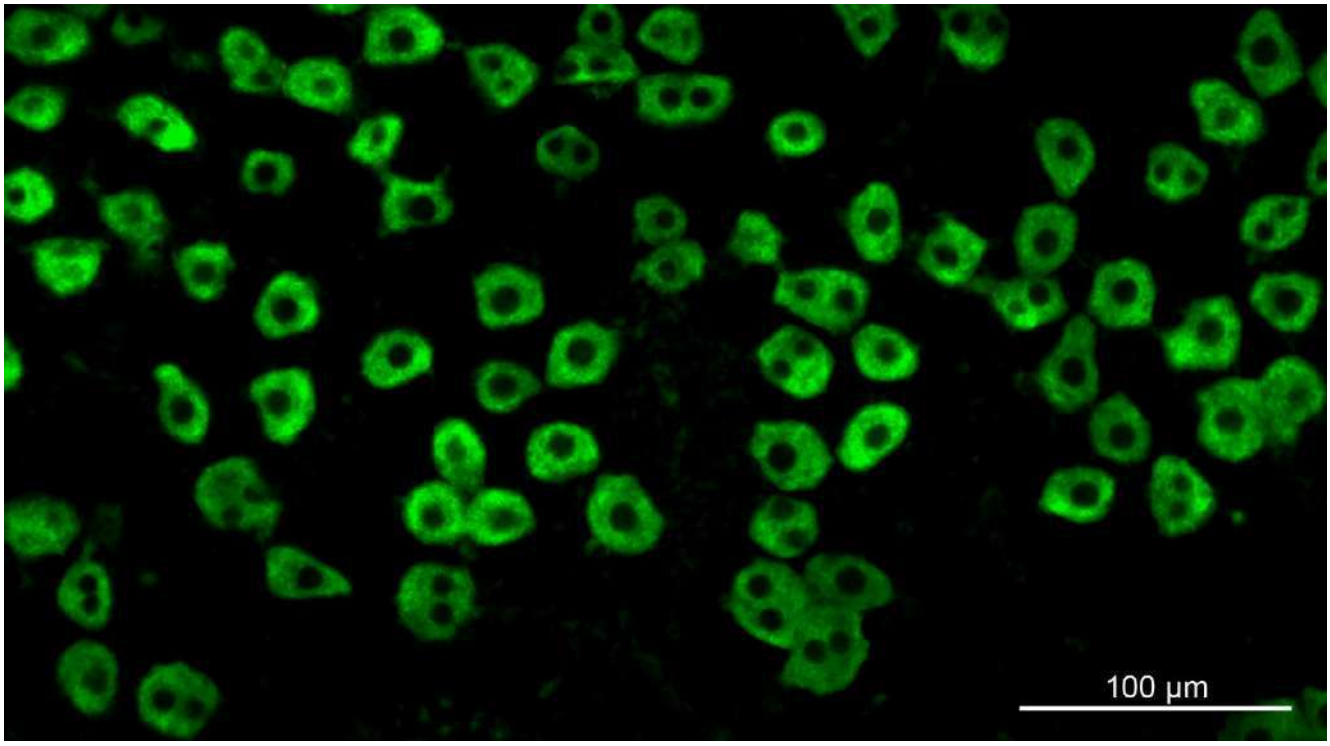


Figure 2.3 — Detection of glycogen in hepatocytes using auramine-SO_2.

To excite the fluorescence of preparations stained with Au-SO_2 and to record their fluorescence, Filter Set 10 (Carl Zeiss, Germany) was used. The Plan-NEOFLUAR 20×/0.50 objective was used for measurements. Fluorescence intensity of cells stained with Au-SO_2 was evaluated using ImageJ programme (NIH, USA) (Bezborodkina et al., 2021).

2.3.2.2. Determination of DNA content in hepatocyte nuclei

DNA in hepatocyte nuclei was detected on smears using fluorescent Feulgen reaction with Schiff's reagent Au-SO_2 (Kudryavtsev, Rozanov, 1974). The preparations were hydrolysed in 6 N HCl at 20–22ºC for 8 min. After hydrolysis, the preparations were rinsed thrice in distilled water and stained with 0.3% Au-SO_2 solution containing 0.2 ml of $SOCl_2$/100 ml in the dark at 4°C for 1.5 h. Then the preparations were rinsed thrice in distilled water cooled to 4°C, passed through sulphur water cooled to 4°C (3 changes, 3 min each), washed in running water for 20 min, rinsed with distilled water and dehydrated in an ascending ethanol series (70°, 96°, 100°) (2 changes of ethanol of each concentration, 5 min each). The preparations were then air dried and stored in the dark. Immediately before measuring the DNA content in cells, the preparations were embedded in non-fluorescent vaseline oil (Bezborodkina et al., 2016).

Images of Au-SO_2-stained hepatocyte nuclei were obtained using an Axioskop microscope (Carl Zeiss, Germany) (Plan-NEOFLUAR 40×/0.75 objective) equipped with a DFC360FX digital camera (Leica Microsystems Inc., Germany). Filter Set 10 (Carl Zeiss, Germany) was used to excite the fluorescence of Au-SO_2 and record it. Fluorescence intensity of Au-SO_2-stained nuclei was determined using ImageJ programme (NIH, USA).

Rat peripheral blood lymphocytes stained with Au-SO_2 after Feulgen were used as a diploid (2c) standard for determining the ploidy of hepatocytes. The average ploidy of hepatocytes N(c) was calculated according to the formula (Delone et al., 1987):

$$\mathbf{N(c)} = \sum \mathbf{n_i} \times \mathbf{2^i} \qquad \textbf{(3)}$$

where: $\mathbf{n_i}$ - relative number of hepatocytes of i-th ploidy class (**i** = 1 - diploid class, **i** = 2 – tetraploid class, etc.) (Bezborodkina et al., 2016).

2.3.2.3. Combined cytochemical method for quantifying several components in the same cell

In order to obtain quantitative data on several parameters such as dry mass, glycogen content and ploidy degree in the same cell, we used a combined cytochemical method (Kudryavtsev et al., 1979). This method makes it possible to identify and measure one parameter after another in such a way that the unmeasured components remain unaffected. The

accuracy of determination of the content of each component is almost the same as during separate determination of the components.

The combined cytochemical method requires the use of several measuring devices. To determine the location of one and the same cell repeatedly, a coordinate grid with the size of squares of about 1 mm² was cut on the slides using a diamond glass cutter. An Axioskop microscope (Carl Zeiss, Germany) (Plan-NEOFLUAR 20×/0.50 objective) equipped with a DFC360FX digital camera (Leica Microsystems Inc., Germany) was used to obtain images of selected areas of the preparation (about 100). Intact hepatocytes lying separately were selected on the images, and each was assigned a number according to its location on the slide (Fig. 2.4). From 100 to 500 cells were marked on each preparation, depending on the study task (Bezborodkina et al., 2016).

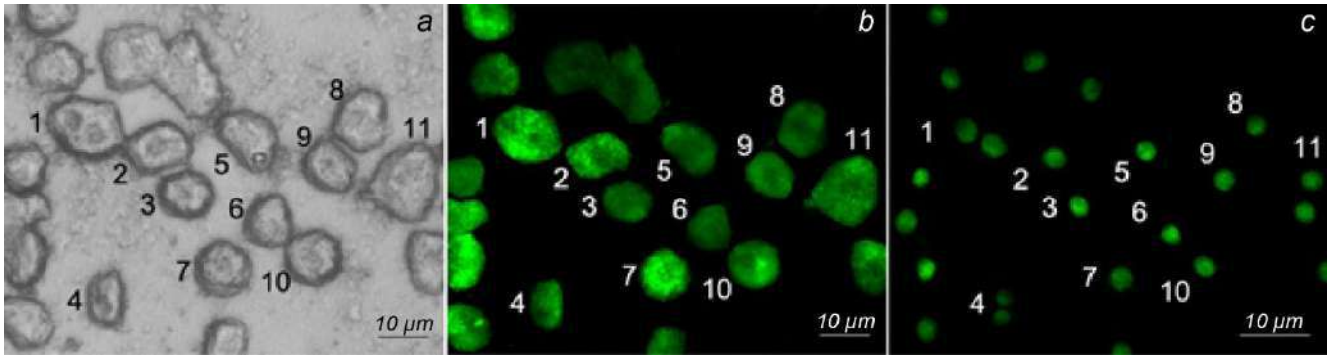


Figure 2.4 — An example of using the combined method: *a* — isolated hepatocytes with assigned numbers, phase interference contrast, *b* — the same hepatocytes stained with fluorescent PAS reaction for glycogen, *c* — the same hepatocytes whose nuclei were stained with fluorescent Feulgen reaction for DNA. Axioskop microscope (Carl Zeiss, Germany), DFC360FX digital camera (Leica Microsystems Inc., Germany). Plan-NEOFLUAR 20×/0.50 objective lens.

First, DM of each of the marked cells was measured (see Section 2.3.1). After that, the coverslip was taken off, glycerol was removed by passing the preparation through an ascending ethanol series, and the glycogen content in hepatocytes was determined (see Section 2.3.2.1). Next, the coverslip was taken off and vaseline oil was removed using xylene and ethanol of increasing concentration. Staining for glycogen was then removed by treating the preparations with 0.025% sodium borohydride ($NaBH_4$) for 20 min. As a result of this treatment, aldehyde groups (-CHO) formed during oxidation of glucose residues in glycogen by KIO_4 converted to primary alcohol groups (-CH_2OH), lost their ability to stain Au-SO_2 and did not affect either the DNA content in the cells or the accuracy of its measurement (Fig. 2.5). Finally, after the $NaBH_4$ treatment and staining after Feulgen, the DNA content of hepatocyte

nuclei was measured (see section 2.3.2.2). The DNA content in the cell was used to estimate the degree of its ploidy. The number of nuclei in a cell indicated whether it was mononucleate, binucleate or multinucleate (Bezborodkina et al., 2016).

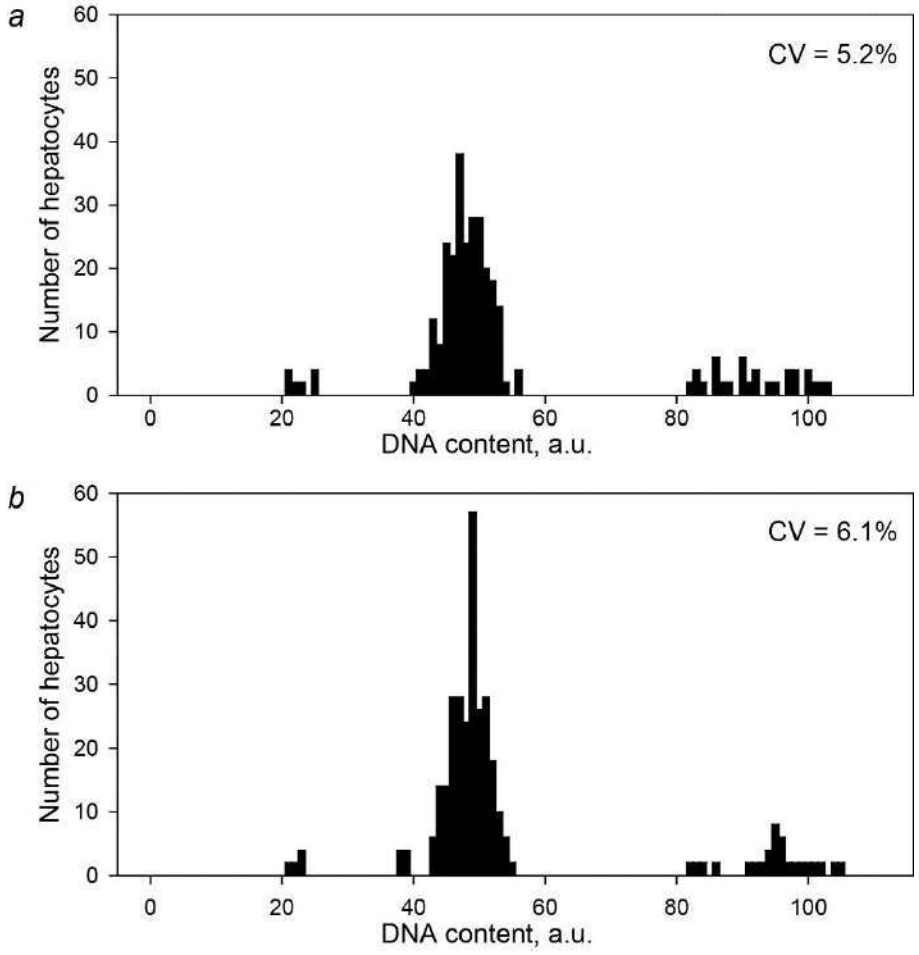


Figure 2.5 — Histograms of hepatocyte distribution in the normal rat liver based on their DNA content: *a* — hepatocytes stained after Feulgen, *b* — hepatocytes stained with the use of PAS reaction for glycogen, treated with 0.025% sodium borohydride and stained after Feulgen. Hepatocytes with DNA content 4c (D.I. = 2) are shown, because they are the dominant class of cells in the rat liver.

2.4. Use of confocal microscopy and FRET (AB) method for the study of the structure of glycogen molecules

Registration of $\mathbf{E_{FRET}}$ in several areas of a hepatocyte (usually 3–4 areas) (Fig. 2.6) was performed with a laser scanning confocal microscope Leica TCS SP5 (Leica Microsystems Inc., Germany) using the FRET AB (acceptor bleaching) programme (Zal, Gascoigne, 2004; Piston, Kremers, 2007). Auramine (Au) was used as the donor (Don) and ethidium bromide

(EtBr) was used as the acceptor (Ac). During bleaching, the luminescence intensity of Ac decreased by ~50%.

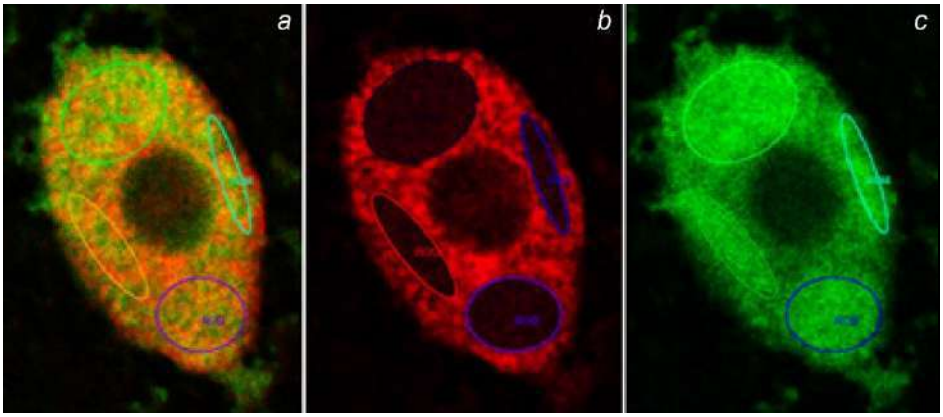


Figure 2.6 — Hepatocyte: *a* — with marked areas; *b* — after acceptor bleaching in marked areas; *c* — increase in fluorescence of donor (Don) after acceptor (Ac) bleaching. Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Inc., Germany). HCX PL APO 40×/1.25 Oil objective.

Two lasers were used in the determination of $\mathbf{E_{FRET}}$: 405 nm (Don excitation) and 514 nm (Ac bleaching). The power of the 514 nm laser was 30% of the maximum. An HCX PL APO 40×/1.25 Oil lens was used in the measurements. Immersol 518F was used as immersion and non-fluorescent vaseline oil was used as the embedding medium. At least 25 cells were measured on each preparation (Bezborodkina et al., 2011).

The distance between Don (Au) and Ac (EtBr) in glycogen molecules was calculated using the formula:

$$\mathbf{r = R_0 \times \sqrt[6]{\frac{1-E_{FRET}}{E_{FRET}}}} \qquad \mathbf{(4)}$$

where: $\mathbf{E_{FRET}}$ is the FRET efficiency value; $\mathbf{R_0}$ is the critical distance at which $\mathbf{E_{FRET}}$ = 0.5.

$\mathbf{E_{FRET}}$ is known to depend on the physical properties of Don and As, namely, the degree of overlap between their excitation and fluorescence spectra, their polarisation, the quantum yield of the donor etc., and most importantly, the distance between them (Piston, Kremers, 2007; Fessenden, 2009):

$$\mathbf{E_{FRET} = \frac{1}{\left(1+\left(\frac{r}{R_0}\right)^6\right)}} \qquad \mathbf{(5)}$$

where: $\mathbf{E_{FRET}}$ is FRET efficiency; $\mathbf{r}$ is the distance between Don and Ac; $\mathbf{R_0}$ is the critical distance at which $\mathbf{E_{FRET}}$ = 0.5.

Figure 2.7 shows absorption and fluorescence spectra of cells stained with EtBr-SO_2 or Au-SO_2 during PAS reaction. Fluorescence spectra were obtained using a Leica TCS SP5 microscope (Leica Microsystems Inc., Germany). The absorption spectra of Au and EtBr are given according to the literature data (Gadella, 1999). It is important to note that the fluorescence spectrum of hepatocytes stained with Au-SO_2 ($\lambda_{max} \approx 526$ nm) and the absorption spectrum of hepatocytes stained with EtBr-SO_2 ($\lambda_{max} \approx 540$ nm) overlap.

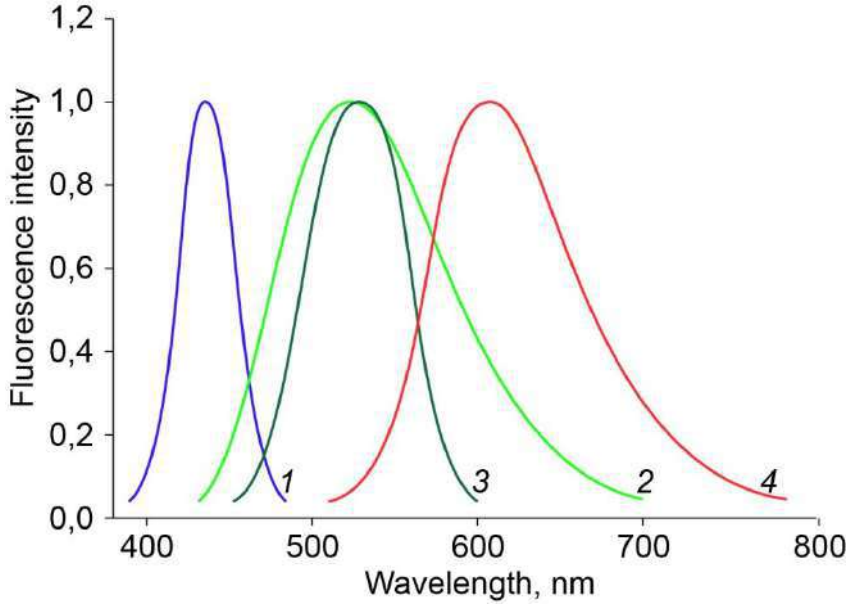


Figure 2.7 - Absorption spectra of Au (***1***) and EtBr (***3***) and fluorescence spectra of Au (***2***) and EtBr (***4***).

Our calculations with the use of PhotochemCAD software (www.photochemcad.com) showed that $\mathbf{R_0}$ value for the Au-EtBr pair used in our work is 3.2 nm. In the calculations, Au quantum yield value was taken to be 0.3 (Vieira Ferreira et al., 2004) and EtBr extinction coefficient was taken to be 5680 M^{-1} cm^{-1} (Lee et al., 2001). Since the radius of the 12-tier β-particle of glycogen is about 21 nm (Rybicka, 1996; Melendez et al., 1999), it can be assumed that the Förster interaction involves Don (Au) molecules bound to glucose residues of the 7th and the 8th internal tiers of the β-particle and Ac (EtBr) molecules located on the 9th and the 10th external tiers of the β-particle (Fig. 2.8) (Bezborodkina et al., 2011).

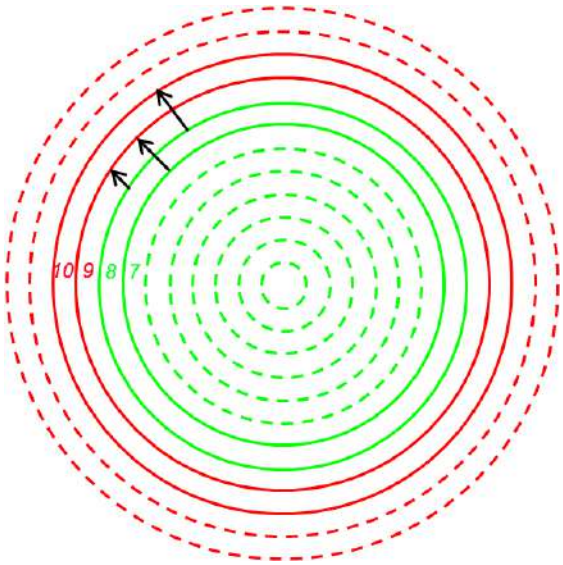


Figure 2.8 — Schematic representation of a glycogen molecule. Numbers indicate tiers between which energy transfer is possible. The distance between each tier in the β-particle of glycogen with a diameter of ~ 42 nm is ~ 1.9 nm.

2.5. Biochemical methods

2.5.1. Biochemical study of rat and human blood serum

Rat and human blood samples obtained during the postabsorptive period of the food cycle were centrifuged at 3500 rpm for 10 min at 4˚C. The glucose concentration, total bilirubin and total protein, alanine aminotransferase (AlAT), aspartate aminotransferase (AsAT) and alkaline phosphatase (ALP) activities were determined in the serum using automated biochemical analysers SMA-12/16 (Technicon Instruments Co., USA) and Abbot-spectrum (Abbot Laboratories, USA).

LP products were determined by the oxidation rate with lipohydroperoxides of divalent iron, which reacts with ammonium thiocyanate to form a compound determined by spectrophotometry at a wavelength of 480 nm. The concentration of diene conjugates (DC) was estimated according to Romanova and Stalnaya (1977) and that malonic dialdehyde (MDA), according to Uchigama and Michera (1978). Reduced glutathione was determined after Seldak and Linday (1968). Superoxide dismutase (SOD) activity was determined by inhibition of nitroblue tetrazolium reduction in the presence of NADH and phenazine methasulphate (Dubinina, 1983). The activity of catalase was determined by the rate of

hydrogen peroxide decomposition. The activity of all the above enzymes was related to the protein content in the samples determined using the Lowry method (Lowry, 1951).

2.5.2. Determination of glycogen concentration in rat liver

Liver pieces were dissolved in 30% KOH in a boiling water bath for 60 min, centrifuged at 1000g for 30 min, then washed sequentially with 70, 80 and 96% ethanol and centrifuged again. The resulting glycogen precipitate was hydrolysed with 2n H_2SO_4 on a boiling water bath for 2.5 h. The hydrolysate was neutralised with 5n NaOH to pH 7.8–8.0 and the amount of glucose formed in it was determined by glucose oxidase method using standard kits. Glycogen concentration was expressed in μM glucose units per g of raw liver mass (Lo et al., 1970; Bezborodkina et al., 2014; Bezborodkina et al., 2021b).

2.5.3. Determination of activity of the key enzymes of glycogen metabolism in rat and human liver

Rat livers and biopsy samples of patients were homogenised in 50mM Tris-HCl buffer (pH 7.4) containing 5mM EDTA, 200mM sucrose, 0.01M β-mercaptoethanol and 0.2M FMSF on ice (1:10). The homogenate was centrifuged at 1000g, 4˚C, for 10 min. The resulting supernatant was centrifuged at 14000g, 4˚C, for 10 min. The newly obtained supernatant was used to determine the activity of cytoplasmic enzymes. For determination of G6Pase activity, the supernatant was additionally centrifuged at 106000g, 4˚C, for 60 min to isolate the microsomal fraction (Berteloot et al., 1991).

Glycogen synthase (GS) activity was measured based on the amount of [U-^{14}C]-glucose incorporated into glycogen using UDP-[U-^{14}C]-glucose (300 mCi/mM, Amersham, USA) as a substrate (Vardanis, 1992). We added 40 μl of the sample to 75 μl of 10 mM Tris-HCl buffer (pH 7.5) containing 0.17 mM EDTA, 100 mM NaF, 2.5 mM $MgSO_4$, 10% glycogen and 0.25 mM UDP-glucose (40000 imp/min). The mixture was incubated in 7.2 mM glucose-6-phosphate at 30˚C for 10 min to determine the activity of the D-form of GS, and in 0.17 mM glucose-6-phosphate to determine the activity of the I-form. The reaction was arrested by application of 0.5 ml of 96% ethanol to 1.5 cm^2 filters (Whatman 3 mm) containing 50 μl of the reaction mixture. The filters were then washed off the non-included label in two changes of

66% ethanol for 30 min each time and in one change of acetone for 5 min. The dried filters were placed in vials containing ZS-7 scintillator, and the number of impulses was recorded using a counter (Beckman, Indianapolis, USA) (Bezborodkina et al., 2014).

Glycogen phosphorylase (GP) activity was determined using the inverse reaction of glycogen synthesis *in vitro*. The amount of [U-^{14}C]-glucose incorporated into glycogen was measured using [U-^{14}C]-glucose-1-phosphate (286 mCi/mM, Amersham, USA) as a substrate (Vardanis, 1992). The reaction was carried out in 50 mM Tris-HCl buffer (pH 6.7) containing 100 mM NaF, 10% glycogen, and 10 mM glucose-1-phosphate (35000 imp/min). When *a*-form of the enzyme was detected, 0.5 mM caffeine was added to the reaction mixture; *b*-form was activated by adding 10 mM AMP. Incubation was carried out at 37˚C for 5 min after addition of 35 µl of the sample. The subsequent operations for impulse counting were performed as described above (Bezborodkina et al., 2014).

Glucose-6-phosphatase (G6Pase) activity was determined in resuspended precipitate of the microsomal fraction using [U-^{14}C]-glucose-6-phosphatase (49mCi/mM, Sigma-Aldrish, USA) as a substrate. For this purpose, 20 µl of the microsomal suspension and 80 µl of the reaction mixture (pH 6.5) containing 50mM cacodylic acid, 2mM EDTA and 10mM glucose-6-phosphate (30000 imp/min) were incubated for 20 min at 30˚C. After incubation, 50 µl of the mixture was loaded into a 1.2 cm high column with Dowex-1 anion exchange resin (x8, in acetate form) and washed with two 0.5 ml portions of water. Effluents were collected in vials and used for impulse counting (Bezborodkina et al., 2013).

Glucokinase (GK) activity was measured in a mixture containing 30mM Tris-HCl (pH 7.5), 50mM glucose, 30mM $MgCl_2$, 50mM ATP, 1mM NADPH and glucose-6-phosphate dehydrogenase (2U/ml). The rate of NADPH reduction was recorded every 15 sec for 3 min at 340 nm wavelength after adding 1 ml of the reaction mixture and 20 µl of the homogenate (Ueda et al., 1974) to the cuvette of a Specol 11 spectrophotometer (Carl Zeiss, Jena, Germany) (Bezborodkina et al., 2013).

Phosphofructokinase (PFK) activity was determined in 75mM Tris-HCl buffer (pH 7,5) containing 3mM $MgCl_2$, 15mM $(NH_4)_2SO_4$, 30mM fructose-6-phosphate, 15mM ATP, 1.5mM NADH, 10U/ml aldolase, 8U/ml triosophosphate isomerase and 4U/ml glycerophosphate dehydrogenase. The reaction rate was determined every minute for 5 min based on the decrease rate of NADH after its addition to a Specol 11 spectrophotometer cuvette (Carl Zeiss,

Jena, Germany) containing 1.6 ml of the mixture and 0.1 ml of the sample (Uyeda, 1979) (Bezborodkina et al., 2013).

To determine *fructose-1,6-bisphosphatase (F1,6BPase) activity,* 50mM Tris-HCl buffer (pH 7.5) containing 2mM $MgCl_2$, 40mM $(NH_4)_2SO_4$, 0.15mM fructose-1,6-bisphosphate, 0.3 mM NADPH, 0.1 mM EDTA, 0.7 U/ml glucose-6-phosphate dehydrogenase and 1 U/ml glucose phosphate isomerase. A cuvette containing 1 ml of incubation mixture and 20 µl of the sample was pre-incubated at 37˚C for 5 min. After that, NADPH content was measured every minute for 5 min (Tejwani et al., 1976) (Bezborodkina et al., 2013).

Total protein was determined according to Bradford (Bradford, 1976) using Coomassie brilliant blue G-250. Protein concentration was calculated using a standard calibration curve. The data obtained by photometry of different amounts of BSA (λ = 595 nm) were used to construct the calibration curve.

2.6. Morphometry of rat and human liver histological sections

Relative volume of the connective tissue and the liver parenchyma was determined on histological sections stained with hematoxylin-picrofuchsin or picrosirius using a "VideoTest" image analyser equipped with a 10×/0.30 objective lens and an interference light filter λ_{max} = 500 nm. For each animal and patient, 20–30 fields of view were analysed. The ratio of the connective tissue was calculated according to the formula:

$$\mathbf{Q} = \frac{\mathbf{S_{c.t.}}}{\mathbf{S_{f.w.}} - \mathbf{S_{r.}}} \quad \textbf{(6)}$$

where: **Q** — ratio of the connective tissue to the cut area; $\mathbf{S_{f.w.}}$ — area of the field of view of the microscope (µm^2); $\mathbf{S_{c.t.}}$ — area of the connective tissue (µm^2); $\mathbf{S_{r.}}$ — area of lumen of vessels and ruptures in tissues (µm^2).

The number of hepatocytes in the liver was determined using the formula:

$$\boldsymbol{N} = \boldsymbol{P} \times \boldsymbol{R} \times \frac{\boldsymbol{f}}{\boldsymbol{M}} \quad \textbf{(7)}$$

where: **N –** number of hepatocytes in the liver; **P** – wet weight of liver mass, g; **R** – ratio of the liver parenchyma; **f** – transition coefficient from wet weight of liver mass to dry mass; **M –** average dry mass of one hepatocyte, g. The coefficient **f** was taken to be 0.27 (Bezborodkina et al., 2016; Bezborodkina et al., 2021b).

2.7. Morphometry of the mitochondrial apparatus of rat hepatocytes

Using the "VideoTest" image analyser, the perimeters of mitochondria and the length of their cristae were measured on electronograms with a magnification of 20000x. The concentration of mitochondrial inner membranes (MIMC) was calculated using the formula:

$$\text{MIMC} = (\mathbf{p} + \mathbf{2l}) \times \mathbf{n} \qquad \textbf{(8)}$$

where: **p** – average perimeter of mitochondria; **l** – average length of cristae in one mitochondrion; **n** – number of mitochondria per unit area.

Electron microphotographs taken at 8000x magnification were used to determine the number of mitochondria and their volume per unit area of the cell.

Five rats from the control group and five rats from the experimental group were used for electron-microscopic study. For each animal, 20 hepatocytes were analysed (Bezborodkina et al., 2008).

2.8. Determination of the contribution of proliferation, polyploidization and hypertrophy to liver mass increase

Relative contribution of proliferation ($\mathbf{Q_1}$), polyploidization ($\mathbf{Q_2}$) and hypertrophy ($\mathbf{Q_3}$) of hepatocytes during the normal and the reparative growth of rat and human liver was calculated using the following formulae (Bogdanova et al., 1990):

$$\mathbf{Q_1} = \frac{\mathbf{M} \times (\mathbf{P_1}/\mathbf{P_2}) - \mathbf{1}}{\mathbf{M} - \mathbf{1}} \qquad \textbf{(9)}$$

$$\mathbf{Q_2} = \frac{\mathbf{M} \times \mathbf{m_1} \times (\mathbf{g_2} - \mathbf{g_1})}{(\mathbf{M} - \mathbf{1}) \times \mathbf{P_2}} \qquad \textbf{(10)}$$

$$\mathbf{Q_3} = \frac{\mathbf{M} \times \mathbf{g_2} \times (\mathbf{m_2} - \mathbf{m_1})}{(\mathbf{M} - \mathbf{1}) \times \mathbf{P_2}} \qquad \textbf{(11)}$$

where: **M** — repetition factor of liver parenchyma mass change during the study period (6 months). Based on the data on the value of mitotic index, duration of mitosis in hepatocytes and the level of parenchyma necrotisation during repeated exposure to CCl_4, it was calculated that the loss of parenchyma mass during 6 months of exposure exceeds the initial mass approximately 5-fold. In case of physiological regeneration the loss of parenchyma mass due to cell death during the same period is equal to its initial mass (Sakuta, Kudryavtsev, 1996); $\mathbf{P_1}$ and $\mathbf{P_2}$ — dry mass of hepatocyte before the beginning of poisoning of rats with CCl_4 and at the end of the experiment, respectively; $\mathbf{m_1}$ and $\mathbf{m_2}$ — average dry mass calculated per diploid

hepatocyte before the beginning of poisoning of rats with CCl_4 and at the end of the experiment, respectively; $\mathbf{g_1}$ and $\mathbf{g_2}$ — average ploidy of hepatocytes divided by 2.

2.9. Statistical processing of the obtained data

Statistical processing of the results was performed on a personal computer using the standard software package Microsoft Excel (Microsoft Corporation, USA) and SigmaPlot v. 9.0, v. 11.0 (Systat Software Inc., USA). Data in charts and graphs were presented as the mean value and its error (X ± Sx). Reliability of differences between values was assessed using Student's t-criterion. Correlation analysis with determination of correlation coefficients (r) was performed to assess the dependence between the parameters. Linear regression was calculated using the formula:

$$\bar{\mathbf{y}}_{\mathbf{x}} = \mathbf{a} + \mathbf{b} \cdot \mathbf{x} \quad \textbf{(12)}$$

Where: **a** — free term

$$a = \frac{\sum y \cdot \sum x^2 - \sum x \cdot \sum yx}{n \cdot \sum x^2 - (\sum x)^2}$$

b — regression coefficient

$$b = \frac{n \cdot \sum xy - \sum x \cdot \sum y}{n \cdot \sum x^2 - (\sum x)^2}$$

n — cell count.

Pearson's table of critical correlation values was used to assess the validity of the results.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Morphofunctional changes during chronic liver disease

3.1.1. Structure of the normal and the cirrhotic liver in rats and humans

Chronic hepatitis (CH) is a common inflammatory disease of the human liver. It can be caused by various toxins, long-term alcohol abuse, autoimmune diseases, genetic factors, and a number of metabolic disorders. One of the most frequent causes of CH are infections caused by hepatotropic viruses A (RNA-containing virus), B, C, D, G (DNA-containing viruses) and others. According to WHO data for 2019, carriers of hepatitis B and hepatitis C (HBV and HCV) made up more than 350 (296 and 58, respectively) million people worldwide (World Health Organisation, 2021). Chronic hepatitis is a continuous process of inflammation, destruction and regeneration of the liver parenchyma that lasts for more than 6 months and causes liver fibrosis. Tissue fibrosis is common in liver pathologies. During long-term liver damage, Ito cells, fibroblasts, and extracellular matrix gradually replace damaged hepatocytes, leading to the development of the final irreversible stage of CH, liver cirrhosis (LC). In turn, LC usually ends in liver failure and/or hepatocellular carcinoma (Kalaitzakis et al., 2011; Ganne-Carrié, Nahon, 2019).

Liver cirrhosis is characterised by nodular transformation of the parenchyma and the formation of fibrous septa connecting the portal system with the hepatic vein system. The main diagnostic features of LC, in addition to fibrosis of much of the parenchyma, are considered to be pronounced regeneration of hepatic tissue, unusually small portal tracts and excessive number of central veins in lobules, as well as intensive deposition of lipofuscin in pericentral hepatocytes (Schuppan, Afdhal, 2008).

Analysis of histological liver sections of rats showed that the structure of liver parenchyma of control animals was typical for the normal organ: it had radially located trabeculae of hepatocytes around central vessels, a small amount of connective tissue, and well-defined borders of portal vessels and bile ducts (Fig. 3.1, *a, b*). Liver sections of cirrhotic rats showed diffuse leukocytic inflitrations indicating activation of inflammatory processes and signs of fibrotisation (Fig. 3.1, *c*). Strands of connective tissue grow into the parenchyma,

violating the lobular structure of the organ. Picrosirius staining revealed connective tissue fibres along the sinusoids (Fig. 3.1, *d*) (Bezborodkina et al., 2013b, 2014, 2021).

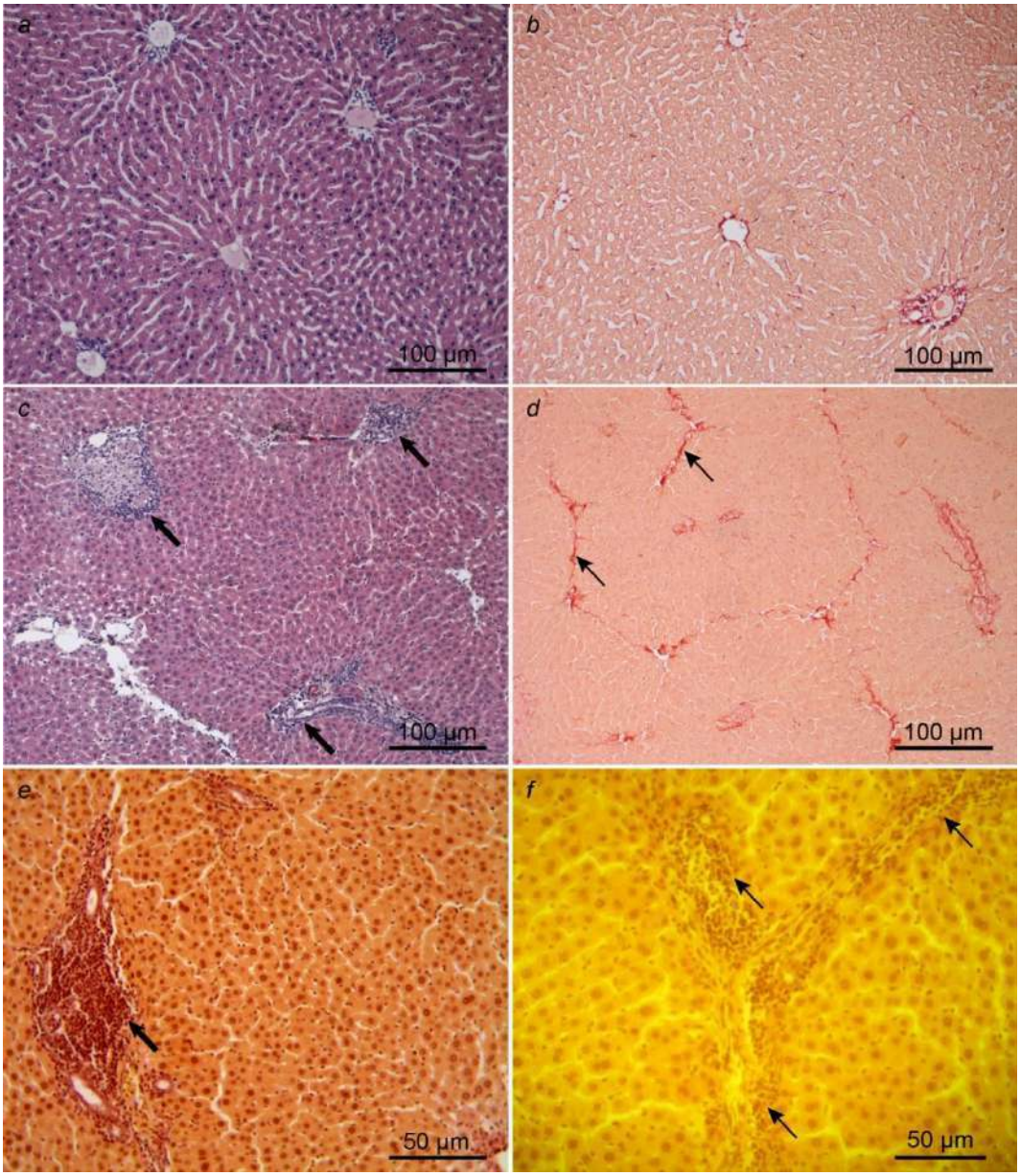


Figure 3.1 — Rat liver sections: *a*, *b* – normal; *c*, *d, e*, *f* – cirrhotic. *a*, *c*, *e* - hematoxylin-eosin staining; *b*, *d* - picrosirius staining, *f* – van Gieson staining. Note: bold arrows indicate foci of inflammation (*c*, *e*), thin arrows indicate connective tissue bands along the sinusoids (*d*, *f*).

The morphological picture of liver damage during CCl_4 exposure has been described in considerable detail (Zimmerman, 1978; Onori, 2000). As a result of CCl_4 intoxication, centrilobular necrosis of hepatocytes leads to the replacement of necrotic zones by the connective tissue and stimulation of cell proliferation in regeneration nodules. In this case, connective tissue is formed not only at the central veins, but also along the course of the entire

venous channel. Inflammatory reaction in portal fields is also accompanied by connective tissue outgrowth along the course of portal vessels. Proliferation of hepatocytes stimulated by their death leads to the appearance of multi-row beams and then to disorderly clusters of hepatic cells.

It is believed that LC in rats caused by prolonged repeated exposure to CCl_4 is similar to cirrhotic liver damage in humans (Kohno et al., 1991; Skobeleva, 1994; Datsko et al., 2020). Microscopic analysis of human liver sections indicates that in the norm the structure of human liver parenchyma, similarly to that in the rat, is characterised by radially located trabeculae of hepatocytes around central veins, well-defined portal vessels and bile ducts (Fig. 3.2, *a*). During development of cirrhosis**,** the lobular structure of the human liver parenchyma, as in the rat, is impaired. The so-called "false lobules" are formed, in which cellular and nuclear polymorphism is sharply expressed, mass death of hepatocytes is observed in the parenchyma, and the proportion of the connective tissue increases (Fig. 3.2, *b*) (Kudryavtseva et al., 2002a; Okovity et al., 2010; Bezborodkina et al., 2021b).

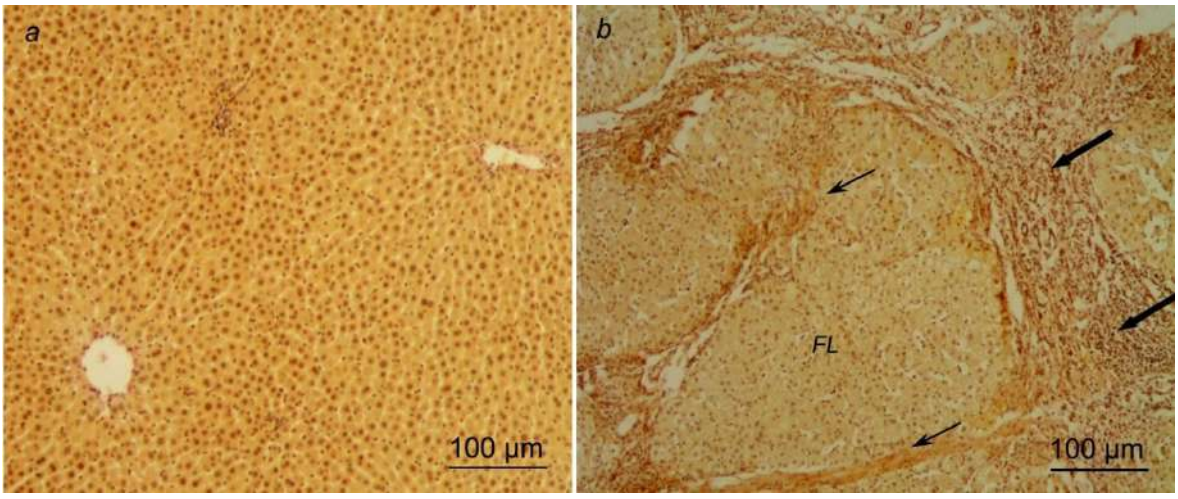


Figure 3.2 — Human liver sections: *a* - normal, *b* - cirrhotic. Haematoxylin-picrofuchsin staining. Notes: 1) FL - false liver lobe; 2) foci of necrosis are marked by bold arrows, thin arrows - connective tissue layers (*b*).

Fibroplasia dramatically changes the ratio of parenchymal and non-parenchymal elements in favour of the latter. The mutually exclusive dynamics of the connective tissue and the parenchyma content during LC development is a characteristic feature of this pathology (James et al., 1986; Reichen et al., 1987).

Morphometry of the normal and the cirrhotic rat liver has established that during LC the proportion of the connective tissue increases 7.7-fold (Table 3.1). At the same time, the mass

of rat liver during LC development increases from 9.72±0.37 g to 11.56±0.55 g ($p < 0.05$). Therefore, despite the fact that the proportion of the parenchyma during cirrhosis in our experiments decreased by 8.8% (Table 3.1), its absolute mass did not differ from the mass of the parenchyma of control animals (Bezborodkina et al., 2021b). A slight increase or stability of the mass of the rat liver in case of its pathologies has also been noted by other authors (Xu et al., 2010; Wang et al., 2015; Rasineni et al., 2021).

Table 3.1 — Some morphometric parameters of the normal rat liver and the cirrhotic rat liver (LC) ($X \pm S_x$, n = 5).

Parameter	Control	LC
Liver weight, g	9.72±0.37	11.56±0.55 [1]
Relative liver weight, %	3.00±0.06	4.33±0.09 [2]
Proportion of parenchyma, %	88.0±1.1	79.2±1.3 [2]
Proportion of the connective tissue, %	0.84±0.07	7.31±0.27 [2]
Number of hepatocytes in 1 g of liver	$2.52 \pm 0.15 \times 10^8$	$1.81 \pm 0.21 \times 10^{8}$ [1]

[1,2] Significantly different from the value in the control ([1] $p < 0.05$, [2] $p < 0.001$).

The results presented in Table 3.2 show that, in contrast to the cirrhotic rat liver, the proportion of the liver parenchyma in the cirrhotic human liver decreases as compared to the norm by 40.4%, while the proportion of the connective tissue increases almost 20-fold. In contrast in rats, the mutually exclusive dynamics of the connective tissue and the parenchyma during LC development in humans can be traced very clearly (Bezborodkina et al., 2021b).

Table 3.2 — Some morphometric parameters of the normal liver and the cirrhotic liver (LC) in humas ($X \pm S_x$).

Parameter	Control (n = 10)	LC (n = 13)
Proportion of parenchyma, %	90.6±0.9	54.0±2.5[2]
Proportion of the connective tissue, %	1.79±0.08	36.5±3.3[2]
Number of hepatocytes in 1 g of liver	$4.81 \pm 0.27 \times 10^8$	$2.32 \pm 0.29 \times 10^{8}$ [2]

n - number of patients in the group; [1,2]significantly different from the value in the control ([1]$p < 0.05$, [2]$p < 0.001$).

The reasons for such a strong fibrotisation of the liver parenchyma and a more intensive death of hepatocytes during cirrhosis development in humans as compared with rats are unclear. Apparently, they are associated not only and not so much with the nature of agents causing liver damage, nor with the difference in the methods of obtaining experimental LC. In explaining the reasons for the considerable differences in the degree of pathological changes between the human and the rat liver, we should first of all pay attention to the fundamental difference between these mammalian species, their size, and thus a different metabolic rate. The body mass of adult rats used in our experiments was ~0.3 kg, while the human body mass was ~70 kg. The specific metabolic rate of a rat with a body mass of 0.3 kg is 94.6 kcal/kg/24 h, whereas that of an adult human with a body mass of ~70 kg is 24.2 kcal/kg/24 h (Schmidt-Nielsen, 1987), i.e. the metabolic rate in the rat is almost 4 times higher. This significant difference means that the rate of various processes, including proliferative ones, is much higher in rats than in humans. It has been shown that in the normal human liver the ratio of hepatocytes in the S-phase of the cell cycle is 0.02–0.05% (Leevy, 1967; Takehara, 1975), while in rats it is 0.2–1.5% (Ryabinina, Benyush, 1973). Another indicator of proliferative activity, the mitotic index, varies in the normal human liver between 0.0001–0.0007% (Kudryavtsev et al., 1991), whereas in the normal rat liver it is 0.005–0.1% (Ryabinina, Benyush, 1973; Mangnall et al., 2003). Small connective-tissue cells (fibroblasts, macrophages, etc.) have a higher proliferative activity than hepatocytes. Therefore, it can be assumed that the rate of replacement of dying hepatocytes by these cells during the development of LC in humans will be much higher than in rats. It is probably due to this fact that the proportion of fibrous tissue in the human cirrhotic liver is much higher than in the rat cirrhotic liver.

In any case, the increased ratio of the connective tissue negatively affects the vital activity of hepatocytes, which is expressed, first of all, in the impairment of metabolic processes between cells. In addition, an increased fibrosis of the liver leads to the disruption of the normal functioning of the hepatic lobule due to the formation of septa connecting the centrilobular zone with the periportal zone by vascular shunts. Finally, the connective tissue growth promotes the formation of the basal membrane and the transformation of sinusoids into stiff capillaries. All these processes result in a significant rearrangement of the lobular structure of the liver (Fig. 3.1, *c, d, e, f,* Fig. 3.2, *b*) and a lower supply of hepatocytes with oxygen, nutrients and energy sources (Bezborodkina et al., 2021b; Okovity et al., 2022).

3.1.2. State of the mitochondrial apparatus of hepatocytes in the normal and the pathological liver of rats

In hepatocytes, as in many other cell types, ATP is a universal source of energy. Approximately 90% of the oxygen consumed by mammalian cells is used by mitochondria to produce ATP by oxidative phosphorylation (Javadov et al., 2018). The rate of oxidative processes in cells is closely related to the number and structure of mitochondria. The liver is one of the most mitochondria-rich rich organs. One hepatocyte is known to contain about 2000 mitochondria (David, 1977), which have unique properties as they integrate the metabolism of carbohydrates, lipids and proteins into a single centre (Degli Esposti et al., 2012).

Electron-microscopic study of mitochondria in hepatocytes of the cirrhotic rat liver revealed changes in the shape and an increased in the size of these organelles (Fig. 3.3).

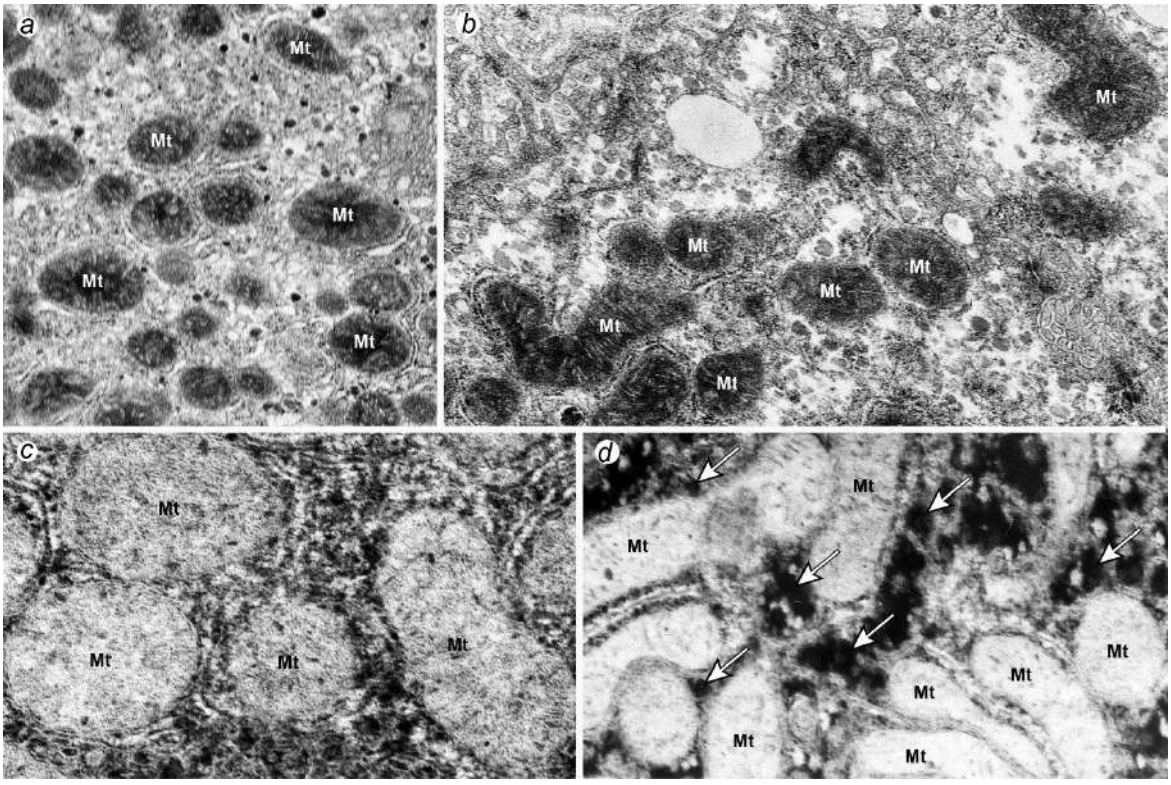


Figure 3.3 — Mitochondria of rat hepatocytes of control (*a*, *c*) and experimental group (*b*, *d*). *a*, *b* - 8000x, *c*, *d* - 20000x. Note: Mt - mitochondria. Arrows mark glycogen granules.

However, these changes could have been the result of treatments applied to tissues in preparation to electron-microscopic studies. Therefore, we investigated the morphology of

mitochondria in the primary culture of hepatocytes isolated from the livers of control rats and rats with toxic hepatitis. It was found that an intragastric administration of CCl_4 for a month and the replacement of drinking water with 5% ethanol led to fibrosis and fatty degeneration of the liver, accompanied by inflammation and disruption of its lobular structure (Okovity et al., 2007). Visual analysis of hepatocytes stained with rhodamine 123 showed that mitochondria in the cells of the control and the experimental animals were slightly different. Basically, these differences were expressed in the fact that many mitochondria had a more elongated shape and a more uniform distribution in the cytoplasm of the control hepatocytes as compared with those of experimental animals (Fig. 3.4) (Baidyuk et al., 2009).

Morphometry of the mitochondrial apparatus in the hepatocytes showed that mitochondrial volume density (MVD) in the control rats was 19.5±0.7%, which agrees well with the data of other authors (David, 1977; Anatskaya, 1999; Das et al., 2012). At the same time, MVD in the liver parenchyma of the entire liver of the control rats was 1.667±0.136 arbitrary units (Fig. 3.5, *b*) (Bezborodkina et al., 2008).

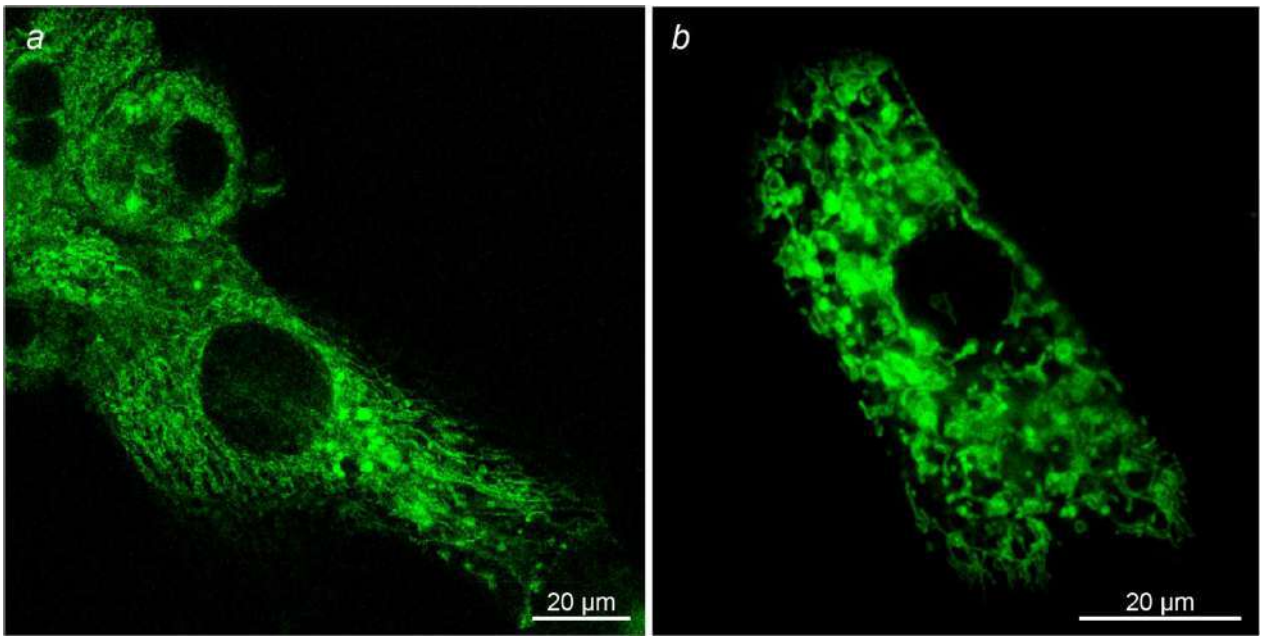


Figure 3.4 — Mitochondria of live hepatocytes stained with rhodamine 123. *a* - hepatocytes of intact rats, *b* - hepatocytes of rats after exposure to CCl_4 and ethanol.

CCl_4-induced cirrhosis does not lead to a change in the number of mitochondria in the parenchyma (Fig. 3.5, *a*) but causes an increase in MVD: on average, up to 25.0±1.0% per hepatocyte ($p < 0.01$). A similar value of MVD was found in rat hepatocytes after prolonged ethanol exposure (Das et al., 2012). The results presented in Fig. 3.5, *b* indicate that MVD in

the entire parenchyma of the cirrhotic liver exceeds the control value by 37.4% ($p < 0.01$) (Bezborodkina et al., 2008).

The increase of MVD in cells can be associated both with an increased oxidative metabolism in mitochondria and with their swelling, which is accompanied by a decrease in ATP production (Kaasik et al., 2007; Javadov et al., 2018). Therefore, to identify the nature of the increase in MVD in hepatocytes during LC, we performed morphometry of the inner membranes of mitochondria, where specific proteins catalysing oxidative reactions in the respiratory chain are situated. These proteins are required for oxidative phosphorylation, through which most ATP is synthesised in the cell.

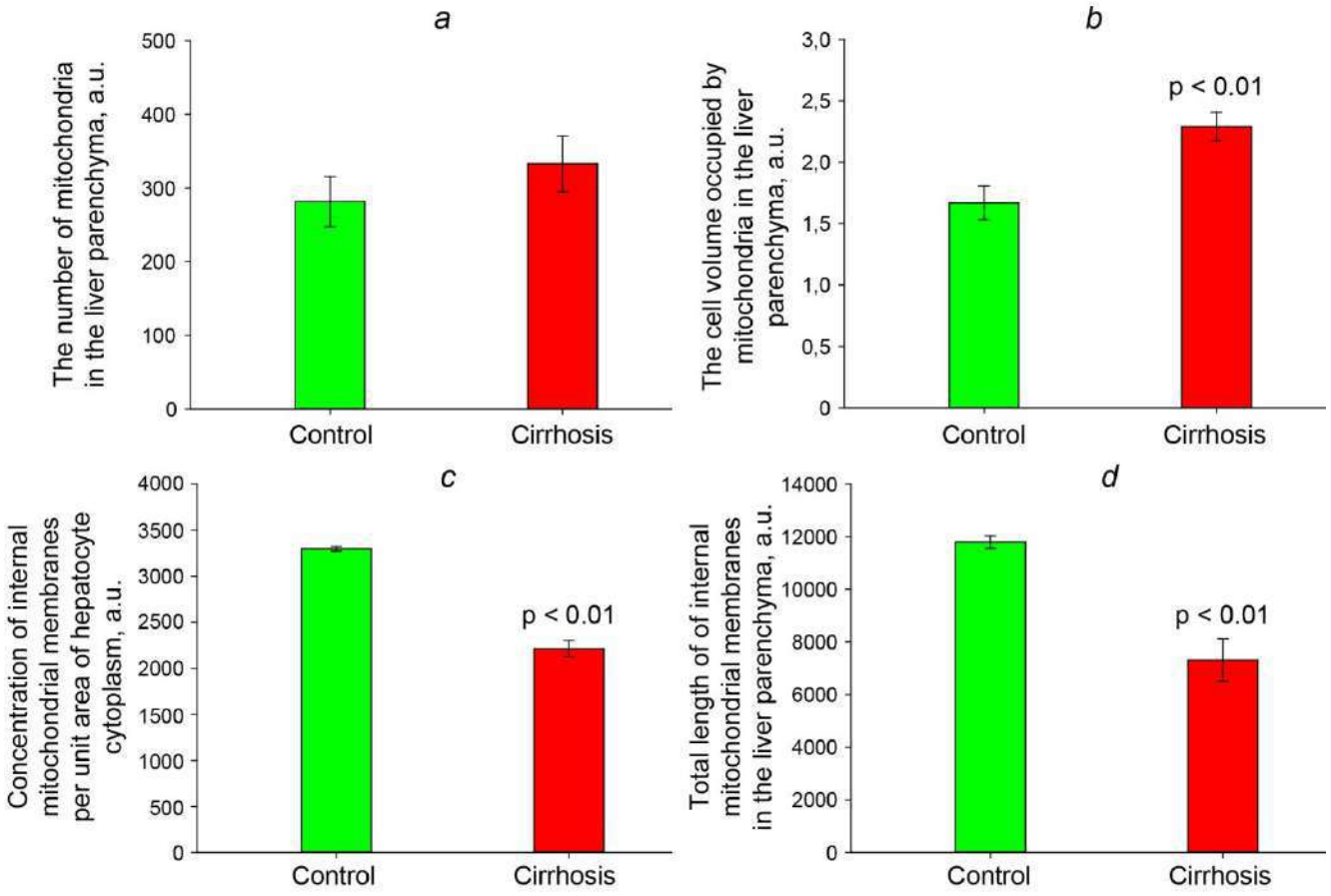


Figure 3.5 — Some morphometric indices of mitochondria in hepatocytes of normal rats (control) and rats with liver cirrhosis (LC) ($X \pm S_x$, $n = 5$).

The concentration of mitochondrial inner membranes per unit area of hepatocyte cytoplasm during LC decreased by 30.7% (Fig. 3.5, *c*), while the total length of inner membranes per one mitochondrion decreased almost twofold (Fig. 3.5, *d*) (Bezborodkina et al., 2008).

Thus, the data obtained indicate that the respiration rate and the level of ATP production in mitochondria of the hepatocytes are significantly reduced during LC as compared with the norm. They also indicate that the concentration of inner membranes of mitochondria reflects the levels of respiration and ATP formation in hepatocytes more adequately than MVD.

An increase in the size of mitochondria in hepatocytes and a decrease in the number of their cristae in case of liver diseases have been noted by many authors (Krähenbühl, Reichen, 1992; Krähenbühl et al., 2000; Welt et al., 2004; Shami et al., 2021). Studies of the activity of the mitochondria respiratory chain enzymes such as NADH dehydrogenase (I-complex), succinate dehydrogenase (II-complex), cytochrome oxidase (IV-complex) and ATP synthase (V-complex) in case of chronic liver diseases has shown that the function these organoids is impaired (Fernandez-Checa et al., 1993; Liu et al., 1996; Krähenbühl et al., 2000; Huang et al., 2003; Yang et al., 2004).

Based on the above considerations, we can conclude that chronic liver diseases such as non-alcoholic fatty liver disease, alcoholic and viral hepatitis, primary hepatocarcinoma, thioacetamide- and CCl_4-induced liver cirrhosis etc., while strongly different in their pathophysiology, cause the same kind of defect of the mitochondrial apparatus of hepatocytes. Its consequence is a decrease in the respiration rate of mitochondria. Decreased respiration rate, in turn, leads to a significant drop of the rate of oxidative phosphorylation, a decreased ATP production and an increased concentration of reactive oxygen species (Harvey et al., 1999; Fukumura et al., 2003; Middleton, Vergis, 2021). The fall in ATP production affects the ATP-dependent sodium pump leading to swelling and death of the cells (Chapa-Dubocq et al., 2018).

3.1.3. Characterisation of tissue-specific functions of rat and human liver during cirrhosis

It is well known that the efficiency of hepatocyte performance is closely related to the rate of ATP formation. Since the rate of oxidative phosphorylation and ATP concentration in hepatocytes during CCl_4-cirrhosis decrease noticeably, it can be expected that the lack of energy-rich molecules will inevitably affect the intensity of tissue-specific liver functions. Their level is usually assessed based on the activity of the corresponding enzymes in its tissue or the concentration of the marker products of metabolism in the blood serum.

The data presented in Figure 3.6 indicate that the concentration of total bilirubin in the serum of rats of the experimental group and in patients with cirrhosis increases while that of the total protein decreases as compared to the norm. Changes in these indices during cirrhosis indicate the disturbance of pigment metabolism in the liver and a decrease in its protein synthetic function. The duration of hexenalum sleep, which reflects the ability of the microsomal oxidation system in hepatocytes to metabolise various xenobiotics, including hexenalum, is increased in rats with LC more than 5-fold ($p < 0.001$) as compared to the norm. The data obtained indicate a decreased efficiency of the microsomal oxidation system in hepatocytes in the pathological liver (Kudryavtseva et al., 2003; Okovity et al., 2006; Bezborodkina et al., 2014).

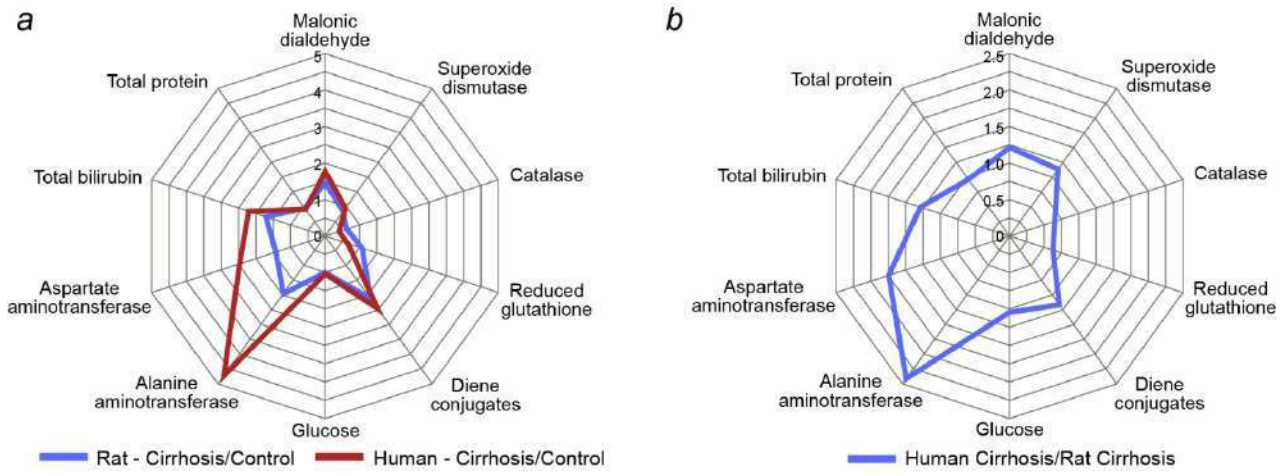


Figure 3.6 — Some serum biochemical parameters in the normal liver (Control) and the cirrhotic liver (Cirrhosis) of rats and humans.

Thus, we can conclude that the development of LC is accompanied by a slump of the protein synthesis and the detoxifying function of the liver. Similar results were obtained by other authors (Krähenbühl, Reichen, 1992; Welt et al., 2004; Young et al., 2006; Nishikawa et al., 2014; Wilson et al., 2014; Ju et al., 2016; Kietzmann, 2019). It has been shown that hypoxia during cirrhosis leads to the deterioration of several liver functions, including the barrier function and the protein synthesis (Skulachev, 1999; Harvey et al., 1999; Schuppan, Afdhal, 2008).

Hypoxia accompanies chronic liver impairments of any aetiology, increasing the production of free radicals and reactive oxygen species (ROS) by mitochondria (Poyton et al., 2009; Wilson et al., 2014; Ju et al., 2016; Prieto, Monsalve, 2017). Enhanced ROS production is a common characteristic of a sustained inflammatory response to liver damage. ROS cause

chain reactions with the accumulation of lipid radicals, resulting in changes in the properties of cell membranes and the formation of various LP products.

Data on the state of pro- and antioxidant systems in rats and humans indicate similar changes during cirrhosis development, but in humans they are more pronounced (Fig. 3.6, *b*) (Okovitiy et al., 2006; Bezborodkina et al., 2008; Bezborodkina et al., 2014; Bezborodkina et al., 2021b).

Concentrations of malonic dialdehyde and diene conjugates in the blood serum of cirrhotic rats increased as compared to the norm approximately 2.1-fold ($p < 0.05$) and 1.5-fold ($p < 0.001$), respectively, the corresponding values in the blood serum of cirrhotic human patients being 2.4-fold ($p < 0.01$) and 1.8-fold ($p < 0.001$), respectively. The increase in the concentration of LP products during the long-term pathological process is associated primarily with the depletion of the antioxidant system reserves. The results of this study indicate a marked decrease in the activity of the antioxidant system during LC. The activity of catalase in human blood serum during LC decreases as compared to the norm 2.5-fold ($p < 0.001$), while the concentration of reduced glutathione decreases 1.5-fold ($p < 0.05$). Rat serum catalase activity is decreased during LC 1.5-fold as compared to the norm ($p < 0.05$). A comparison of rats and humans by indicators such as total protein, MDA, superoxide dismutase and DC showed that the response of the liver to damage during pathophysiological conditions was comparable (Fig. 3.6, *b*). At the same time, the data in this figure suggest that the cirrhotic liver of humans is less capable of neutralising bilirubin, free radicals and peroxides. As a consequence, the human liver seems to be more vulnerable to the impact of damaging agents than the rat liver (Kudryavtseva et al., 2003; Okovitiy et al., 2006; Bezborodkina et al., 2008; Bezborodkina et al., 2014; Bezborodkina et al., 2021b).

When the capacity of the antioxidant system of the liver becomes insufficient to counteract the continuous flow of ROS, the membrane structures of cells are damaged (Ohyashiki et al., 1995; Skulachev, 1999), the function of hepatocytes deteriorates and they finally die by cytolysis. Cytolysis in the liver is usually indicated by an increase of the levels of indicator enzymes such as alanine aminotransferase (AlAT) and aspartate aminotransferase (AsAT) in the serum (Siegel et al., 2000). Our data (Fig. 3.6, *a*) indicate that the activity of AlAT and AsAT in rats with CCl_4-cirrhosis is increased as compared to the norm by the factor of 2.0 ($p < 0.001$) and 1.4 ($p < 0.01$), respectively. In patients with chronic hepatitis and cirrhosis, the levels of AlAT and AsAT increased as compared to the norm by the factor of 4.8

($p < 0.001$) and 2.0 ($p < 0.001$), 4.7 ($p < 0.001$) and 2.5 ($p < 0.001$), respectively (Fig. 3.6, *a*) (Kudryavtseva et al., 2003; Bezborodkina et al., 2014; Bezborodkina et al., 2021b). Comparison of AlAT and AsAT levels in the rat and the human liver showed that LC in humans is accompanied by a significantly more pronounced release of these enzymes into the blood than in rats. An intensive cytolysis of hepatocytes in the cirrhotic liver of humans is confirmed by a greater loss of hepatocytes during cirrhosis formation (Table 3.2).

The available data on the number of hepatocytes in the rat liver obtained using various methods indicate that their number varies from 1.29×10^8 to 2.64×10^8 cells/g of wet liver weight (Iype et al., 1965; Marcos et al., 2006; Sohlenius-Sternbeck, 2006; Baidyuk, 2013). According to our data, the number of hepatocytes in 1 g of the normal rat liver is 2.52×10^8 cells (Table 3.1). Chronic poisoning with CCl_4 for 6 months leads to a decrease in the number of hepatocytes to 1.81×10^8 cells/g liver (Table 3.2). The number of hepatocytes in 1 g of the normal human liver is 4.81×10^8, and in 1 g of the cirrhotic human liver, 2.32×10^8 cells (Table 3.2).

Data on the absolute number of hepatocytes in the normal and the cirrhotic liver of rats and humans show the number of hepatocytes in 1 g of liver decreases as cirrhosis develops: by 28.2% in rats and by 51.8% in humans (Table 3.1, Table 3.2) (Bezborodkina et al., 2021b). Taking into account that the liver mass in humans during cirrhosis decreases by 10–18% as compared to the norm (Apisarnthanarak et al., 2014; Simon et al., 2020), the total loss of hepatocytes during LC development in humans makes up about 45%.

Thus, our data indicate that the development of LC in humans is accompanied by deeper structural and functional changes than in rats, resulting, in particular, in a greater loss of hepatocytes, which progresses in the course of the pathological process.

3.1.4. Polyploidy and hypertrophy of rat and human hepatocytes in the norm and during chronic liver diseases

Continuous cell death during LC development stimulates liver regeneration. Its main cellular mechanisms are proliferation, polyploidisation and hypertrophy of hepatocytes (Bogdanova et al., 1990; Sakuta, Kudryavtsev, 1996; Sakuta, Kudryavtsev, 2005). The leading role in the reparative growth of the liver under acute or chronic action of agents of different aetiology is played by DNA-synthetic processes: proliferation and polyploidisation of

hepatocytes. They result in an increase in the number of hepatocytes in the liver and/or the number of genomes in the cells (Sakuta, Kudryavtsev, 1996; Sakuta, Kudryavtsev, 2005).

The proliferative response of the liver parenchyma to chronic exposure to hepatotoxins is characterised by a decrease in the probability of hepatocytes completing the cell cycle. As a result of incomplete cell divisions, binucleate cells and cells with polyploid nuclei appear in the parenchyma.

Cytophotometric analysis of hepatocyte distribution by ploidy classes in rats with CCl_4-cirrhosis of the liver showed that the composition of the cell population of the liver parenchyma in this group of animals significantly differs from the norm (Table 3.3) (Bezborodkina et al., 2016).

Table 3.3 — Distribution of rat hepatocytes by ploidy classes in the cell populations of the normal (control) and the cirrhotic liver (LC) ($X \pm S_x$, n = 5).

Group of rats	Proportion of hepatocytes of different ploidy classes, %						
	2c	2c×2	4c	4c×2	8c	8c×2	Average cell ploidy, c
Control	0.63±0.24	3.62±0.48	81.84±3.14	9.38±2.90	3.53±1.79	-----	4.46±0.15
LC	2.86±0.91[1]	2.13±1.45	68.51±3.95[1]	12.68±2.94	12.37±2.82[1]	1.45±0.62	5.12±0.17[1]

[1]Significantly different from the value in the control at $p < 0.05$.

The parenchyma of the cirrhotic rat liver is characterised by a decrease in the ratio of binucleate hepatocytes with diploid nuclei (2c×2-cells) and an increase in the relative number of cells with a high ploidy. The ratio of mononucleate octaploid (8c) hepatocytes increases 3.5-fold ($p < 0.05$) compared to intact rats of the same age. In addition, binucleate hepatocytes with two octaploid nuclei (8c×2-cells) are observed during LC; no such cells were found in the normal liver. Some cells in the cirrhotic liver reach a ploidy of 16c and even 32c (16c×2). As a result, the average ploidy level of hepatocytes of rats of the experimental group increases by 14.8% ($p < 0.01$) as compared to the norm (Table 3.3).

In contrast to rats, in humans the modal class of hepatocytes is represented by mononucleate diploid (2c) cells. The average ploidy of hepatocytes of the normal human liver was 2.21±0.05c, while in patients with chronic hepatitis it increased by 13.1% and 15.8%,

respectively. In the normal and the pathological liver parenchyma, mononucleate cells with diploid nuclei predominated (Table 3.4).

Table 3.4 — Distribution of human hepatocytes by ploidy classes in the normal liver, in the liver of patients with chronic hepatitis (CH) and in the cirrhotic liver (LC) ($X \pm S_x$).

	Proportion of hepatocytes of different ploidy classes, %					Average cell ploidy, c
	2c	2c×2	4c	4c×2	8c	
Control (n = 7)	89.57±2.28	4.70±1.47	5.73±1.38	-	-	2.21±0.05
CH (n = 5)	76.90±4.83[1]	15.46±2.96[1]	6.82±1.65	-	0.82±0.36	2.50±0.11[1]
LC (n = 7)	75.19±5.23[1]	16.22±3.81[1]	7.07±1.89	1.07±0.58	0.45±0.17	2.56± 0.17[1]

[1]Significantly different from the value in the control at $p < 0.05$.

Increased polyploidisation of hepatocytes in response to pathological effects is characteristic of mammals and is accompanied by changes in the ratio of cell ploidy classes (Maurer et al., 1973; Zavadskaya, 1989; Sakuta, Kudryavtsev, 1996; 2005). In particular, it has been found that the relative number of 2c- and 2c×2-hepatocytes decreases in the liver of rats and mice after chronic exposure to CCl_4 and cells with high ploidy, absent in the normal organ, appear (Factor, Uryvaeva, 1980; Brodsky, Uryvaeva, 1981). As a result, the ploidy of hepatocytes increases significantly (Zavadskaya, 1989; Kudryavtsev et al., 1993; Melchiorri et al., 1993; Sakuta, Kudryavtsev, 1996; 2005).

An increased proliferative activity and ploidy level of hepatocytes in the damaged liver is also characteristic of humans. For example, CH and LC of various aetiology, fatty hepatosis, extrahepatic cholestasis are also accompanied by a marked increase in the ploidy of hepatocytes (Koike et al., 1982; Fang et al., 1994; Melchiorri et al., 1994). At the same time, it should be noted that polyploidisation of hepatocytes during chronic liver damage in humans is never as great as in rats and mice. In addition, in contrast to rats and mice, the ratio of 2c×2-hepatocytes in humans is markedly increased in case of chronic liver pathologies (Table 3.4), which is in agreement with the earlier data (Kudryavtsev et al., 1993).

An important feature of the cell population of the liver parenchyma during cirrhosis is a 2.2-fold ($p < 0.05$) increase in the ratio of mononucleate diploid (2c) hepatocytes in rats and the maintenance of a high percentage of these cells (about 75%) in humans (Table 3.3, Table 3.4). Enhanced proliferation of 2c-hepatocytes in the cirrhotic rat liver and a high proportion

(more than 80%) of these cells in the cirrhotic human liver have been previously observed by other authors (Kudryavtsev et al., 1993; Sakuta, Kudryavtsev, 2005). This fact seems to be related to the higher ability of 2c-hepatocytes, compared to polyploid cells, to enter the mitotic cycle (Uryvaeva, Marshak, 1969; Watanabe et al., 1970). Earlier it was suggested that reparative regeneration of the liver mainly occurs due to proliferation of mononucleate diploid hepatocytes, while polyploidisation of cells during reparative liver growth plays a noticeable role only in some patients (Kudryavtsev et al., 1993).

In the process of physiological regeneration, the number of hepatocytes formed in intact rat liver after 6 months exceeds their initial level about 1.8-fold, the corresponding value for reparative regeneration being 3.1-fold. Calculations based on the values of the thymidine-labelled nuclei index and the mitotic activity of hepatocytes available in the literature demonstrate even greater differences between these regeneration methods. The loss of parenchyma after 6 months of experimental LC development is five times the weight of the liver in the initial state (Sarkisov et al., 1975; Brodsky, Uryvaeva, 1981; Macdonald et al., 1986), while the loss of parenchyma in intact liver during the same period is equal only to the initial weight of the organ (Ryabinina, Benyush, 1973; Ottensen et al., 1980). Thus, each hepatocyte during 6 months of chronic exposure to CCl_4 performs approximately 2–2.5 times more divisions than in the intact liver in the course of physiological regeneration. Nevertheless, despite the high proliferative activity of hepatocytes in the course of LC development, the number of cells in the damaged liver of rats and humans was eventually much lower than in the intact liver (Table 3.1, Table 3.2), which indicates that cell death prevails over the increase in cell number.

Along with proliferation and polyploidisation, an important role in the normal and reparative growth of the liver is played by the hypertrophy of hepatocytes (Shalakhmetova et al., 1981; Arefieva et al., 1993; Arosio et al., 1993; Sakuta, Kudryavtsev, 2005). It can have two causes: 1) an increase in cell ploidy, which usually leads to a proportional increase in cell volume and mass (James et al., 1979; Brodsky, Uryvaeva, 1981; Shalakhmetova et al., 1981; Gaub et al., 1981; Zavadskaya et al., 1983) and 2) an increase in the cytoplasm volume, which is accompanied by a decrease in the nucleoplasmic ratio but is not associated with an increase in cell ploidy. The hypertrophy of the cell cytoplasm is believed to be based on the intensification of intracellular regeneration processes (Sarkisov et al., 1975). These processes

lead to an increase in the number and size of various cellular structures and organelles and, ultimately, to an increase in the volume and mass of the cells.

Cell hypertrophy caused by an increase in the cytoplasmic volume is considered as a special form of regeneration that increases the number of specific structures inside cells and compensates the impaired functions (Ryabinina, Benyush, 1973). The analysis of cell hypertrophy is important for understanding the mechanisms of normal and reparative growth of an organ. The hypertrophy of a cell unrelated to its ploidy can be accurately assessed, e.g., by determining its ploidy level and mass.

Cell mass is usually determined by cytophotometry based on the total protein content after staining with appropriate dyes or by measuring the dry mass (DM) of unstained cells using an interference microscope (Brodsky, 1966). Unlike cytophotometric methods with dyes, interferometry makes it possible to obtain data on the DM, of which approximately 80% is due to the protein content (Giese, 1959), in absolute units, picograms (10^{-12} g), and to calculate the absolute number of cells in an organ without resorting to complex and labour-intensive techniques.

Measurement showed that DM of one hepatocyte in the control rats averaged 943±21 pg, while that in the cirrhotic rats, 1184±56 pg, i.e. DM increased by 25.6% during cirrhosis ($p < 0.001$). An increase of DM of rat hepatocytes and protein content in them during the development of cirrhosis was also noted by other authors (Tongiani et al., 1976; Zavadskaya et al., 1983).

The results of interferometry showed that in the normal human liver DM of hepatocytes was on average 546.0±29.6 pg, while during LC it increases by 23.7% as compared to the norm ($p < 0.05$), reaching 675.4±41.3 pg (Bezborodkina et al., 2021b). It has been previously found that DM of hepatocytes is 431.4±22.1 pg in the normal human liver and 640.0±49.2 pg in the cirrhotic human liver (Zavadskaya et al., 1983). Lower values of DM of hepatocytes in the normal human liver obtained by Zavadskaya et al. seem to be due to the fact that DM measurements were performed on biopsied material from young people with an average age of 29 years, whereas the average age of patients in our study was 44 years.

Thus, the loss of hepatocytes during the pathological process in the rat liver is fully compensated by an increase in the ploidy of cells and the hypertrophy of their cytoplasm. Compared with rats, the development of cirrhosis in humans causes deeper disturbances of

liver architectonics, and there is no complete compensation for the loss of hepatocytes and liver parenchyma (Bezborodkina et al., 2021b).

It should be noted that even a complete restoration of the parenchyma volume and the number of hepatocytes in the cirrhotic liver does not guarantee the restoration of its function. The reason is a strong transformation of the liver architectonics, as a result of which hepatocytes perform their multiple functions under conditions of hypoxia, acute shortage of energy sources and necessary substrates. Therefore, an assessment of the key liver functions and an understanding of the mechanisms regulating these functions under pathological conditions is one of the primary tasks for prognosis and therapy of LC.

3.2. Characterisation of glycogenesis in the normal and the pathological liver of humans, rats and mice at different stages of the food cycle

It is obvious that carbohydrate metabolism and, in particular, glycogen and glucose metabolism in the liver, which is closely involved in food processing, is very different at different stages of digestion. Therefore, in order to determine the functional capabilities of the cirrhotic liver, to identify deviations from the norm and to explain their causes, it was necessary to compare glucose and glycogen metabolism in the normal and the pathological liver during the food cycle and to reveal the cellular mechanisms of its regulation.

3.2.1. Prolonged starvation. Blood glucose concentration, glycogen content and the activity of key enzymes of its metabolism in the normal and the cirrhotic rat liver after 48 h of starvation

Starving rats rapidly loose body and liver weight. According to our data, after 24 h of starvation the body weight of animals decreased by 12.5% and the liver weight, by 17.9%, the corresponding values after 48 h being 20.8% and 27.7%, respectively (Fig. 3.7).

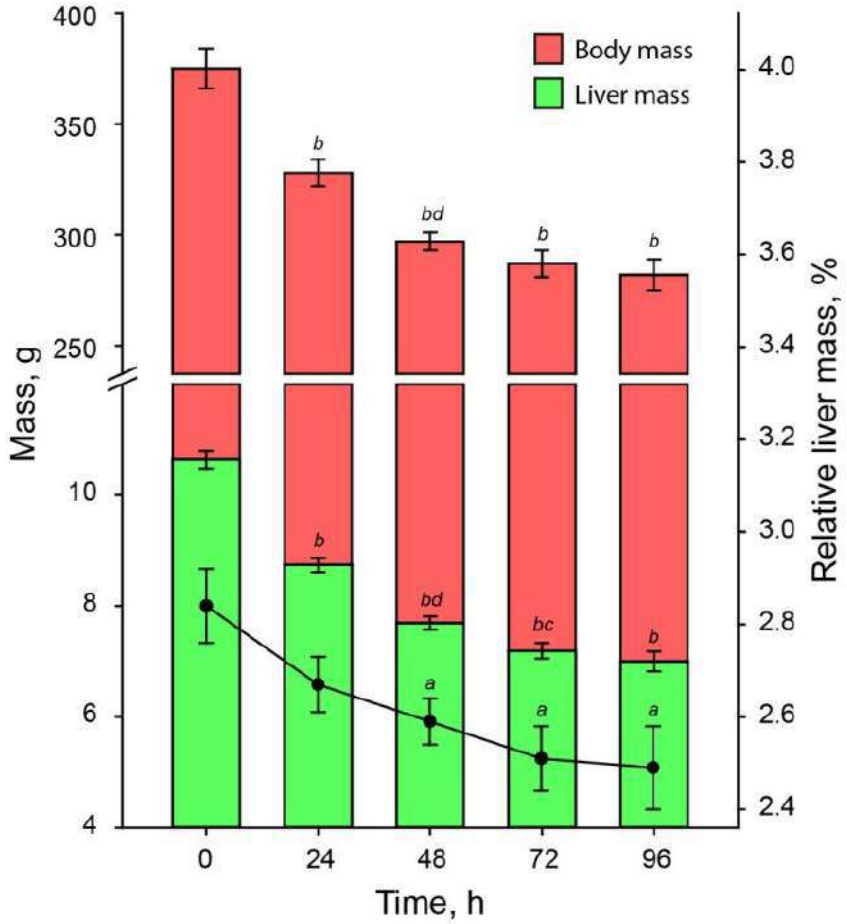


Figure 3.7 - Change in body weight, liver weight and relative liver weight of rats during starvation, $X \pm S_x$. Notes: 1) differences from the value before the start of starvation (0 h) at significance level: *a* — $p < 0.01$, *b* — $p < 0.001$; 2) differences from the previous value at significance level: *c* — $p < 0.01$, *d* — $p < 0.001$.

However, after 48 h these parameters change much more slowly than at the beginning of starvation. Similar data were obtained by Soberon and Sanchez (1961), who found that rats starving for 48 h lost 20–25% of the liver weight. In addition to a decrease in liver weight, starvation also results in a significant decrease of the hepatocyte volume and protein content (Belloni et al., 1988).

During prolonged starvation, carbohydrate metabolism changes profoundly, with the liver glycogen reserves being severely depleted (Bois-Joyeux et al., 1990; Jungermann, 1992). The results presented in Table 4.5 indicate that the glycogen content in the normal liver of rats after 48 h of starvation is very low. These results are in agreement with the literature data (Giardina et al., 1994; Minassian et al., 1999).

Table 3.5 — Some indices of glucose and glycogen metabolism in blood and liver of rats after 48 h of starvation in the norm and during LC ($X \pm S_x$, n = 3).

Indicator	Control	LC
Glucose concentration in serum, mmol/L	3.24±0.28	3.16±0.14
Glycogen concentration in liver, μmol glucosyl units/g raw weight of liver	15.4±0.2	10.4±0.5[2]
Glycogen content in hepatocytes, arbitrary units	2.09±0.06	1.87±0.02[1]
Glucose-6-phosphatase activity, nmol-min^{-1} -mg protein^{-1}	44.2±0.6	43.4±1.3
Glucokinase activity, nmol-min^{-1} -mg protein $^{-1}$	11.2±0.2	1.9±0.1[2]
Glycogen synthase activity, -G6P/+G6P	0.19±0.01	0.20±0.01
Activity of glycogen phosphorylase *a*, nmol-min^{-1} -mg protein^{-1}	99.0±2.4	44.1±1.6[2]
Total glycogen phosphorylase activity, nmol-min^{-1} -mg protein^{-1}	162.4±3.1	158.9±1.5

[1,2] Significantly different from the value in the control ([1] $p < 0.05$,[2] $p < 0.001$).

However, in the liver of cirrhotic rats, the glycogen content after 48 h of starvation was even lower than in the control animals (Bezborodkina et al., 2013a; Bezborodkina et al., 2014; Bezborodkina et al., 2016). Despite the fact that glycogen concentration in the liver decreased sharply after 48 h starvation, most of the cells in its parenchyma did contain glycogen. Analysis of the histogram presented in Figure 4.8, *a* showed that after 48 h of starvation glycogen was present in about 70% of hepatocytes of the normal rat liver, and in some of the hepatocytes its content was very high. In contrast to the control animals, in cirrhotic rats glycogen was present in only a half of the hepatocyte population, though in some of them glycogen content reached the same level as in fed control rats (Fig. 3.8, *b*).

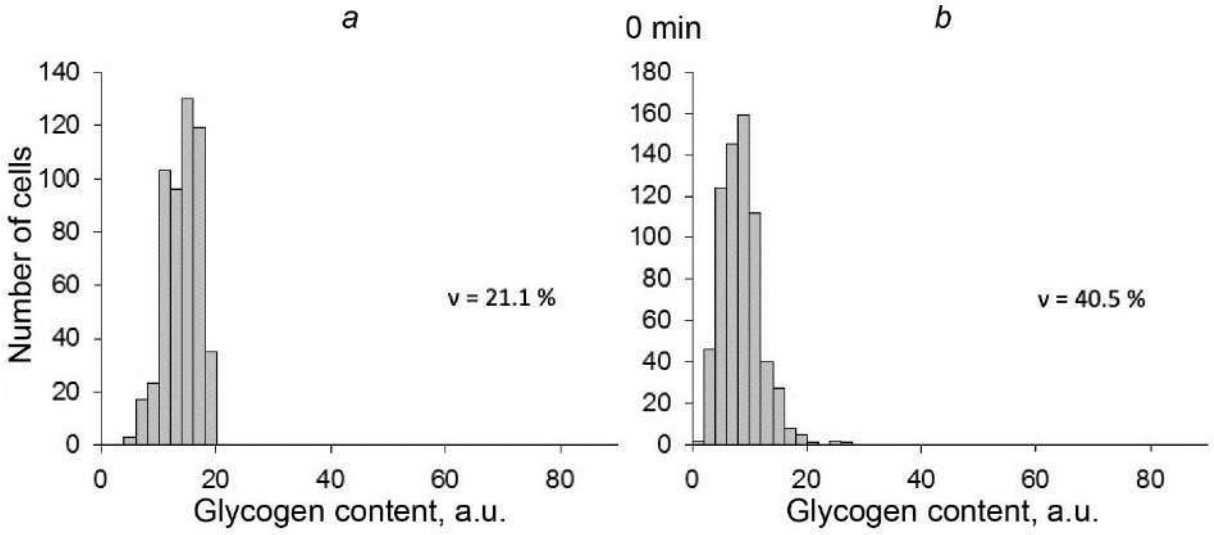


Figure 3.8 — Histograms of glycogen content distribution in hepatocytes of the mononucleate 4c-cell class in the control (*a*) and the cirrhotic (*b*) rat liver after 48 h of starvation.

Glycogen content in hepatocytes is regulated by the activity of enzymes of its synthesis and breakdown, and blood glucose concentration *per se* plays an important role for them. The data presented in Table 3.5 demonstrate that glucose concentration in peripheral blood of the control and the cirrhotic rats is at a similarly low level after 48 h of starvation (Bezborodkina et al., 2014). Similar data for rats starved for 40–48 h were obtained in other studies (Mlekusch et al., 1981; Calder, Geddes, 1992). Nevertheless, the normal level of glucose in the blood of cirrhotic rats does not imply that the processes associated with its production are undisturbed. Several studies have shown that after overnight fasting, glucose production by the cirrhotic liver is 20–40% lower than the norm (Reichle et al., 1978; Owen et al., 1981; Merli et al., 1986; Johansson et al., 1994). However, other studies have found that glucose production during LC is normal, being maintained by increased glucagon concentrations (Keller et al., 1982; Shmueli et al., 1993; Petrides et al., 1994). An analysis of the activity of the key enzymes of glycogen metabolism might elucidate the conflicting information on glucose production by the cirrhotic liver.

The study of G6Pase, the key enzyme responsible for glucose production by the liver, revealed no differences in its activity in starved cirrhotic rats from the control values (Table 3.5). Our data indicate that after 48 h of starvation glucose production by the cirrhotic rat liver is within the normal range. Since glycogen stores in the liver after 48 h of starvation are very low (Table 4.5) (Bezborodkina et al., 2014; Bezborodkina et al., 2016), it is obvious that glucose production during LC is almost completely carried out by gluconeogenesis (Bezborodkina et al., 2013a).

It has been shown that glucose controls the activity and amount of GK in the liver (Brocklehurst et al., 2004). During starvation, the activity of this enzyme decreases, while in the fed state it increases in accordance with the increase in the blood glucose concentration (Taketa et al., 1976). Since the K_m of GK for glucose is about 20 mM (Lowes et al., 1998), the rate of glucose phosphorylation under physiological conditions depends only on its intracellular concentration. It is natural to expect that a low blood glucose concentration during starvation would inhibit GK activity and promote the channelling of the substrate flux in the liver to glucose production.

Determination of GK activity in the normal liver of starved rats showed that when blood glucose concentration is reduced, GK activity is also low (Table 3.5) (Bezborodkina et al., 2013a). This result confirms the data of many authors that after 48 h of starvation GK activity

in the normal rat liver makes up 40–50% of the "fed" level (Vinuela et al., 1963; Perez et al., 1964; Bois-Joyeux et al., 1990; Minassian et al., 1999). As the duration of starvation increases, the activity of this enzyme decreases further (Minassian et al., 1999) down to its complete loss (Vinuela et al., 1963; Panserat et al., 2014). Though in our experiment the glucose concentration in the blood of control and experimental rats after 48 h of starvation was at the same level, GK activity in the cirrhotic liver was only 16% ($p < 0.001$) of the control values (Table 3.5) (Bezborodkina et al., 2013a).

Tissue intolerance to glucose is one of the characteristic manifestations of LC (Mion et al., 1996), which links it to diabetes. GK activity is markedly reduced in these diseases (Rossetti et al., 1993; Caro et al., 1995; Aiston et al., 2001; Wu et al., 2005). Impaired insulin secretion and/or insensitivity of cells to insulin is considered to be the main mechanism for the development of tissue glucose intolerance. Since GK activity in the liver is regulated by insulin-mediated expression levels of the enzyme (Iynedjian, 1993) and its intracellular compartmentalisation (Agius, 1994), an impaired secretion of this hormone or its binding by cells may lead to the dramatic decrease in GK activity during LC.

Determination of GS activity in cirrhotic rats after 48 h of starvation showed that it did not differ from that in the normal liver (Table 3.5) (Bezborodkina et al., 2014). In contrast to our data, it has been shown using the thioacetamide LC model that after 24 h of starvation GS activity in the cirrhotic rat liver was reduced as compared to the norm (Giardina et al., 1994). Similar data were obtained for rats with biliary LC induced by bile duct ligation. In this case, the decrease in the total and the active forms of GS correlated with the decrease in the number of hepatocytes in the liver (Krähenbühl et al., 1996). On the other hand, it was found that during CCl_4-induced cirrhosis in rats the level of the active form of GS did not differ from the norm (Krähenbühl et al., 1991).

According to our data, GP*a* activity in the cirrhotic rat liver after 48 h of fasting was lower than normal by more than 50% ($p < 0.001$) (Table 3.5) (Bezborodkina et al., 2014). Since GP activation is carried out by a cAMP-dependent mechanism (Rath et al., 2000; Roach, 2002) and since the level of cAMP production falls during LC (Francavilla et al., 1978; Vysotskaya et al., 1998), it can be assumed that the GP levels in the cirrhotic liver are affected by the decrease in adenylate cyclase activity and cAMP concentration (Bezborodkina et al., 2014).

Thus, carbohydrate metabolism in cirrhotic rats starved for 48 h differs significantly from that starved control rats. According to our data, the main differences are an approximately 6-fold decrease in GK activity and an almost twofold decrease in GP*a* activity.

3.2.2. Absorption period. Blood glucose concentration, glycogen content and activity of the key enzymes of its metabolism in the normal and the cirrhotic rat liver after glucose administration to starved animals

Food intake after starvation causes significant metabolic changes in various organs, including the liver. The absorptive period of the food cycle is characterised by the deposition of glycogen and the predominant use of glucose for the body's energy needs. The duration of the absorptive period after carbohydrate ingestion is thought to be about 2 hours (Niewoehner et al., 1984). During this time, glucose is almost completely absorbed from the intestine and its concentration in the plasma increases.

According to our data, oral administration of 30% glucose to starved rats leads to a rapid increase in its concentration in the peripheral blood in both the control and the experimental groups. However, after 2 h glucose concentration in cirrhotic rats was 14.2% ($p < 0.05$) lower than in the control animals (Fig. 3.9) (Bezborodkina et al., 2014). Glucose reached the maximum concentration, ~9 μM/l, in the blood of control rats 90 min after *per os* administration. Earlier it has been shown that administration of 50% glucose to starving rats causes an increase in blood glucose concentration up to 9 μM/ml in 10 min and up to 12 μM/ml in 20 min (Niewoehner et al., 1984).

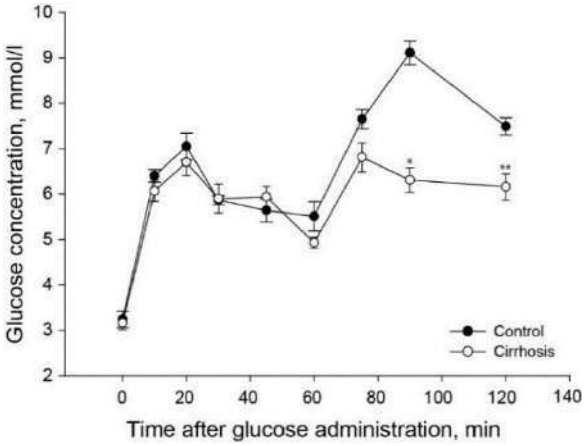


Figure 3.9 — Glucose concentration in blood serum of control (Control) and experimental (Cirrhosis) rats at different time intervals after glucose administration to starved animals. Significantly different from the value in the control: * $p < 0.01$, ** $p < 0.05$. For each point n=3.

Glucose concentration in the blood after a meal is determined mainly by the rate of absorption in the intestine and subsequent utilisation by various tissues. For example, the brain utilises 45–60% of glucose, red blood cells, 5–10%, kidneys, 10–15%, and skeletal muscles, 15–20% (Dimitriadis et al., 2021). However, blood glucose concentration depends not only on glucose utilisation but also on its production by the liver and adrenal glands. Therefore, the reduced blood glucose concentration in cirrhotic rats after refeeding with glucose may be due to different reasons. It may be associaetd with a slower glucose entry from the intestine into the blood, a higher rate of its utilisation by various tissues (primarily skeletal muscles), as well as a lower glucose production, mainly by the liver and adrenal glands (Bezborodkina et al., 2014).

It is generally accepted that glucose intolerance of various tissues is a characteristic feature of many patients with LC (Muller et al., 1992; Riggio et al., 1997; Kruszynska, 1999; Schneiter et al., 1999). In such a situation, one cannot expect a high rate of absorption and utilisation of glucose from food. On the other hand, absorption and transport of sugars and other substances in the intestine are impaired during LC (Beloborodova, 1978; Stratton et al., 2003) and the morphology of this organ is altered (Onori et al., 2000). Therefore, structural and functional disorders observed in the intestine during LC may be one of the reasons behind a weaker increase in blood glucose concentration in rats with CCl_4-cirrhosis as compared to the norm.

Glucose administration stimulated glycogen synthesis in the liver of both control and experimental rats (Fig. 3.10). Nevertheless, during the absorption period glycogen

accumulation in hepatocytes was slower in the cirrhotic liver than in the normal one (Bezborodkina et al., 2013b).

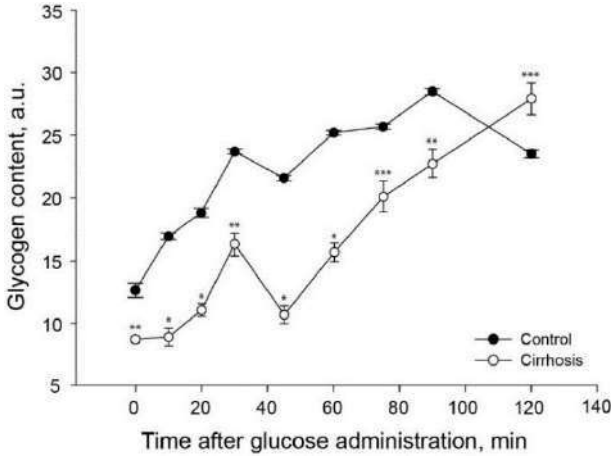


Figure 3.10 — Dynamics of glycogen accumulation in hepatocytes in the normal liver (Control) and the cirrhotic liver (Cirrhosis) of rats at different time intervals after glucose administration to starved animals. Notes: 1) vertical sections — error of the mean; 2) mean values during LC differ from normal values at the significance level * $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$.

The reasons of the lower rate of glycogen accumulation and lower glycogen content in hepatocytes of the cirrhotic liver in the absorptive period are unclear. They may be associated with the metabolism (activity of enzymes in the liver directly or indirectly involved in its synthesis and degradation) or with the number of hepatocytes and their characteristics (ploidy, size, proliferative activity, etc.).

Undoubtedly, glycogen content in hepatocytes is strongly influenced by the level of activity of the enzymes catalysing equilibrium and non-equilibrium reactions of glucose production, glycolysis, gluconeogenesis, glycogenogenesis and glycogenolysis. Glucose administration causes a weaker rise in GK activity in the cirrhotic rat liver as compared to the normal one: from 1.5 to 5.2 nmol-min^{-1} -mg protein^{-1} *vs* from 11.2 to ~ 20.0 nmol-min^{-1} -mg protein^{-1}, respectively (Fig. 3.11) (Bezborodkina et al., 2013a). As a result, the ratio of GK activity in the liver of the experimental and the control rats (LC/C) was only ¼, indicating an extremely low GK phosphorylation activity during cirrhosis (Fig. 3.12).

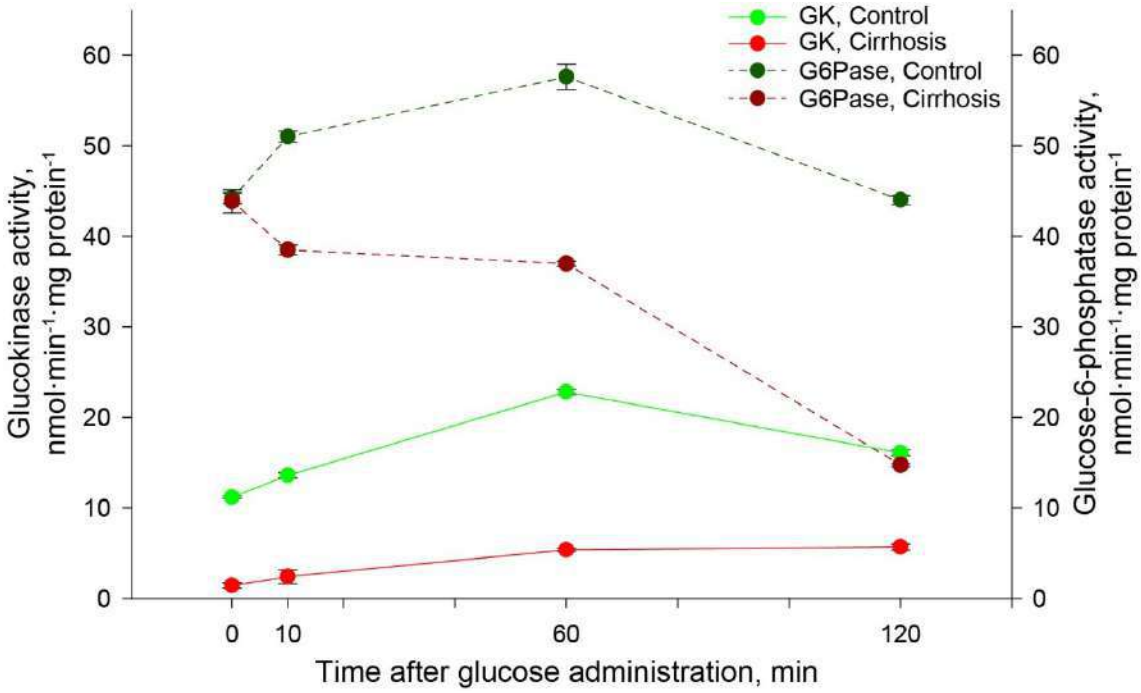


Figure 3.11 — Glucokinase (GK) and glucose-6-phosphatase (G6Pase) activity in the liver of the control and the experimental rats at different time intervals after glucose administration to starved animals.

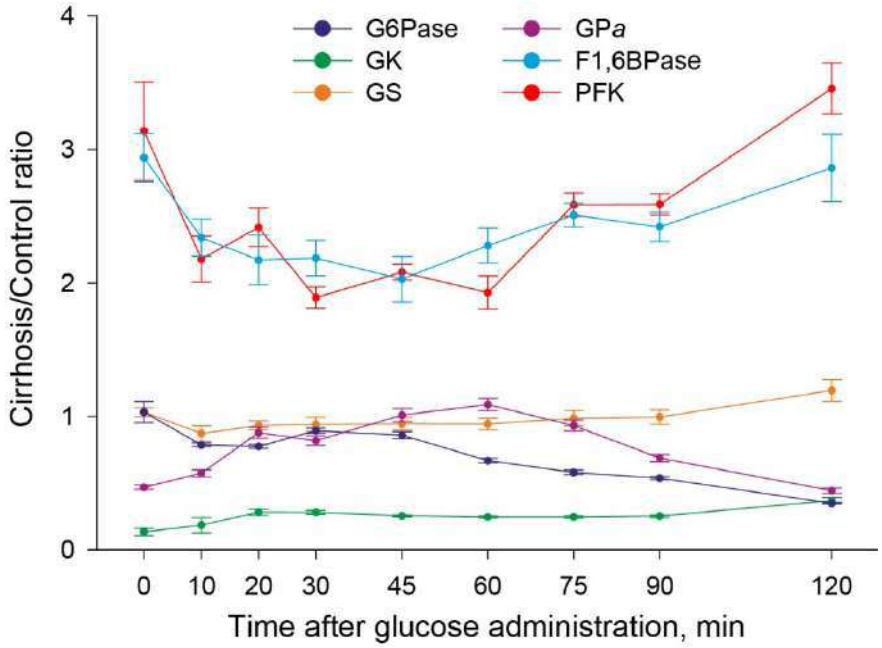


Figure 3.12 — Activity ratios of glucose and glycogen metabolism enzymes in the normal and the cirrhotic rat liver during the absorptive period after glucose administration to animals starved for 48 h.

It is assumed that when GK activity is weak, the liver may use other metabolic pathways that can partially or completely compensate for the insufficient phosphorylation activity of GK in LC. Indeed, the activity of other hexokinases capable of phosphorylating

glucose has been found to be increased in the cirrhotic human liver (Lowes et al., 1998; Taketa et al., 1976, 1988). In addition, in some cases, glucose phosphorylation in the liver involves G6Pase. For example, in experimental diabetes or prolonged starvation, when GK activity is very low, an intense phosphorylation of glucose due to the transferase activity of G6Pase is observed (Nordlie, Arion, 1964; Alvares, Nordlie, 1977; Nordlie et al., 1979). Therefore, an increased activity of hexokinases with a high affinity for glucose and the transformation of the hydrolase function of G6Pase into the transferase function can be considered as an adaptive response to the extremely weak phosphorylation capacity of GK during cirrhosis.

In the normal liver, about 85% of the G6P formed during glucose phosphorylation is used almost equally for glycogen synthesis and for glycolysis (O'Doherty et al., 1996). In the cirrhotic liver, due to a low GK activity, almost all G6P is metabolised via glycolysis or the pentose phosphate pathway (shunt).

It has been shown that the loss of liver parenchyma during 6 months of experimental cirrhosis development is equal to five times the initial liver mass (Peters, 1962; Sarkisov et al., 1975; Brodsky, Uryvaeva, 1981; Smejkalova et al., 1985; Macdonald et al., 1986). In intact liver, the loss of liver parenchyma during the same time corresponds only to its initial mass (Ryabinina, Benyush, 1973; Ottensen et al., 1980). According to our data (Table 3.1), 1 g of the normal liver of adult rats contains ~ 2.5×10^8 hepatocytes (Bezborodkina et al., 2021b). Consequently, the loss of hepatocytes resulting from chronic exposure of rats to CCl_4 for 6 months would be about 10×10^8 cells/g liver. This means that approximately the same number of hepatocytes must be generated by compensatory reparative regeneration in order to maintain the multiple functions of the cirrhotic liver at an adequate level. The replenishment of such a significant number of hepatocytes in the course of cirrhosis development requires vast amounts of energy and plastic materials, which are mainly derived by glycolysis and the pentose phosphate pathway. Our data on the high rate of glycolysis during LC in rats (Fig. 3.12) (Bezborodkina et al., 2013a) confirm the results of other authors obtained from cirrhotic patients (Taketa et al., 1976, 1988; Nishikawa et al., 2014; Lee et al., 2018). The available data suggest that regenerative processes in the cirrhotic liver are provided with energy and necessary intermediate products due to a high rate of glycolysis and the pentose phosphate pathway.

Intense catabolic processes during LC in skeletal muscle, liver, and adipose tissue (Marchesini et al., 1981; Weber et al., 1992; Kalafateli et al., 2015; Gangitano et al., 2022)

cause an increase in blood concentrations of amino acids, glycerol, and lactate, which are during destruction of cells and cellular structures. Increased concentration of these catabolic products is a powerful stimulus for the increase of the rate of gluconeogenesis in the liver. It is worth noting that in addition to the transformation of non-carbohydrate substances into glucose, the function of gluconeogenesis is to remove lactate from cells and tissues functioning under conditions of oxygen deficiency, thus protecting them from acidosis.

Determination of the activity of the key enzymes of gluconeogenesis, G6Pase and F1,6BPase, in the liver of rats of the control and the experimental group in the absorptive period yielded unexpected and paradoxical results. On the one hand, we found that F1,6BPase activity during LC was increased approximately 2.4-fold ($p < 0.001$) as compared to the norm (Bezborodkina et al., 2013a). This result confirms the data of many authors that the rate of gluconeogenesis is markedly increased during LC (Newsholme, 1976; Owen et al., 1983; Krähenbühl, Reichen, 1993; Bugianesi et al., 1998; Petersen et al., 1999; Changani et al., 2001). On the other hand, during the absorptive period G6Pase activity in the cirrhotic rat liver was, on average, about one third ($p < 0.01$) lower than in the liver of the control rats (Fig. 3.11, Fig. 3.12) (Bezborodkina et al., 2013a).

There are two points of view on G6Pase activity in the liver during the absorptive period. According to one, glucose production in the normal liver decreases after food intake (Newgard et al., 1984; Minassian et al., 1999; van Dijk et al., 2001; Moore et al., 2012). According to the other, it does not decrease and even increases (Rossetti et al., 1993; Massillon et al., 1995; Rognstad, 1996; Rossetti, 1996).

Our data (Fig. 3.12) indicate that both an intensive gluconeogenesis and a high glucose production are maintained in the normal liver during the absorptive period (Bezborodkina et al., 2013a). These results are supported by the findings that intensive gluconeogenesis and glucose production occur for at least 2 h after glucose administration to starved rats, even though exogenous glucose is absorbed by the liver and gluconeogenic substrates are used for glycogen synthesis (Niewoehner et al., 1984; Niewoehner, Nuttall, 1988). The contradictory data on G6Pase activity and the level of glucose production during the absorptive period may be due to differences in the time intervals studied by the authors. For example, the conclusion about the inhibition of G6Pase during refeeding was made on the basis of the analysis of the dependence between the enzyme activity and the liver glycogen content plotted at only three points: 90, 180 and 360 min after the beginning of refeeding (Minassian et al., 1999). In other

words, the authors investigated the end of the absorptive period and the beginning of the postabsorptive period, whereas we investigated the absorptive period only (0–2 h).

The reasons for such a low G6Pase activity in cirrhotic rats during the entire absorptive period (Fig. 3.11) (Bezborodkina et al., 2013a) cannot be explained only by a 9% decrease of the liver parenchyma (Table 3.1) (Bezborodkina et al., 2021b). One of the reasons for the decrease of the G6Pase activity is the disruption of RER membranes after the damaging impact of LP products (Benedetti et al., 1980; de Groot et al., 1985; Ohyashiki et al., 1995). In this regard, a decreased activity of SOD, catalase, and, accordingly, an increased concentration of LP products in blood and in the cirrhotic liver (Fig. 3.6) is an evidence in favour of the hypothesis that the enhancement of LP processes is one of the main mechanisms behind the low G6Pase activity in the cirrhotic liver.

Changes in the activities of GK, PFK, F1,6BPase, and G6Pase lead to a decrease or increase in the concentration of G6P in the liver, indirectly affecting glycogen levels in the liver cells. However, it is GS and GP that directly affect the glycogen content in hepatocytes, because these enzymes are closely involved in its synthesis and degradation by attaching or removing glucose residues in glycogen molecules.

The results presented in Figure 3.13 demonstrate a rapid increase in GS activity in the liver of the control and the experimental group of rats after glucose administration, confirming the information that the activity of this enzyme is stimulated after feeding (Niewoehner and Nuttall, 1988; Niewoehner et al., 1984; Ercan et al., 1994). As a consequence, the ratio of GS activities in the liver of the control and the experimental group of rats during the absorptive period was close to 1.0 (Fig. 3.12). Nevertheless, the enzyme activity during the absorptive period was 6.2% lower in the experimental group ($p < 0.01$) than in the control group (Bezborodkina et al., 2014).

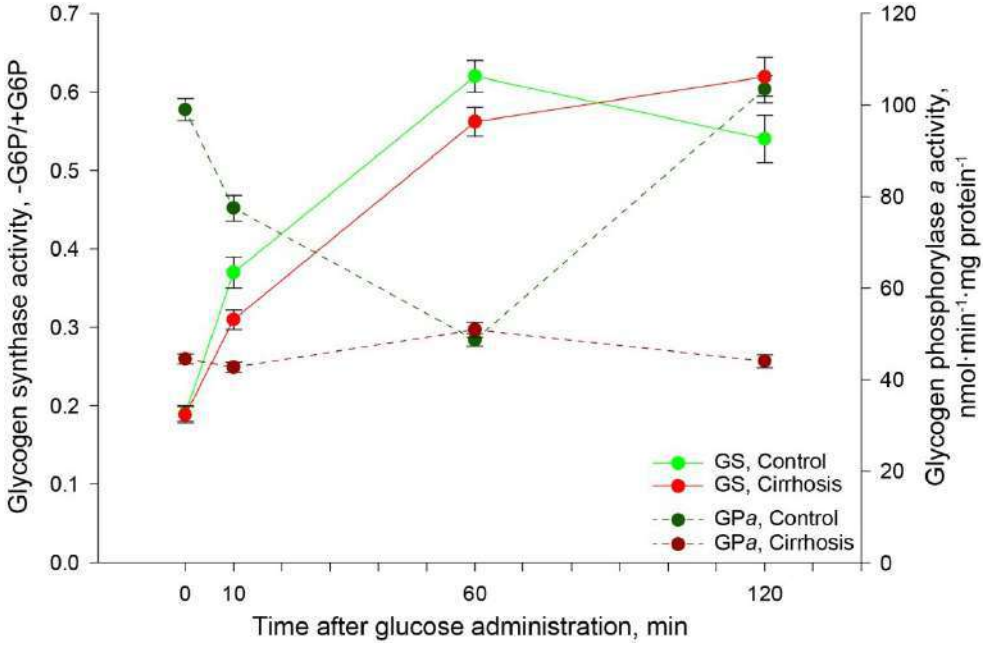


Figure 3.13 — Activity of glycogen synthase (GS) and glycogen phosphorylase *a* (GP*a*) in the normal liver and the cirrhotic liver (LC) of rats in the absorptive period at different time intervals after administration of glucose to starved animals.

In contrast to GS, whose activity rapidly increased after glucose administration and then reached a plateau, the activity of GP*a* underwent more complex changes during the absorptive period. In rats of the control group, glucose administration caused a rapid drop in GP*a* activity, but it began to increase 1 h after the start of refeeding, reaching the initial level by the end of the absorptive period (Fig. 3.13) (Bezborodkina et al., 2014).

Since the absolute activity of GP is much higher than that of GS, the former plays a leading role in the control of glycogen metabolism in the liver (Roach, 2002; Ferrer et al., 2003). GP*a* is a strong inhibitor of GS phosphorylase and therefore a reduction in its activity was considered a necessary condition for the initiation of glycogen synthesis in the liver (Hers, 1990). The results presented in Figure 3.13 indicate that a decrease in GP*a* activity in the normal liver at the beginning of the absorptive period activates GS and leads to rapid glycogen synthesis in hepatocytes (Figure 3.10) (Bezborodkina et al., 2014). Our data demonstrate the reciprocal interaction between GS and GP, which has been shown earlier in other studies (Minassian et al., 1999; Aiston et al., 2003). However, this pattern of enzyme interaction only persists for a short time interval. Sixty minutes after the start of refeeding, when the glycogen content in hepatocytes becomes sufficiently high (Fig. 3.10), GP activity begins to increase, reaching the initial "starvation" level by the end of the absorption period (Fig. 3.13) (Bezborodkina et al., 2014). As a result, in the second half of the absorptive period, both GS

and GP become very active (Fig. 3.13), i.e. glycogen synthesis and degradation in the normal liver occur simultaneously. Thus, the increase in glycogen content may be an important factor stimulating glycogen turnover in the liver and preventing its uncontrolled accumulation (Bezborodkina et al., 2014). The presence of glycogen turnover in the liver is confirmed by the direct correlation between the rate of glycogenolysis and the concentration of glycogen in the liver (Magnusson et al., 1994).

In contrast to the control group, the initiation of glycogen synthesis and its further accumulation in the liver of the cirrhotic rats occurred against the background of a low GP*a* activity throughout the absorptive period (Fig. 3.12, Fig. 3.13) (Bezborodkina et al., 2014).

As mentioned above, glycogen can be synthesised by direct and indirect pathways. In the latter case, the substrate for GS is G6P synthesised from gluconeogenic C_3-precursors (Newgard et al., 1983; Katz, McGarry, 1984; Kuwajima et al., 1986; Napoli et al., 1992). In the normal liver, the contribution of the direct and the indirect pathway to glycogen synthesis varies depending on the stage of the food cycle, the amount and composition of food, the animal species, and a number of other factors. It is thought that in case of *ad libitum* feeding, the ratio of the direct and indirect pathway is approximately 50/50 (Soares et al., 2009); however, during the absorptive period, the contribution of the direct pathway can increase up to 77% (Magnusson et al., 1989).

A weak phosphorylation activity of GK (hexokinase IV) during LC (Fig. 3.11, Fig. 3.12) can be compensated by the activity of other hexokinases, primarily I and II (Taketa et al., 1976, 1988; Lowes et al., 1998). At the same time, a high activity of these isoenzymes in the liver does not necessarily entail an increased GS activity and an intensive glycogenogenesis. This paradox is associated with the presence of two pools of G6P in hepatocytes (Seoane et al., 1996; Gomis et al., 2002). One of them is formed with the participation of GK and in the normal liver activates the hepatic type of GS (GYS2), stimulating glycogen synthesis in hepatocytes. Another pool of G6P is formed by the activity of hexokinase (**not** glucokinase!) and stimulates GYS1 (muscle-type GS) but not GYS2 (Meijer, 2002). Therefore, an increased glucose phosphorylation in the cirrhotic liver due to an increase in hexokinase activity does not increase hepatic GYS2 activity and, consequently, does not stimulate glycogen synthesis in hepatocytes.

Analysis of GK and GS dynamics in the absorptive period showed that the correlation coefficients between the activity of these enzymes in the control group were 0.93 ($p < 0.001$)

and in the experimental group, 0.95 ($p < 0.001$). This result suggests that the direct pathway of glycogen synthesis (GK→G6P→GS→glycogen) also functions in the cirrhotic liver, but its contribution to glycogen accumulation in hepatocytes is small as compared with gluconeogenesis. According to our data, the contribution of the direct pathway to glycogen synthesis in the cirrhotic rat liver is about 25% and that of the indirect pathway, about 75%.

Almost all liver glycogen is concentrated in the cells of the liver parenchyma. Therefore, the characteristics of glycogenesis in the normal and the cirrhotic liver are largely determined by the number and properties of hepatocytes. At the same time, the influence of cell and tissue factors on the glycogen content and its dynamics in hepatocytes at different stages of the food cycle has been studied insufficiently. In particular, the role of the cell size and ploidy in the process of glycogenesis is unclear. It is also unknown how different subpopulations of hepatocytes influence the rate of glycogen accumulation in the liver during feeding. Using the methods of quantitative cytochemistry, we studied in detail the influence of these cellular factors on glycogenesis in hepatocyte populations in the normal and the pathological liver of rats and humans.

An analysis of the histograms presented in Figure 3.14, *a* and Figure 3.14, *b* showed that glycogenesis in the hepatocyte population of the cirrhotic liver is characterised by a considerable asynchrony. In the normal liver the coefficients of variation of hepatocyte distribution by glycogen content vary in the range of 20–30%, while in the cirrhotic they vary in the range of 40–55%. These data indicate that different cells in the liver parenchyma synthesise glycogen at different rates, with the differences in the ability of cells to accumulate glycogen increasing with the increased duration of the absorptive period.

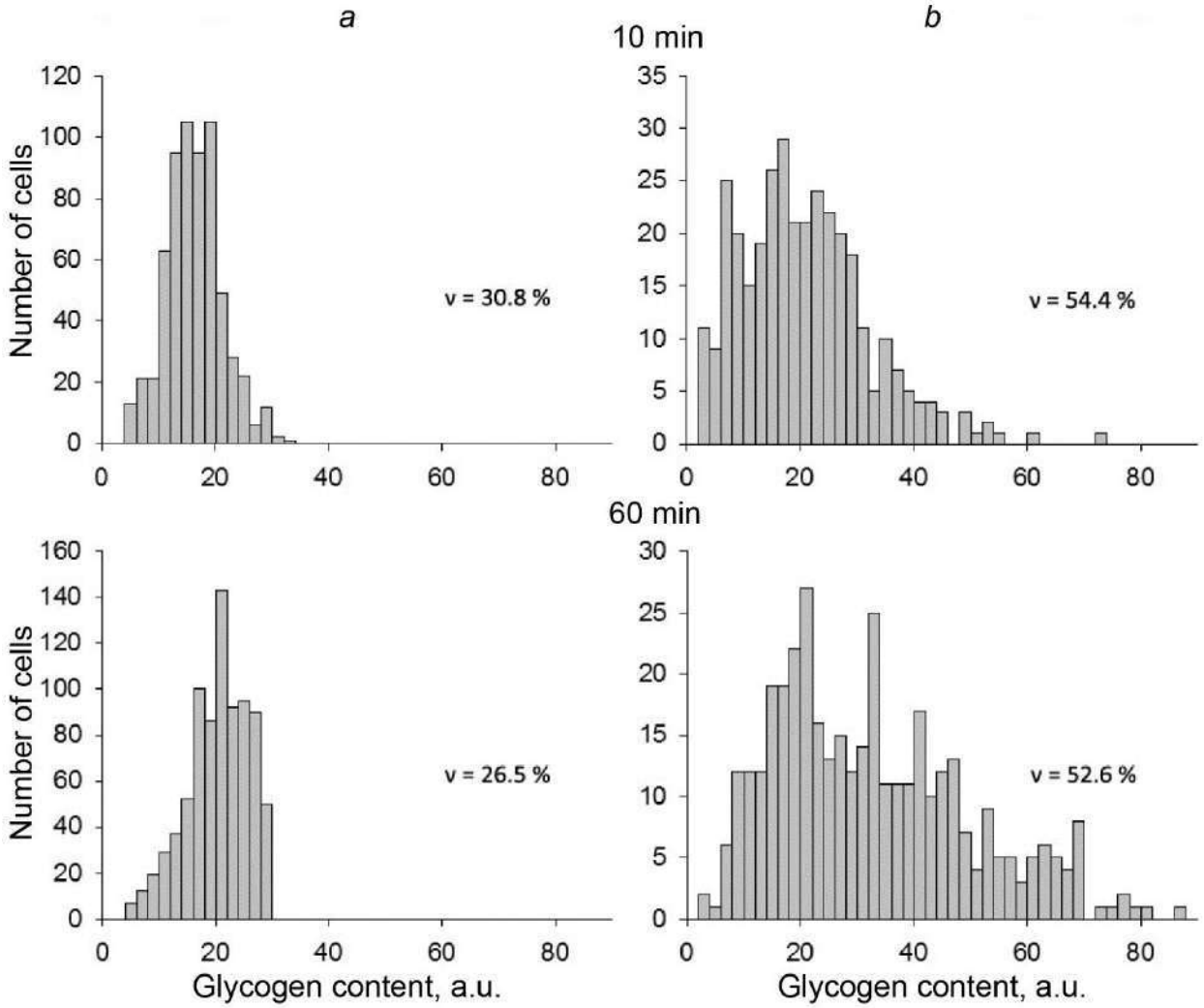


Figure 3.14 — Histograms of distribution of glycogen content in hepatocytes in the class of mononuclear 4c cells in the control (*a*) and the cirrhotic (*b*) rat liver 10 and 60 min after glucose administration to fasted animals.

The presence of polyploid cells in the parenchyma of the mammalian liver is a characteristic feature of this organ. In some species, polyploidy may affect almost the entire population of hepatocytes, reaching high levels (Brodsky, Uryvaeva, 1981). It has been found that the number of polyploid cells increases in case of damage and various diseases of this organ (Kohno et al., 1991; Kudryavtsev et al., 1993: Tarao et al., 1993, 1994; Melchiorri et al., 1994). Therefore, the effect of ploidy levels on glycogen content in hepatocytes of the cirrhotic liver is a fundamental question. It has been shown that in the postabsorptive period of the food cycle, the glycogen content in the hepatocytes of the normal liver increases in proportion to the ploidy level of the cells (Kudryavtsev et al., 1979, 1980; Shalakhmetova et al., 1981; Mayetsyan, 1983). In this work, we investigated the relationship between the glycogen content in hepatocytes and their ploidy level during the absorptive period, in the course of intensive glycogenesis, in the normal and in the cirrhotic liver.

Our data confirmed that the rate of glycogen accumulation by hepatocytes of the cirrhotic liver is lower than of the normal hepatocytes (Table 3.6) (Bezborodkina et al., 2009a; Bezborodkina et al., 2016). Glycogen content in the normal liver cells 10 min after glucose administration to starved rats was 23% higher than in the cells of the cirrhotic liver, and after 60 min it was by 69% higher (Table 3.6).

Table 3.6 — Glycogen content (arbitrary units) in mononucleate and binucleate hepatocytes of different ploidy classes in the normal and the cirrhotic (LC) rat liver after 48 h of starvation (0 min) and 10 and 60 min after glucose administration to starved animals ($X \pm S_x$, n = 3).

Time, min	Ploidy classes, c					
	2c	2c×2	4c	4c×2	8c	8c×2
Norm						
0	4.7± 0.3 (10)	9.9± 0.2 (46)	9.6± 0.1 (637)	19.3± 0.3 (116)	18.2± 0.4 (44)	-
10	8.8± 0.5[3] (12)	16.3± 0.6[3] (54)	17.2± 0.1[3] (740)	32.9± 0.8[3] (168)	34.3± 0.9[3] (64)	-
60	17.3± 1.8[3] (6)	33.2± 0.8[3] (50)	33.5± 0.2[3] (1078)	65.9± 0.8[3] (183)	63.8± 1.7[3] (39)	-
LC						
0	3.7± 0.8 (16)	7.2± 0.8 (27)	7.6± 0.2 (903)	14.9± 0.6 (185)	14.4± 0.6 (138)	27.1± 10.8 (8)
10	6.8± 2.2 (12)	13.4± 3.9 (34)	13.2± 0.7[3] (599)	27.1± 3.2[3] (183)	26.4± 3.1[3] (86)	54.9± 6.1[1] (14)
60	10.4± 1.0[3] (43)	21.0± 2.0[3] (47)	22.3± 0.8[3] (593)	44.1± 4.7[3] (67)	41.5± 3.1[3] (93)	76.2± 9.1[2] (10)

Notes: 1) results for each time point are a weighted average of data obtained for 3 rats; 2) number of cells measured is in parentheses; 3) glycogen content in hepatocytes differs from the corresponding value in starved animals at the significance level [1] — $p < 0.05$, [2] — $p < 0.01$, [3] — $p < 0.001$.

Although hepatocytes in the normal liver accumulate more glycogen at different stages of refeeding than in the cirrhotic liver, the glycogen content in mononucleate and binucleate hepatocytes of different ploidy classes in both the normal and the cirrhotic liver matched the gene dosage, i.e. it was the same per genome (Figure 3.15) (Bezborodkina et al., 2009a; Bezborodkina et al., 2016).

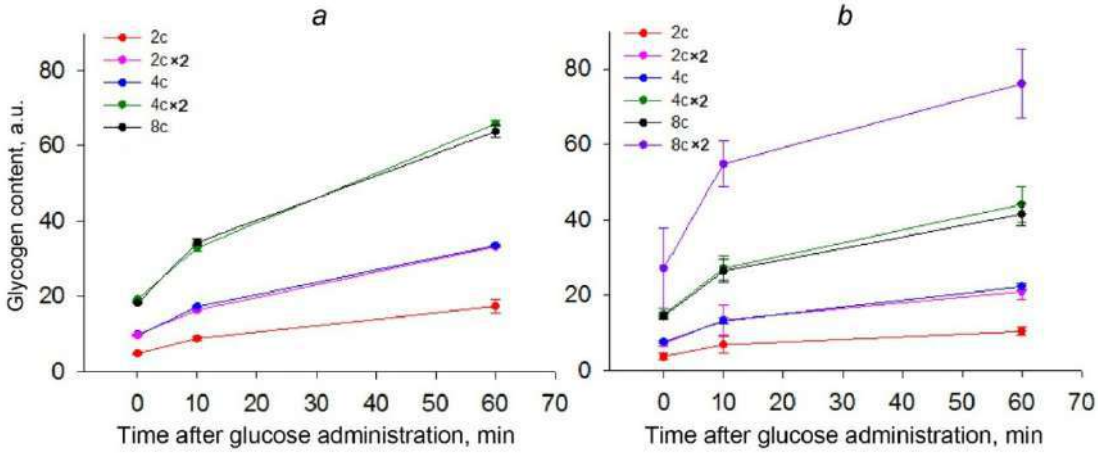


Figure 3.15 — Dynamics of glycogen content in hepatocytes of different ploidy classes in control (*a)* and cirrhotic liver (*b*) during refeeding of rats with glucose. Note: vertical sections — error of the mean.

Thus, the conclusion that the glycogen content in hepatocytes is proportional to the level of cell ploidy during glycogenesis (Fig. 3.16) (Bezborodkina et al., 2016) fully coincides with an earlier conclusion derived from the study of this pattern in a relatively stable postabsorptive period of the food cycle (Kudryavtsev et al., 1979, 1980; Shalakhmetova et al., 1981; Maytesyan, 1983). Consequently, the general principles of regulation of transcription and translation in hepatocytes of the cirrhotic liver are unaffected by the pathological process, although the absolute level of these processes may differ in the normal and the pathologically altered organ.

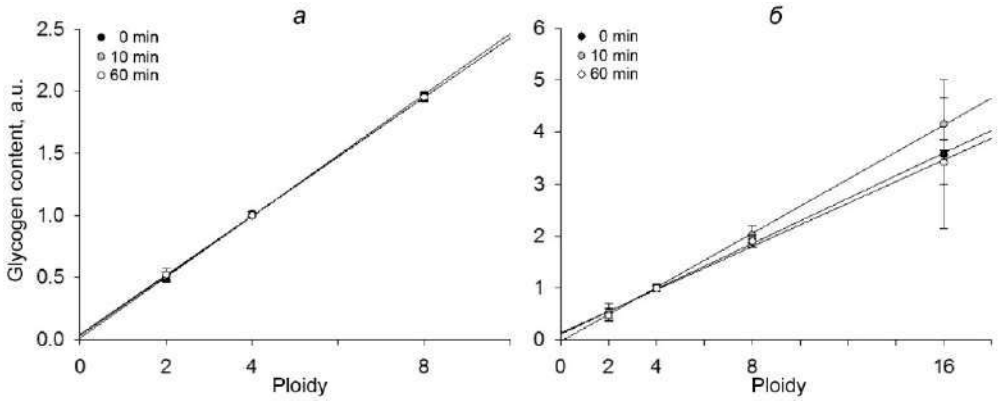


Figure 3.16 - Dependence of glycogen content in hepatocytes of the control (*a*) and the cirrhotic (*b*) rat liver on cell ploidy during starvation (0 min), as well as 10 and 60 min after glucose administration to starved animals. Note: vertical sections are the error of the mean.

The size of cells is another fundamental parameter that significantly affects their structure and function (Jorgensen, Tyers, 2004). Most of studies on the relationship between the cell size and the cell function are devoted to the influence of ion-dependent fluctuations of

the cell volume on metabolism. Changes of the cell volume due to hydration or dehydration have been shown to have a strong influence on various metabolic pathways, including protein synthesis and degradation, lipogenesis, glycolysis, and urea synthesis (Waldegger, Lang, 1998). However, rapid oscillations of the hepatocyte size caused by swelling or dehydration have a small amplitude and are not accompanied by any changes in the number of organelles or other structures in the cells (Espelt et al., 2008). In contrast, the interval of static changes of the hepatocyte size in the mammalian liver is incomparably higher than the cell volume fluctuations caused by hyper- and hypo-osmotic conditions.

Interferometry determination of the dry mass (DM) of hepatocytes[*)] in the normal (Bezborodkina et al., 2022) and the cirrhotic liver showed that this parameter could change 10-fold during the absorptive period (Bezborodkina et al., 2016). The differences in the DM of cells are partly due to the differences in their ploidy, but DM of hepatocytes differed greatly even within one ploidy class. For example, in starved rats, the DM of mononucleate 4*c* cells varied from 400 to 798 pg (Bezborodkina et al., 2016). The reasons behind this variability are not entirely clear. It is known, however, that hepatocytes perform numerous tissue-specific functions, some of which are related to protein synthesis and secretion. It is also known that cells have circadian, circahoralian and other rhythms of synthesis of proteins and their excretion into the blood plasma (Brodsky, 1992; Brodsky, 2014). At the same time, the variability of cells by protein content, inclusion of labelled amino acids and protein excretion at the maximum and the minimum of the circahoralian rhythm can be as high as 2–4-fold (Novikova, Borovkov, 1987). Based on this information, we can assume that the changes in the DM of hepatocytes in our experiments were mainly associated with fluctuations in protein synthesis and excretion from cells.

Cell size is thought to have a significant effect on the metabolism and glycogen content of hepatocytes. It is believed that there is a relationship between the size of hepatocytes and glycogen content in them: larger hepatocytes have a higher glycogen content (Newsholme, Start, 1975). We tested this hypothesis and found, as can be seen in Figs. 3.17, *a*; 3.18, *a;* 3.19, *a*; 3.20, *a;* 3.21, *a*, that the glycogen content in hepatocytes in the normal rat and human liver did correlate with their size (Bezborodkina et al., 2009a; Bezborodkina et al., 2016). This dependence was observed in each cell ploidy class.

[*)] About 85% of dry mass of hepatocytes is due to the protein content (Brodsky, 1966)

The dependence of glycogen content on the hepatocyte size is clearly observed in the rat liver both during starvation and during intensive glycogen synthesis. It is particularly graphic in the class of mononucleate tetraploid hepatocytes (Fig. 3.20, *a*). Our data indicate that there are three subpopulations of hepatocytes differing in glycogen content in the normal liver (Fig. 3.20, *a*). These subpopulations might be associated with the portal, the intermediate and the zone of the lobule (Bezborodkina et al., 2009a; Bezborodkina et al., 2016).

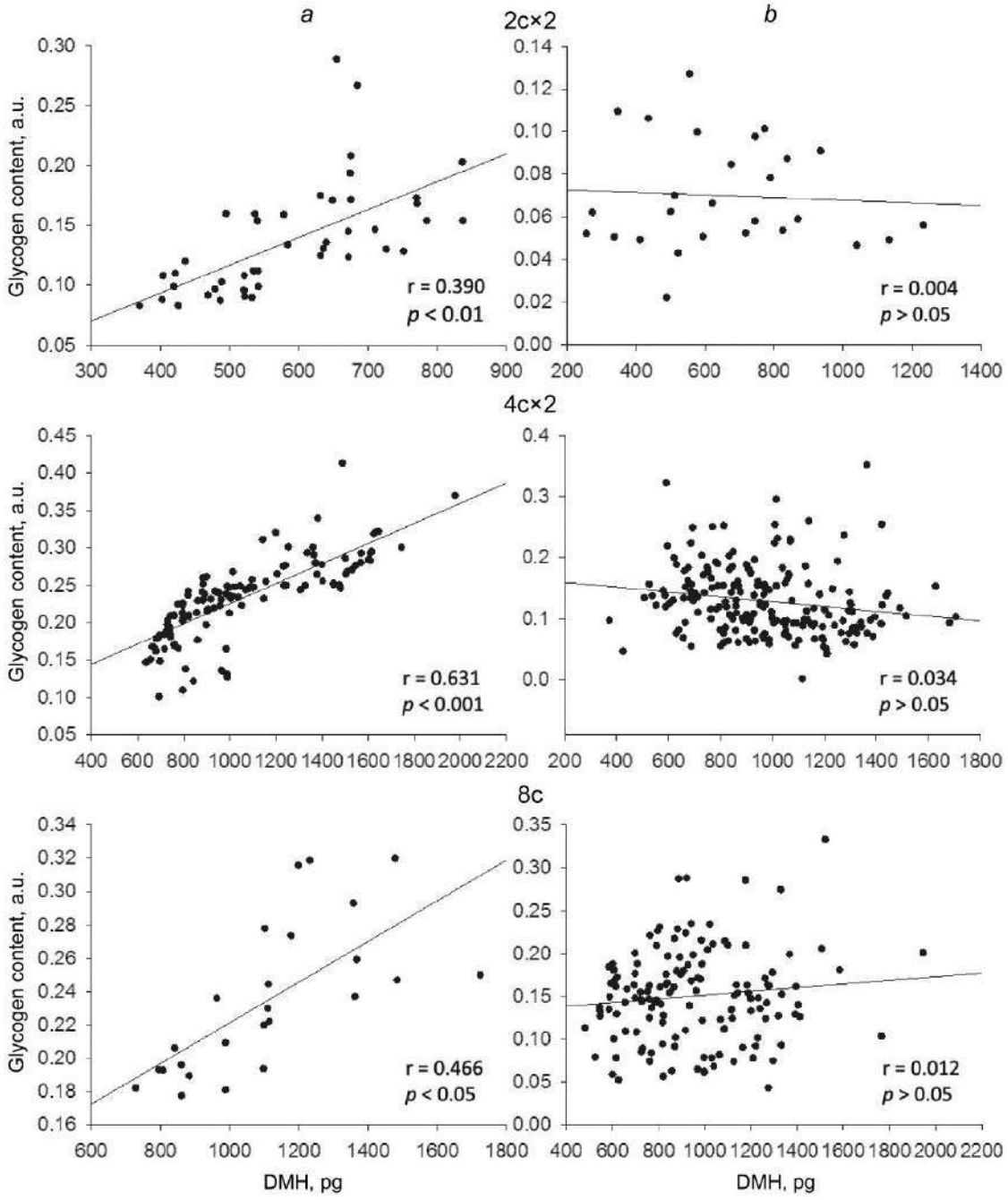


Figure 3.17 — Dependence of glycogen content on dry mass of hepatocytes (DMH) of different ploidy classes of the normal (*a)* and the cirrhotic (*b)* liver in starved rats (0-th min). Note: each point on the graph corresponds to one cell.

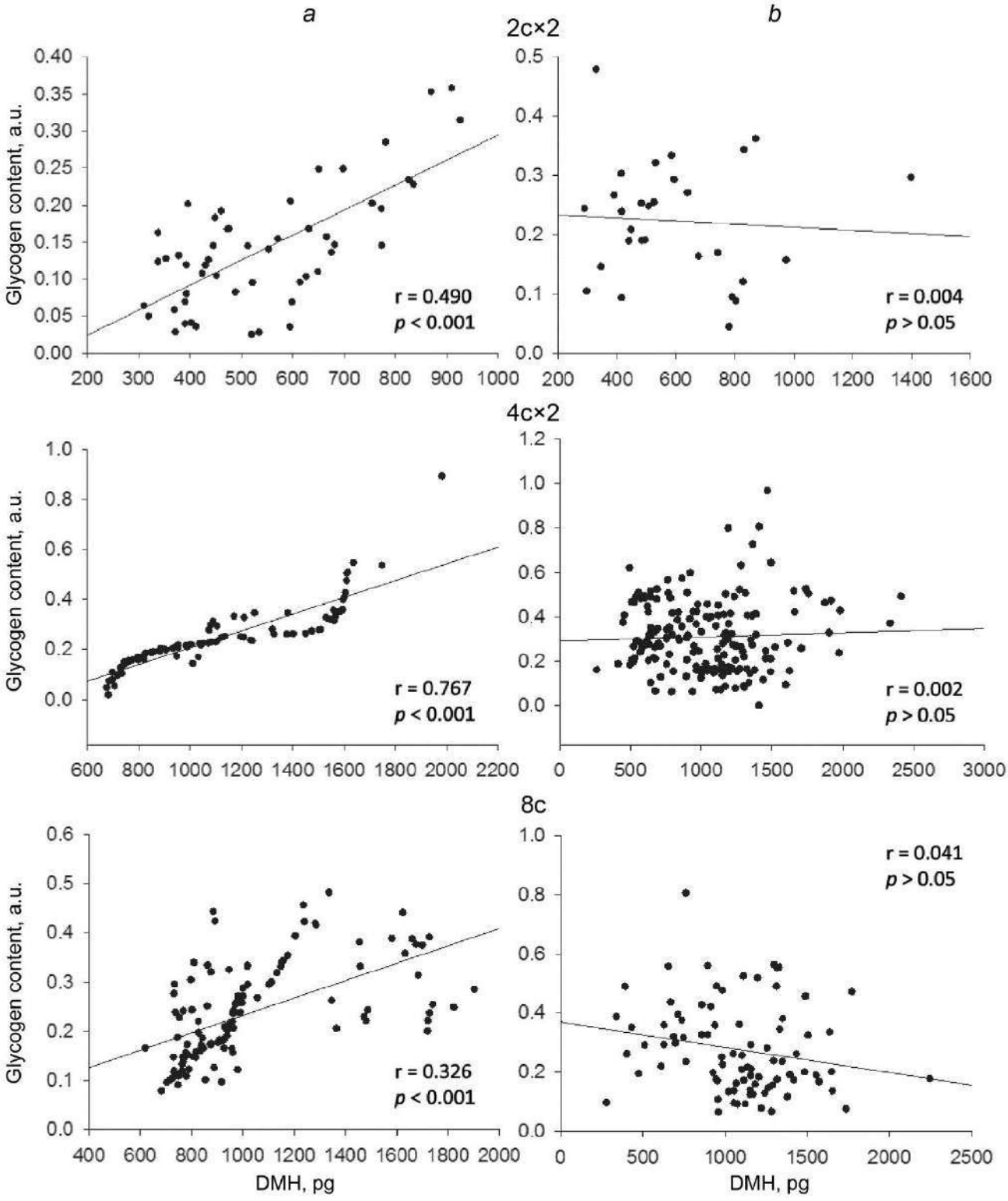


Figure 3.18 — Dependence of glycogen content on dry mass of hepatocytes (DMH) of different ploidy classes of the normal (*a*) and the cirrhotic (*b*) liver 10 min after glucose administration to starved rats. Note: each point on the graph corresponds to one cell.

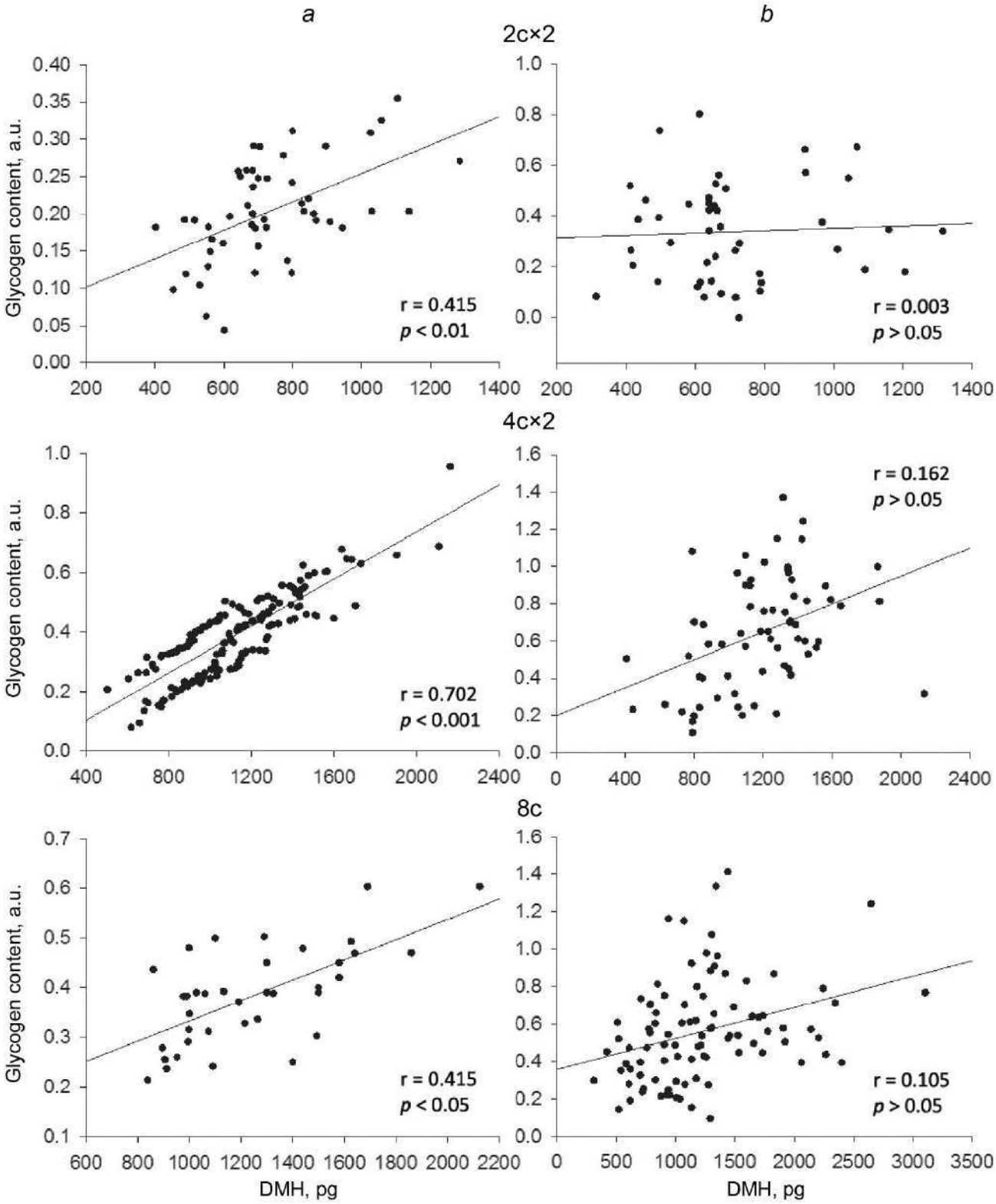


Figure 3.19 — Dependence of glycogen content on dry mass of hepatocytes (DMH) of different ploidy classes of the normal (*a*) and the cirrhotic (*b*) liver 60 min after glucose administration to starved rats. Note: each point on the graph corresponds to one cell.

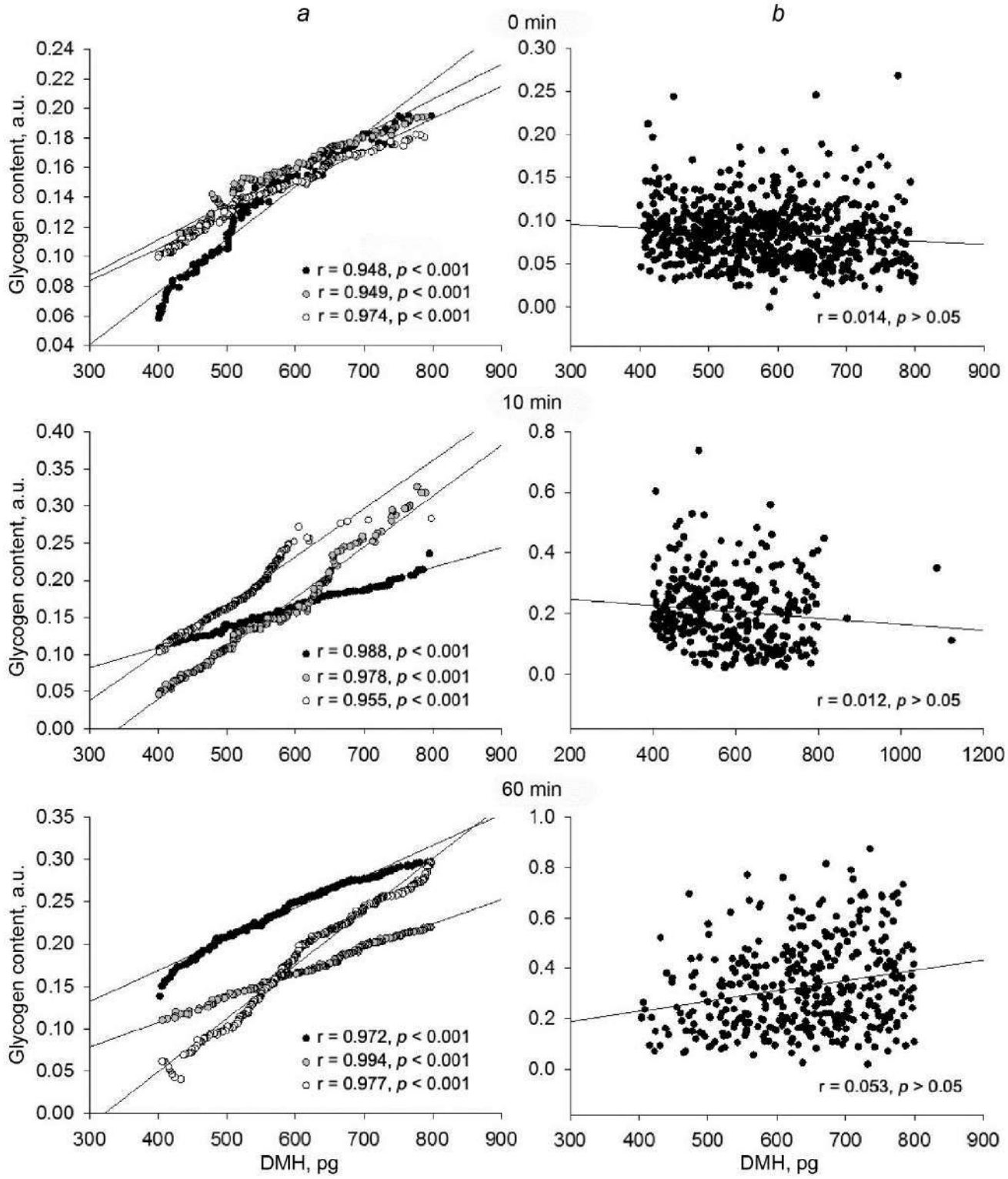


Figure 3.20 — Tetraploid hepatocytes of the control (*a*) and the cirrhotic (*b*) rat liver at different stages of refeeding. Relationship between glycogen content and their dry mass (DMH). Notes: 1) each point on the graph corresponds to one cell; 2) three subpopulations of cells in the control rat liver are shown in black, grey and white.

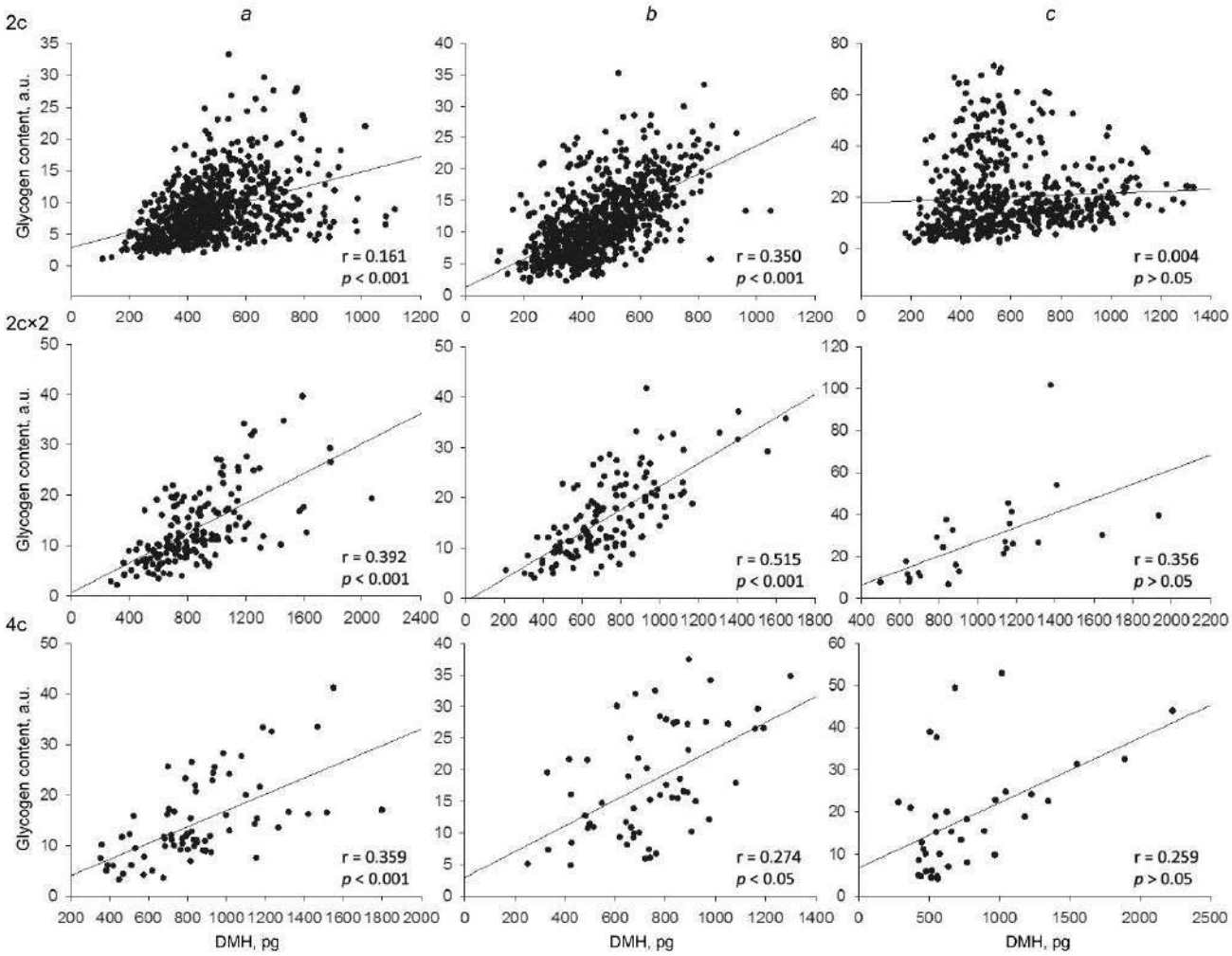


Figure 3.21 — Dependence of glycogen content on dry weight of hepatocytes of different ploidy classes in the control human liver (*a*), the liver of humans with chronic hepatitis (*b*) and the liver of humans with cirrhosis (*c*). Note: each point on the graph corresponds to one cell.

In contrast to the normal liver, there is no dependence of glycogen content in hepatocytes on their size in the cirrhotic liver of rats and humans (Fig. 3.17, *b*; 3.18, *b;* 3.19, *b*; 3.20, *b;* 3.21, *c*). Based on this, we can assume that the lack of dependence between hepatocyte size and glycogen content in the cirrhotic liver is mainly due to the disruption of its lobular structure, which leads to a sharp increase in the heterogeneity of the hepatocyte microenvironment (Bezborodkina et al., 2016).

Thus, we have shown that during the absorptive period of the food cycle the intensity of glycogen accumulation in the cirrhotic liver is lower than in the normal liver and that it differs considerably in different cells of the hepatocyte population. In addition, we found a direct correlation between the size of hepatocytes and their glycogen content in the normal liver. No such correlation was found in the cirrhotic liver.

3.2.3. Blood glucose concentration, glycogen content in hepatocytes and activity of the key enzymes of its metabolism in the normal and the pathologically altered liver of humans, rats and mice in the postabsorptive period after overnight fasting

Most data on the content and metabolism of glycogen in the normal and pathologically altered liver have been obtained for the postabsorptive period, the duration of which is 10–12 hours. For instance, in humans a typical postabsorptive period is the state of the organism after an overnight fasting.

Several studies have been devoted to the analysis of glycogen content in the pathologically altered human and rat liver during the postabsorptive period of the food cycle. Nevertheless, the available data do not provide an unambiguous answer to the question about the level of glycogen in the damaged organ. In particular, using a model of experimental LC in rats (CCl_4 + phenobarbital), it was shown that the glycogen content per hepatocyte in the cirrhotic rat liver was reduced by about 36% as compared to the norm (Krähenbühl et al., 1991). Since GS and GP*a* activity did not change, the authors concluded that the fall in the glycogen content in the cirrhotic liver was due to “intrinsic” causes. In another work, using a model of long-term cholestasis in rats, it was found that the glycogen content per millilitre of hepatocytes was reduced by approximately 61% (Krähenbühl et al., 1996). The authors attributed the decrease of the glycogen reserves to the decrease in the rate of glycogen synthesis due to a low GS activity. Finally, using the model of thiocetamide LC, it was shown that glycogen reserves in the cirrhotic rat liver make up only a half of the those in the control animals (Giardina et al., 1994).

The available data on glycogen content in the cirrhotic human liver are also ambiguous. The study of liver biopsy material from 28 alcoholic LC patients showed that the glycogen content was 25.8±3.5 mg/g of crude mass, while in two healthy people it was higher, 32.0 and 48.8 mg/g of crude mass (Owen et al., 1981). These values correspond approximately to the mean glycogen content (43.7 mg/g) in the livers of 58 healthy volunteers after 12–16 h of overnight fasting (Nilsson, 1973). The decreased glycogen content during LC was explained by an increase in the proportion of fibrous tissue, which was 41±3% in the liver parenchyma of the cirrhotic patients (Owen et al., 1981). It is known that the parenchyma occupies about 90% of the volume of the normal human liver (Snyder et al., 1975). Based on these data, we can conclude that the concentration of glycogen in fibrosis-free parenchyma in cirrhotic patients

will actually be not 25.8±3.5 mg/g, as noted in Owen et al., 1981, but 52.6±7.1 mg/g. This calculated value does not differ from the value of the glycogen concentration in the normal human liver, 43.7±1.8 mg/g, reported by Nilsson (1973).

In patients with alcoholic and biliary cirrhosis the glycogen content per millilitre of hepatocyte is about 50% lower than in the norm (Krähenbühl et al., 2003). At the same time, the levels of GS and GP in patients in the control group and the group with LC did not differ, and the authors attributed the drop in the glycogen content to a ~7-fold decrease in GK activity. However, no mechanism of GK involvement in the decrease of glycogen levels was suggested. Finally, in a study using $^{2}H_2O$ method and ^{13}C-NMR spectroscopy, it was found that glycogen concentration in the liver of cirrhotic patients was by 34% lower than in the control group. Unfortunately, the authors related the glycogen concentration to the total volume of the cirrhotic liver, and so it is not evident that glycogen levels were reduced. Furthermore, the authors showed that the decrease in the glycogen content was accompanied by a 3.5-fold drop in glycogenolysis in the liver of cirrhotic patients (Petersen et al., 1999). This fact is surprising, since such a strong decrease in glycogenolysis should have resulted to an increase in the glycogen content in the liver.

In contrast to the data presented above, the results of glycogen concentration determination in the liver of control rats and rats with CCl_4-cirrhosis in the postabsorptive period, which we obtained using the biochemical method, showed that it doubled in the cirrhotic liver as compared with the norm: 56.5±7.9 vs 28.3±6.5 mg/g of liver crude mass ($p < 0.05$), respectively (Bezborodkina et al., 2022).

One of the possible reasons behind the difference between our data and the results obtained by other authors may be different methods of determining the volume of liver parenchyma in the cirrhotic liver. The accuracy of this determination is very important for a correct estimation of glycogen concentration in the liver with the use of biochemical techniques. Therefore, we used the cytofluorimetric method to measure the glycogen content. In this case, there is no need to take into account the volume of liver parenchyma in the pathologically altered liver, because the glycogen content is measured directly in hepatocytes.

Microscopic examination of hepatocytes stained with the fluorescent PAS reaction showed that normal hepatocytes are characterised by a rather fine structure and distinct contours of the nucleus and the cytoplasm (Fig. 3.22, *a*, *c*). Chronic poisoning of rats with CCl_4 not only causes a fibrous degeneration of the liver parenchyma but also alters the

structure of the hepatocytes. They increase in size, become vacuolated and show signs of dystrophy. A distinctive feature of the cell population of the parenchyma during LC is the polymorphism of its hepatocytes both by size and by the structure of the cytoplasm. At the same time, some cells characteristic of normal liver can also be found (Fig. 3.22, *b*, *d*) (Bezborodkina et al., 2013b; Bezborodkina et al., 2022).

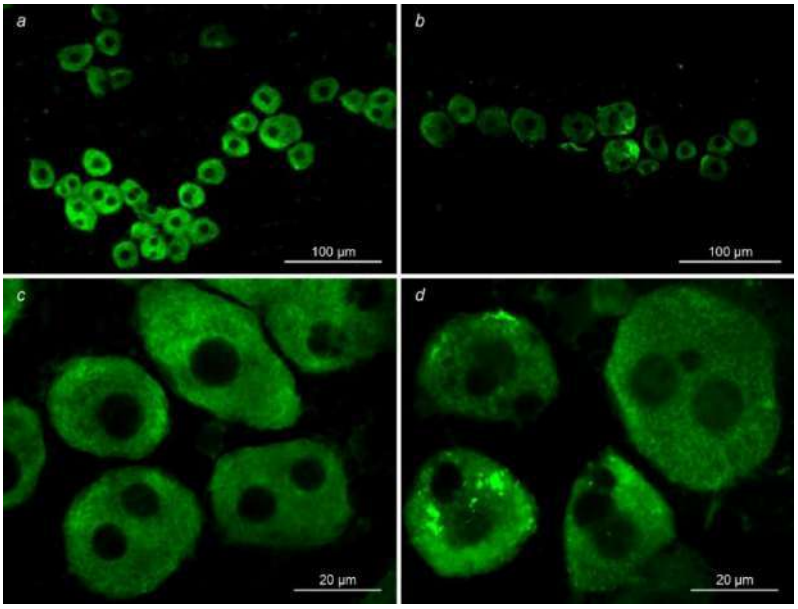


Figure 3.22 — Rat hepatocytes of the control (*a*, *c*) and the experimental group (*b*, *d*). Fluorescent PAS reaction.

Cytofluorometry of glycogen in rat hepatocytes showed that after overnight fasting its content in the cirrhotic liver was significantly higher than in the cells of the normal organ (Fig. 3.23, *a*). This confirms our results of the glycogen content measurements obtained using biochemical analysis (Bezborodkina et al., 2022).

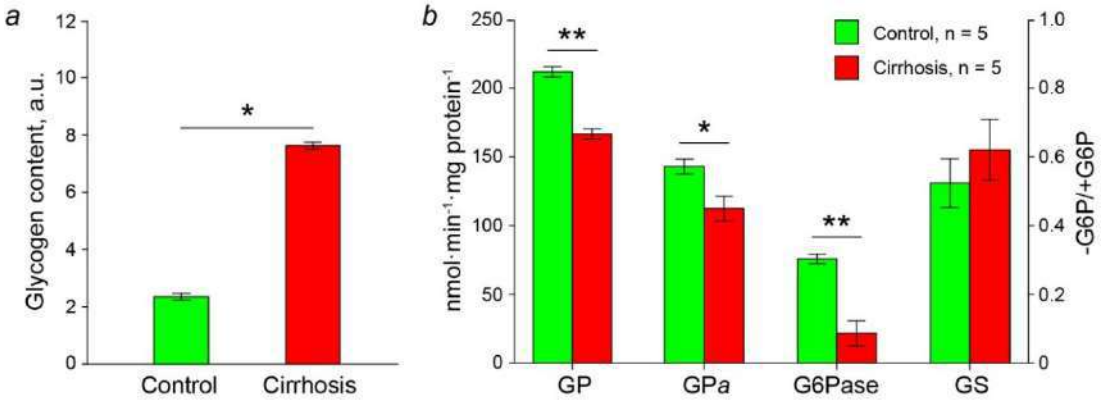


Figure 3.23 — Glycogen content in hepatocytes (*a*) and activity of enzymes of its metabolism (*b*) in the normal and the cirrhotic rat liver. GP — total glycogen phosphorylase; GP*a* — glycogen phosphorylase *a*; G6Pase — glucose-6-phosphatase; * $p < 0.05$; ** $p < 0.001$. Enzyme activity was normalised for fibrotic tissue content.

The study of the activity of the key enzymes of glycogenesis and glycogenolysis in the rat liver showed that the levels of total GP and its active form during LC were about 20% lower than in the normal liver (Fig. 3.23, *b*) (Bezborodkina et al., 2022). GS activity in the cirrhotic liver did not differ from the control values (Fig. 3.23, *b*) (Bezborodkina et al., 2022). These data are in agreement with the results obtained earlier (Kudryavtseva et al., 1994) and suggest that glycogen accumulation in hepatocytes may be caused by a weakening of glycogenolysis due to a decrease in GP*a* activity.

Analysis of micrographs of human hepatocytes stained by fluorescent PAS reaction showed that the cells of CH patients increase in size and become more polymorphic; vacuoles, apparently of lipid origin, appear in their cytoplasm (Fig. 3.24, *b, c*). In cirrhotic patients, the proportion of cells with pathological features increases and the changes become more pronounced (Fig. 3.24, *d*) (Bezborodkina et al., 2022).

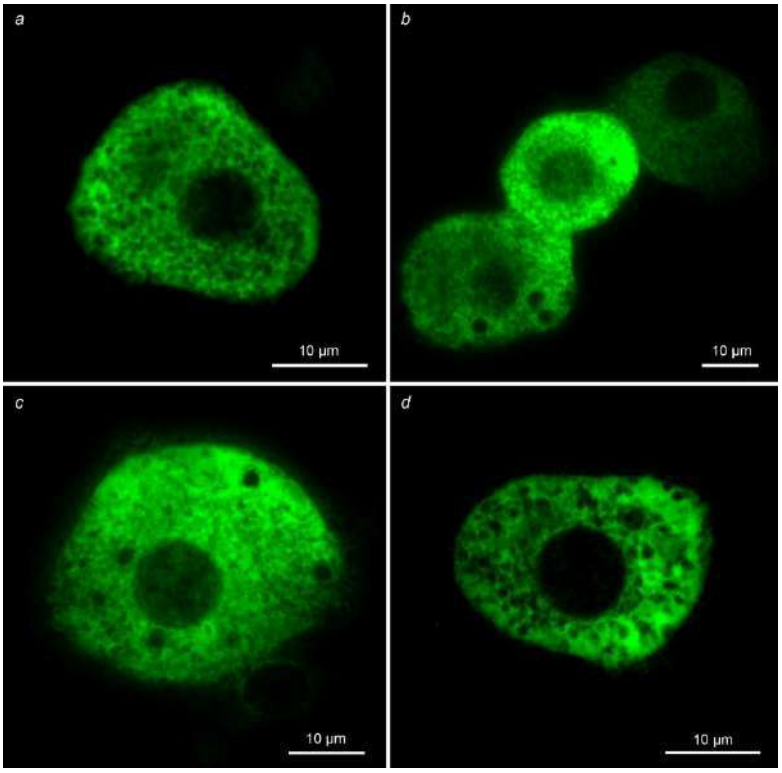


Figure 3.24 — Human hepatocytes. *a* - normal; *b*, *c* - chronic hepatitis; *c* - liver cirrhosis. Fluorescent PAS reaction.

Cytofluorometry of glycogen in the hepatocytes of the normal and the pathological human liver showed that, similarly to rats, its content during LC is much higher than in the norm (Fig. 3.25, *a*) (Bezborodkina et al., 2022). Our data also indicate that the chronic inflammatory process in the human liver is accompanied not only by the rearrangement of the

lobular structure of this organ and changes in the composition and morphology of the parenchyma cells but also by a gradual accumulation of glycogen in hepatocytes with the increasing severity of the liver damage. On average, the glycogen content in hepatocytes of CH patients exceeded the norm by 88%, and in cells of LC patients, by 160% (Fig. 3.25, *a*) (Bezborodkina et al., 2022). At the same time, glycogen accumulation in hepatocytes of LC patients was accompanied by a stronger decrease in the activities of both total glycogen phosphorylase and its active form, GP*a*, than during LC in rats: by 22% and 46%, respectively (Fig. 3.25, *b*) (Bezborodkina et al., 2022). This result confirms the earlier data (Kudryavtseva et al., 1992, 1994) and suggests that the weakening of glycogenolysis due to a low GP*a* activity is particularly important for the increase of glycogen content during LC in humans, similarly to what is observed in rats. To reveal the mechanisms of hepatocyte glycogenolysis in the cirrhotic liver, the main factors regulating GS and GP*a* activity and affecting glycogen levels in the liver should be identified.

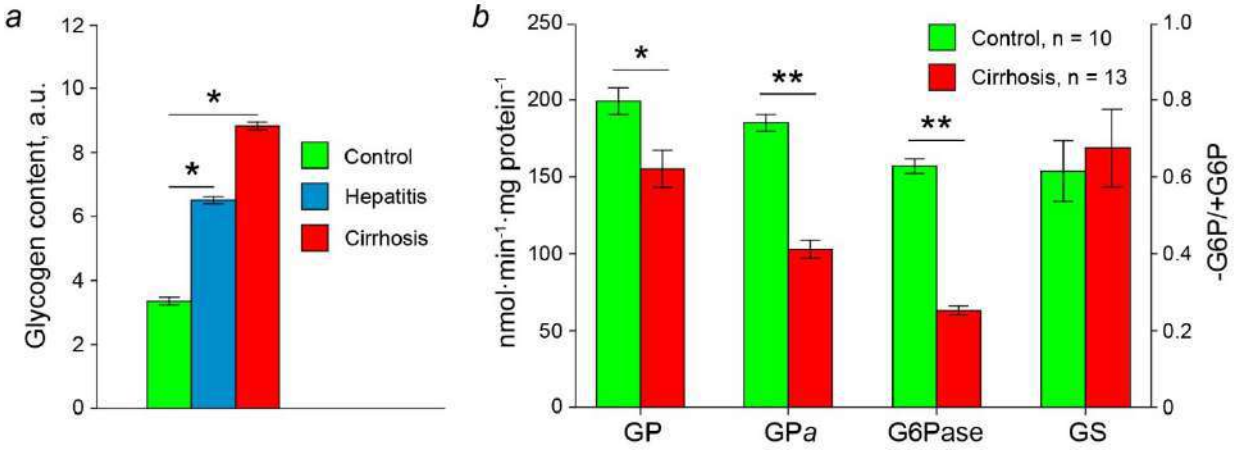


Figure 3.25 — Glycogen content in hepatocytes (a) and activity of enzymes of its metabolism (b) in the normal and the cirrhotic human liver. GP — total glycogen phosphorylase; GP*a* — glycogen phosphorylase *a*; G6Pase — glucose-6-phosphatase; * $p < 0.05$; ** $p < 0.001$. Enzyme activity was normalised for fibrotic tissue content.

Alongside with the hormonal and the energy status of the organism, phosphorylation-dephosphorylation processes, and changes in the localisation in the cell (Biorn et al., 2001; Ferrer et al., 2003; Jurczak et al., 2008; Roach et al., 2012), an important role in the regulation of these enzymes is played by the concentration of G6P in hepatocytes. G6P determines the extent of GS phosphorylation, promotes better interaction with phosphatase synthase (von Wilamowitz-Moellendorff et al., 2013) and affects the localisation of the enzyme in the cell

(Fernandez-Novell et al., 1996). An increase in the intracellular concentration of G6P increases GS activity and stimulates glycogen synthesis (Ciudad et al., 1986; Villar-Palasi, Guinovart, 1997).

Under physiological conditions, GP*a* activity is inversely correlated with the concentration of G6P in cells. In contrast to GS, the increase of G6P in hepatocytes leads to a decrease in GP*a* activity and the inhibition of glycogenolysis, regardless of whether this metabolite is formed by phosphorylation of glucose by GK or in the process of gluconeogenesis (Aiston et al., 2003). Thus, G6P in hepatocytes is a major determinant of the rate of glycogen synthesis and the rate of glycogenolysis. The concentration of G6P in hepatocytes is strongly influenced by the opposite action of GK and G6Pase (Seoane et al., 1997). Therefore, a change in their activities can be expected to impact the activities of GS and GP and, consequently, the glycogen content in the cells.

According to our data, the activity of G6Pase in the liver of rats with CCl_4-cirrhosis in the postabsorptive period made up 29% of its activity in the normal liver (Fig. 3.23, *b*) (Bezborodkina et al., 2022). According to the data obtained earlier, the activity of this enzyme during LC in rats can vary from 23 to 36% of the normal level (Kudryavtseva et al., 1994, 1996). A significant decrease in the activity of G6Pase has also been found by many authors in the liver of cirrhotic patients (Taketa et al., 1976; Owen et al., 1981; Pieniazek et al., 1985; Sotaniemi et al., 1985; Shimamura et al., 1987; Kruszynska, McIntyre, 1991). In particular, it has been shown that in the cirrhotic human liver G6Pase activity after overnight fasting is less than 50% of the normal level (Kudryavtseva et al., 1992). Our study of biopsied clinical material showed that the drop in G6Pase activity depended on the severity of liver damage. In patients with CH, G6Pase activity in the postabsorptive period was 1.2 times lower than the norm, while in patients with LC it was 4.4 times lower than the norm (Fig. 3.25, *b*) (Bezborodkina et al., 2022).

The available data suggest that GK activity in the cirrhotic human liver in the postabsorptive period is also very low (Taketa et al., 1976; Sotaniemi et al., 1985; Lowes et al., 1998; Krähenbühl et al., 2003). This low GK activity cannot be explained solely by the loss of the parenchyma in cirrhotic patients, since its volume is reduced by about a half, while the enzyme activity is reduced by several times. According to some authors, a possible reason behind the decrease in GK activity during LC is an impaired synthesis of the GK protein (Taketa et al., 1976; Sotaniemi et al., 1985). However, this factor is unlikely to be the main

one, since the rate of protein synthesis in hepatocytes of the cirrhotic liver decreases much less considerably than the activity of this enzyme (Tessari, 2003; Katayama, 2020; Guldiken et al., 2021). Therefore, the mechanism of the significant decrease of GK activity remains unclear. Nevertheless, an extremely low GK activity during LC suggests that in the postabsorptive period the rate of glucose phosphorylation and the rate of glycogen synthesis in hepatocytes by the "direct" pathway (glucose→G6P→glycogen) are low.

It is known that hepatocytes can use G6P, which is formed during glycogenolysis and gluconeogenesis, for glucose production. In the normal liver after overnight fasting, 70–80% of glucose is produced by glycogenolysis and only 20–30% by gluconeogenesis (Krusczynska, McIntyre, 1991; Krusczynska, 1999). During LC, the contribution of gluconeogenesis to glucose production increases to 67–87%, while the relative contribution of glycogenolysis sharply decreases (Owen et al., 1981; Bugianesi et al., 1998; Petersen et al., 1999; Perdigoto et al., 2003).

Some authors have suggested that the reduced contribution of glycogenolysis to glucose production during LC is due to a decrease in the glycogen content of hepatocytes (Owen et al., 1981; Schricker et al., 1996; Petersen et al., 1999). However, it has been fairly pointed out that no such conclusion can be made in the absence of data of direct assessment of glycogen content in biopsied cirrhotic liver (Bugianesi et al., 1998). Indeed, our cytofluorimetric analysis of hepatocytes isolated from the cirrhotic liver of rats and humans revealed a high glycogen content in them (Fig. 3.23, *a*, Fig. 3.25, *a*). It may be due, firstly, to an intensive gluconeogenesis, which increases the concentration of G6P in the liver and, as a consequence, weakens the activity of GP, the key enzyme of glycogen degradation, and, secondly, to a low activity of G6Pase. An important role of G6Pase in the regulation of glycogen levels in the liver is confirmed by the fact that in patients with hereditary glycogenosis type I, when this enzyme is inactive, hepatocytes accumulate huge amounts of glycogen (Beaty et al., 2002).

3.2.4. Comorbidity of liver cirrhosis and diabetes. Influence on glycogen content in hepatocytes

Comorbidity is a characteristic feature of many human diseases including LC. Up to 70% of LC patients have clinically significant DM type 2 and hepatogenic diabetes (Garcia-Compean et al., 2009; Kumar, 2018). Diabetes has been shown to reduce the life expectancy in

cirrhotic patients (Garcia-Compean et al., 2015; Kumar, 2018). The mechanism of diabetes development during LC is associated with a decrease in the liver parenchyma mass and structural remodelling of the liver and its vasculature. Under these conditions, the expression of insulin receptors is reduced, which leads to hyperinsulinaemia and insulin resistance (Nishida, 2017).

There is increasing evidence that LC and DM type 2 share a number of similarities. The only major differences between LC and DM type 2 are the metabolic section associated with glucose production by the liver and the histological structure of the organ (Fig. 3.26). Compared to diabetes, cirrhosis appears to be characterised by a much greater liver fibrosis (Anthony et al., 1978; Owen et al., 1981; Doycheva et al., 2013; Zhou et al., 2014; Torbenson, Washington, 2020). However, it should be noted that there are few histological studies of diabetic liver, that no comparative analysis of the liver morphology during LC and DM has been performed and that there are no data on the morphometric indices of the diabetic liver. As for the differences in glucose metabolism in the diabetic and the cirrhotic liver, they are mostly associated with glucose production and G6Pase activity. In contrast to LC, glucose production and G6Pase activity in DM are much higher than the norm (Garfield, Cardell, 1979; Kolterman et al., 1981; Magnusson et al., 1992; DeFronzo et al., 1992; Clore et al., 2000; Westergaard, Madsen, 2001). In other parameters, DM and LC are similar (Alberti, 1981; Petrides, DeFronzo, 1989; Levinthal, Tavill, 1999; Jiang et al., 2020; Loria et al., 2013; Schofield et al., 2017) (Fig. 4.26).

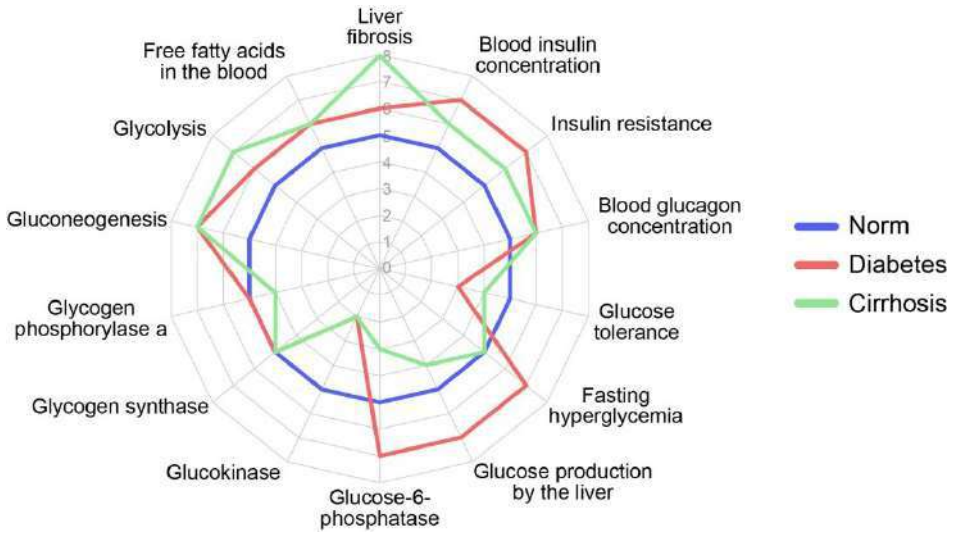


Figure 3.26 — Metabolic and morphological parameters of patients with DM type 2 and cirrhosis (as compared to the norm).

The issue of glycogen content in the liver of diabetic patients has long attracted the attention of researchers. The general conclusion of the studies conducted on the liver of patients with diabetes or depancreatised animals was that diabetes leads to a decrease in glycogen content (Vallance-Owen, 1952) and the rate of its synthesis (Hornbrook, 1970; Whitton, Hems, 1975; Golden et al., 1979) as compared to the norm. The decreased ability of the liver of DM patients to synthesise glycogen was explained by defective GS activation (Bishop, 1970; Tan, Nuttall, 1976; Bollen et al., 1983).

However, the question about the glycogen content in the liver of DM patients has remained open for two reasons. Firstly, it has been found in a number of studies that the livers of many patients who did not receive insulin therapy and those who died of diabetic coma contain normal or increased amounts of glycogen (Geelmuden, 1920; Warren, 1930; Popper, Wozasek, 1931). Secondly, information about the low liver glycogen content has been obtained mainly from animals in which DM was induced for a relatively short time (short-term diabetes). Meanwhile, it is known that DM in humans develops over many months and even years (long-term diabetes). When using models of DM, the condition of duration of exposure of laboratory animals is usually not observed and the factor of adaptation of different substrate fluxes to a long-term pathological condition is usually not taken into account. It is therefore assumed that low liver glycogen levels in diabetes are the exception rather than the rule (Friedmann et al., 1963).

The role of liver glycogen in the pathogenesis of DM remains unclear. To detect early pre-diabetic changes in glycogen content and structure, we used transgenic homozygous OE-NPY$^{D\beta H}$ mice with a phenotype of late-onset obesity, hepatosteatosis and pre-diabetes (Ruohonen et al., 2008; Vähätalo et al. 2015). C57BL/6N mice were used as wild-type phenotype (WT) animals. To reduce blood glucose levels, the mice were treated with the anti-hyperglycaemic agent metformin, which is widely used to treat diabetes.

The data presented in Table 3.7 indicate that during the postabsorptive period, the glycogen content in hepatocytes of OE-NPY$^{D\beta H}$ mice and WT mice is similar (Ailanen et al., 2018). At the same time, the structure of glycogen in hepatocytes of OE-NPY$^{D\beta H}$ mice is dramatically different from that in hepatocytes of wild-type mice. Treatment of homozygous OE-NPY$^{D\beta H}$ mice with metformin leads to an almost twofold decrease in glycogen content in hepatocytes, but at the same time normalises the ratio of its fractions. In WT mice, metformin has no effect on either the glycogen content of hepatocytes or its fractional composition

(Ailanen et al., 2018). The reasons for the different effects of metformin on glycogen content in hepatocytes of WT- and OE-NPY$^{D\beta H}$-mice appear to be associated with the different characteristics of gluconeogenesis in the liver of these murine lines.

It is known that diabetes leads to an increased gluconeogenesis in the liver as compared to the norm (Consoli et al., 1989; Giaccari, Rosetti, 1992; Torres et al., 2011; Jiang et al., 2020). It has also been shown that metformin markedly decreases the rate of hepatic gluconeogenesis (Alengrin et al., 1987; Heishi et al., 2006; Otto et al., 2003). Based on these data, it can be assumed that glycogen synthesis and accumulation in hepatocytes of OE-NPY$^{D\beta H}$ pre-diabetic mice occurs predominantly by the indirect pathway, via gluconeogenesis (C_3-precursors→G6P→glycogen), whereas in the liver of C57BL/6N mice, the direct pathway (glucose→G6P→glycogen) is mainly used for glycogen synthesis (Ailanen et al., 2018). This assumption is also supported by the fact that the indirect pathway of glycogen synthesis is markedly increased in diabetes (Giaccari, Rosetti, 1992; Bischof et al., 2002).

Table 3.7 — Content of total glycogen (TG) and the ratio of its labile and stable fractions (LF/SF) in hepatocytes of C57BL/6N wild-type (WT) mice and OE-NPY$^{D\beta H}$ transgenic homozygous mice before and after metformin action, arbitrary units, ($X \pm S_x$, n = 3).

Genotype	Treatment	TG, arbitrary units	LF/SF
WT	Without metformin	28.67±0.71	1.46±0.07
	Metformin	25.64±0.76	1.37±0.08
OE-NPY	Without metformin	30.74±0.71	0.64±0.03[1]
	Metformin	16.58±0.49[1]	1.61±0.09

[1] Significantly different from the other TG or LF/SF values ([1] $p < 0.05$).

Cytofluorometry of glycogen content in hepatocytes of DM type 2 patients after overnight fasting revealed that it was increased 2.7-fold compared to the cells of the control group (Table 3.8). The data presented in Table 4.8 indicate that the glycogen content in hepatocytes of LC patients does not differ from that in cells of patients with comorbid form of the disease, LC + DM 2.

Table 3.8 — Mean glycogen content in hepatocytes of healthy humans (control), patients with type 2 diabetes mellitus (DM 2), patients with liver cirrhosis (LC) and patients with the combined form of these two diseases (LC+DM 2) (arbitrary units, $X \pm S_x$).

	Control (n = 9)	DM 2 (n = 12)	LC (n = 4)	LC + DM 2 (n = 5)
Glycogen content	1.88± 0.22	4.19± 0.38[1]	6.90± 0.32[1a]	6.12± 0.79[1a]

[1]Significantly different from the value in the control ([1]$p < 0.001$). [a] Significantly different from the value in DM 2 group ([a]$p < 0,05$).

Our data confirm the results obtained previously on patients with DM (Vallance-Owen, 1952; Manderson et al., 1968) and on rats with alloxan or streptozotocin diabetes (Winternitz, Lattanzi, 1956; Van de Werve, Jeanrenaud, 1987; Ferrannini et al., 1990; Giaccari, Rosetti, 1992; de Oliveira et al., 2007; Bhanudas, Gopal, 2016). The glycogen content in hepatocytes of DM type 2 patients was comparable to that of LC patients (Table 3.8). A slightly increased glycogen content in hepatocytes of the cirrhotic liver may be due to the small sample of patients (4 individuals). It should also be noted that the comorbidity of cirrhosis and diabetes does not result in either additive or potentiated synergistic effects, as the glycogen content in hepatocytes of patients in LC + DM 2 group is almost the same as in patients with DM type 2 alone or LC alone (Table 3.8). The reason may be that glycogen synthesis in these diseases occurs via the same metabolic pathway, gluconeogenesis, whose capacity is limited.

Thus, glycogen content in hepatocytes of patients with DM type 2 in the postabsorptive period is increased as compared to the norm, but comorbidity of DM with LC does not increase glycogenosis.

3.3. Spatial structure of glycogen molecules in hepatocytes in the normal and the cirrhotic liver of rats and humans

As shown above, glycogen content in cells may undergo rapid and large-scale changes in response to the demands of the organism. At the same time, changes in glycogen content in cells can be associated not only with the activity of the key enzymes of its metabolism and numerous factors regulating enzyme activity, but also with the number of glycogen molecules and its internal structure. It has been established that the structure of glycogen molecules in the

cells of normal and pathologically altered organs and tissues can be considerably rearranged (Kudryavtseva et al., 1988, 1992; Kudryavtseva, 1987; Shearer et al., 2005; Kishnani, Chen, 2007; Wilson, 2010; Roach et al., 2012; Gilbert, Sullivan, 2014). At the same time, the mechanisms of formation and degradation of glycogen molecules (β-particles), the dynamics of their composition under normal and pathological conditions, the role of structural changes in the regulation of glycogenesis and glycogenolysis are still unclear in many respects. This lack of information is mainly associated with the limitations of modern methods when it comes to the study of the structure of glycogen molecules in cells.

3.3.1. Cytofluorimetric method for studying glycogen structure in individual hepatocytes

The spatial structure of such a large molecule as glycogen cannot be described with sufficient accuracy and completeness by any of the modern methods (Brust et al., 2020). Therefore, a method allowing the investigation of the spatial structure of glycogen molecules (β-particles) in individual hepatocytes is urgently needed. However, it should be noted that glycogen molecules consist of absolutely identical links, glucose residues, and therefore the study of its structure is not an easy task.

The disadvantages of the existing methods of studying the spatial structure of glycogen are as follows:

- they are applied to glycogen isolated from tissues, which entails a risk of disturbing its original structure;

- they have a low sensitivity. The minimum amount of tissue for the determination of glycogen content, let alone the determination of any parameters of its molecules, is 10–100 μg (10^{-6} g). The minimum determinable amount of glycogen in this sample is 3–50 μg (Delaval et al., 1983). According to our data, the wet mass of one hepatocyte in the normal rat liver is approximately 3.5 ng (10^{-9} g), and the minimum amount of glycogen in one hepatocyte is 10-20 pg (10^{-12} g) (Bezborodkina et al., 2022). Consequently, a method for studying glycogen structure in a single hepatocyte should be able to determine with a high accuracy ~100000 times lower glycogen content than the existing methods in a tissue sample that is smaller by ~3000 times;

- they do not take into account the heterogeneity of the cell composition of the liver, which increases significantly in case of liver pathology.

The development of our method for studying glycogen structure in individual cells was based on:

- the hypothesis of the spatial structure of the glycogen molecule currently supported by the largest body of evidence. It is based on the existence of pro- and macroglycogen (PG, MG) and their characteristics. PG includes eight inner tiers of the molecule, contains ~5% of glucose residues and ~10% of glycogenin, is not extracted from tissues by 10% TCA; MG includes four outer tiers (9th to 12th) of the fully formed molecule[*)], contains ~95% of all glucose residues, is extracted from tissues by TCA treatment; PG serves as an intermediate in the pathway of MG formation (Lomako et al., 1993; Alonso et al., 1995; Melendez et al., 1997; Adamo, 1998).

- Periodic Acid-Schiff (PAS reaction), which is widely used for specific detection of glycogen in cells (Lillie, 1965). This reaction can be used to quantify the glycogen content of cells (Kudryavtseva et al., 1970; Gahrton et al., 1975). The PAS reaction consists of two main steps: mild oxidation of preparations in iodic acid solution and their subsequent staining in Schiff's reagent. During oxidation with iodic acid or its salts, aldehyde groups are formed from 1.2-glycol groups in the glucose residues of glycogen, which are then detected using Schiff's reagents. The classic version of Schiff's reagents is a solution of basic fuchsin in sulphurous acid, but other stains, including fluorescent stains, can also be used.

- FRET (Förster Resonance Energy Transfer) method. This state-of-the-art microscopic analysis of interactions between biological macromolecules is highly sensitive to the smallest changes in the distance between fluorophores. Förster resonance energy transfer typically occurs at distances between 1 and 10 nm, which is comparable to the size of most biological macromolecules. FRET is a radiation-free process in which the energy of a fluorescent donor molecule (Don), which is in the excited state, is transferred to the ground state of another fluorescent molecule, the acceptor (Ac), by dipole-dipole interaction (Piston, Kremers, 2007; Grecco, Verveer, 2011).

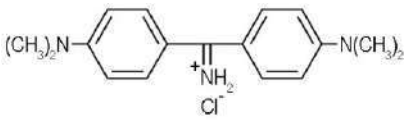
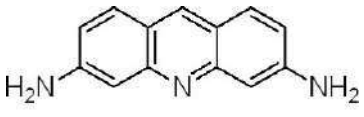
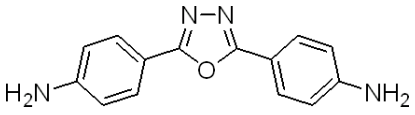
The development of the new method involved several steps: 1) testing different fluorescent stains as substitutes for basic fuchsin in Schiff's reagent; 2) selection of a pair of stains which can be used not only to selectively detect glycogen in cells during PAS reaction

[*)] Some authors consider macroglycogen (MG) as a complete glycogen molecule, which comprises 12 tiers of glucose residues together with 8 tiers of proglycogen (PG), containing 4–5 % of glucose residues in the complete glycogen molecule. We distinguish two parts of the molecule: the 8 tiers of PG and MG, represented by glucose residues on the 9–12th outer tiers of the molecule.

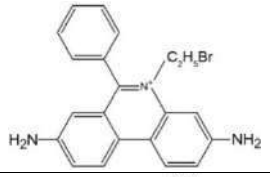
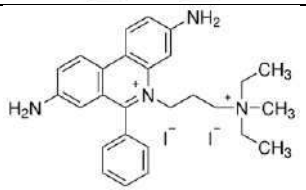
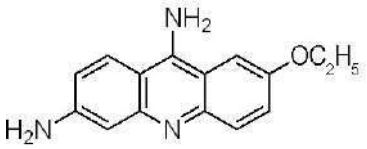
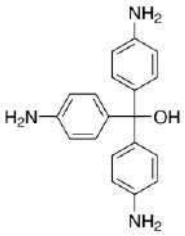
but also as Don and Ac in FRET analysis; 3) calculation of Förster distance R_0 for the selected pair of stains; 4) investigation of the dependence of the glycogen staining intensity in hepatocytes with the use of two selected Schiff's reagents of different colours on the time of staining; 5) determination of optimal conditions for staining preparations to detect and estimate the content of TCA-soluble and TCA-insoluble fractions of glycogen in individual cells; 6) development of a technique for calculating the number of glucose residues on the outer tiers of glycogen molecules.

The choice of stains for cytofluorimetric studies when two or more stains should be used simultaneously is a difficult task. On the one hand, an important condition for using the method to determine the content of TCA-soluble and TCA-insoluble fractions of glycogen in hepatocytes is that the fluorescence spectra of the stains used in Schiff reactions should overlap as little as possible. On the other hand, the application of the FRET method to the analysis of the internal structure of glycogen molecules in cells requires at least partial overlapping of the fluorescence spectra of Don emission and Ac absorption. Therefore, when choosing a pair of stains, we took into account not only the specificity of stain binding to aldehyde groups of glycogen but also its spectral characteristics. The list of stains tested in this work is given in Table 3.9 (Bezborodkina et al., 2009b).

Table 3.9 — Characteristics of fluorescent stains used for the preparation of Schiff's reagents.

Stain (source of information)	Abbre-viated title	Structural formula	Concen-tration, %	λ of excitation, nm	λ_{max} of fluorescence, nm
Auramine OO (Bosshard, 1964; Kudryavtsev, Rozanov, 1974)	Au		0.3	405, 436	526
Acriflavine (Böhm, Sprenger, 1968)	Afl		0.001	546	605
2,5-Bis-(4-aminophenyl)-1,3,4-oxdiazole (Yataghanas et al., 1969)	BAO		0.00001	365	440–450

Continue of table 3.9.

Stain (source of information)	Abbre-viated title	Structural formula	Concen-tration, %	λ of excitation, nm	λ_{max} of fluorescence, nm
Ethidium bromide (Kudryavtsev et al., 1974)	EtBr		0.0004	546	595–610
Propidium iodide (Foglieni et al., 2001)	PI		0.0003	546	615–625
Rivanol (Khachaturov, Smirnova, 1966)	Riv		0.01	380–480	520
Pararosaniline (Böhm, Sprenger, 1968; Fujita, 1973)	pRA		0.01–0.05	570	620

Note: Schiff's reagents were aqueous solutions of stains of appropriate concentration to which thionyl chloride was added (0.2 ml per 100 ml of stain solution).

Examination of preparations stained with Schiff's reagents showed that all reagents detected glycogen in hepatocytes with a high selectivity (Fig. 3.27). The reaction for glycogen with all reagents was negative after pre-treatment of the preparations with α-amylase and when the preparations were not oxidised with iodic acid before being placed in the Schiff's reagent. It turned out, however, that rivanol and especially acriflavin have metachromatic properties, i.e. the fluorescence spectrum of these stains shifted when the concentration of the tested substance was changed. In addition, in cytofluorometry of cells stained with acridine stains, the effect of the internal filter is quite pronounced. In addition, as the concentration of acridine stains and BAO in Schiff's reagents increased, a weak non-specific staining of cells was observed. Another disadvantage of BAO is that the cells treated with this stain fluoresce in the blue-green part of the spectrum, where, as it is known, the intensity of the cells' own fluorescence is particularly high. A number of drawbacks were also noted for other stains. Auramine 00 was insufficiently resistant to excitation light. Schiff's reagents based on auramine 00 and

propidium iodide can be used only once because the precipitate is formed. Pararosaniline can be used only in very low concentrations because of the possible disruption of the proportionality between the stain content and the substance under investigation. In addition, its use requires strict control of the absorption intensity of the excitation light to avoid errors in photometry (Bezborodkina et al., 2009).

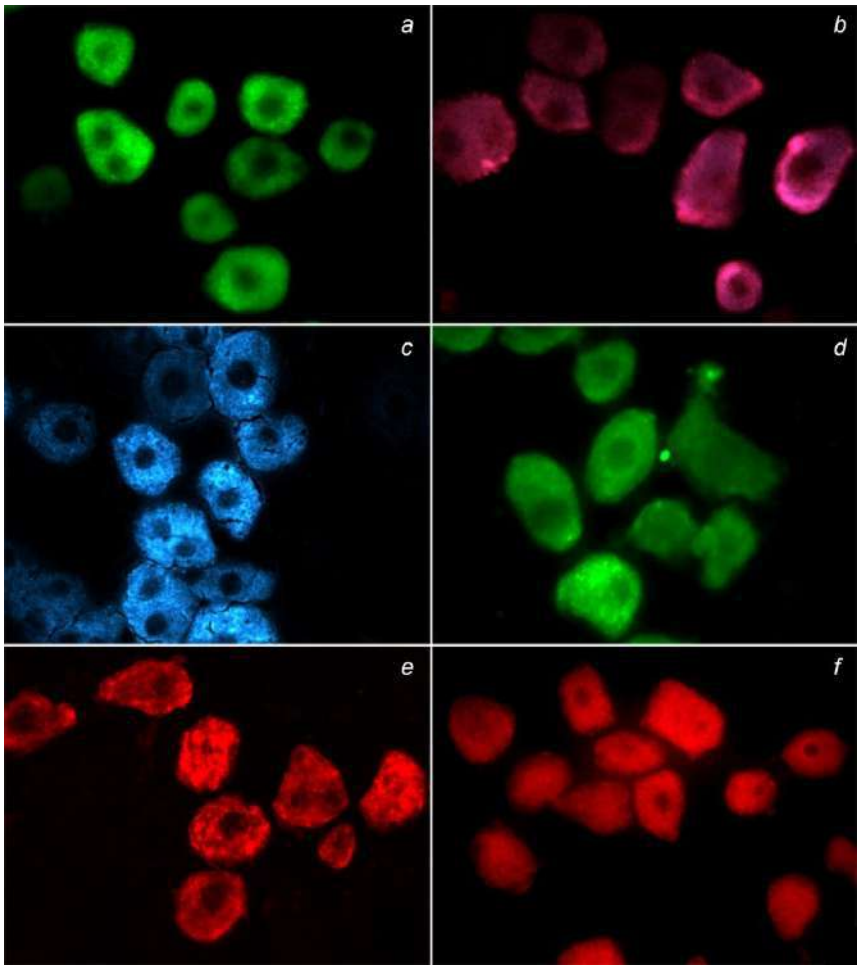


Figure 3.27 — Rat hepatocytes stained by PAS reaction using Schiff's reagents: auramine-SO_2 (*a*), basic fuchsin-SO_2 *(b),* BAO-SO_2 *(c),* rivanol-SO_2 *(d)*, ethidium bromide-SO_2 *(e)* and acriflavine-SO_2 *(f*). Axioskop (Carl Zeiss, Jena, Germany), objective 40x.

As a result, auramine 00 (Au) and ethidium bromide (EtBr) were chosen for further studies. Firstly, Schiff's reagents Au-SO_2 and EtBr-SO_2 used in PAS reaction allow a specific detection of glycogen in hepatocytes. Secondly, the fluorescence spectra of hepatocytes stained with Au-SO_2 (λ_{max} ~ 526 nm) and EtBr-SO_2 (λ_{max} ~ 610 nm) overlap weakly (Fig. 2.7) and do not shift with the change in the glycogen concentration in cells. It would be possible to use pararosaniline as a “red” stain, too, but EtBr was considered preferable, given an extensive and successful experience of its use as a fluorescent stain.

Since aldehydes readily enter into various addition, substitution and condensation reactions, it was necessary to ascertain whether aldehyde groups retain their reactivity during prolonged staining with Schiff's reagent Au-SO_2.

The data presented in Figure 3.28 indicate that there was no loss of aldehyde groups when the preparations were kept for 3 h in an aqueous solution of SO_2 with a concentration identical to that of SO_2 in Schiff's reagent. Taking into account these data, the dependence of the staining intensity of preparations on the time of their treatment with Schiff's reagents was studied (Bezborodkina et al., 2009b).

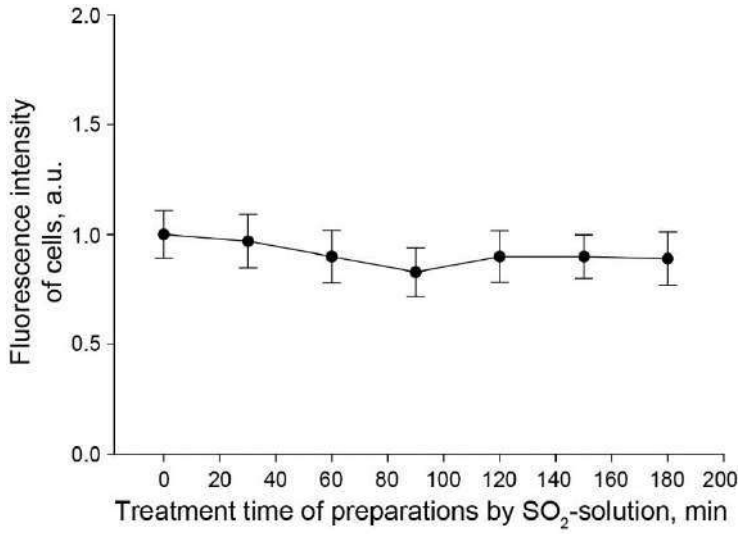


Figure 3.28 — Effect of different incubation time of preparations in SO_2 solution on the stability of aldehyde groups of glycogen in hepatocytes. Note: vertical sections — error of the mean.

To determine the nature of the dependence of the PAS reaction intensity on the duration of cell staining with Schiff's reagents Au-SO_2 and EtBr-SO_2, hepatocytes on coverslips were oxidised in 0.8% KIO_4 solution. Then they were treated for various times at room temperature with either Au-SO_2 (0.3% Au + 0.2 ml $SOCl_2$/100 ml) or EtBr-SO_2 (0.0004% EtBr solution + 0.2 ml $SOCl_2$/100 ml). After completion of the staining, all preparations were simultaneously removed from the reagents and processed according to the procedure described in Section 2.3.2.1.

The results presented in Figure 3.29 show that the dependence of hepatocyte fluorescence intensity on the time of staining during PAS reaction using both reagents has a similar two-stage character. In the interval of 0–40 min there is a rapid binding of both Au-SO_2 and EtBr-SO_2, while in the interval of 40–70 min the binding of both stains decreases sharply

(or stops altogether). After that, a new wave of intensive stain binding is observed. After 90 min the graph of the dependence of cell fluorescence intensity on staining time reaches a plateau, indicating the completion of stain binding to aldehyde groups of glycogen (Bezborodkina et al., 2011; Bezborodkina et al., 2021a).

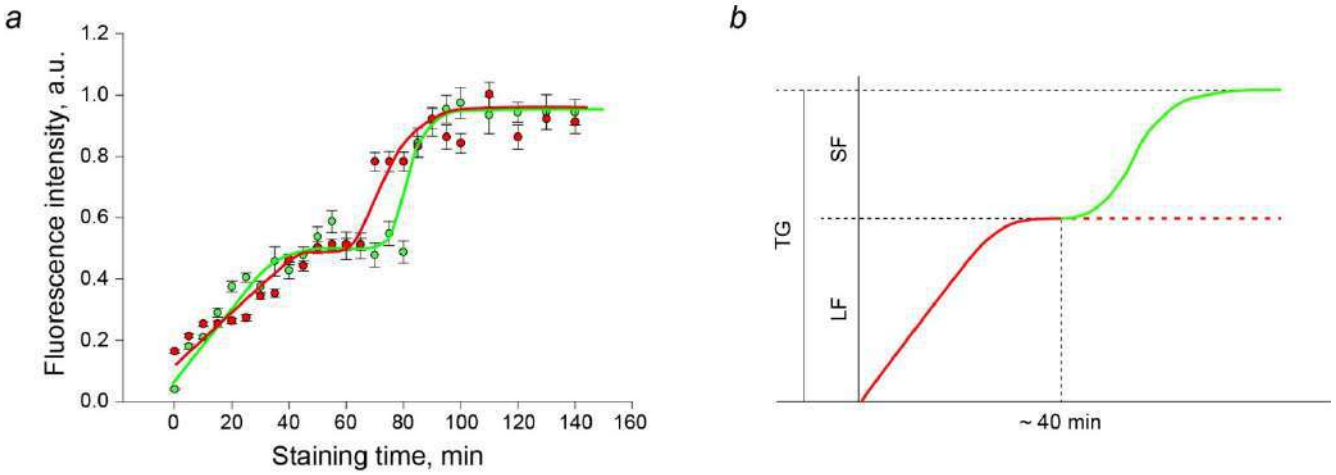


Figure 3.29 — Fluorescent PAS reaction with Schiff's reagents. *a* — dependence of hepatocytes fluorescence intensity on the duration of their staining with EtBr-SO_2 (red line) and Au-SO_2 (green line); *b* — scheme showing sequential staining of cells with EtBr-SO_2 (red line) and then Au-SO_2 (green line). Note: cells derived from different rats were used in the study of the relationships shown in Figure *a*. LF — rapidly staining glycogen fraction. SF — slowly staining glycogen fraction. TG — total glycogen = LF + SF.

To elucidate the nature of the rapidly staining (LF) and slowly staining (SF) glycogen fraction, hepatocyte preparations on slides obtained from the same rat were treated with 10% TCA or 30% KOH before staining with Au-SO_2 or EtBr-SO_2 (Bloom, Knowlton, 1953).

The results of this experiment (Table 3.10) indicate that glycogen in hepatocytes is fully detectable after 90 min of oxidation of the preparations in KIO_4 and subsequent staining with Au-SO_2 or EtBr-SO_2. The fraction of glycogen detected quickly (within 40 min) after the beginning of staining in Schiff's reagent corresponds to the fraction easily extractable with TCA (labile fraction), while the fraction of glycogen detected slowly (in the interval from 40 to 90 min) corresponds to the fraction that is fully extracted only when the preparations are treated with hot KOH (stable fraction). Our data (Table 3.10) indicate that in the hepatocytes of experimental rats the proportion of the fraction sensitive to treatment with 10% TCA is ~72% and the proportion of the fraction not sensitive to TCA treatment is ~28% (Bezborodkina et al., 2011; Bezborodkina et al., 2021a).

Table 3.10 — Effect of different treatments on fluorescence intensity of hepatocytes stained by PAS reaction using Schiff's reagents Au-SO_2 or EtBr-SO_2

Type of treatment	Fluorescence intensity (arbitrary units)
KIO_4 → Au-SO_2 (90 min)	224±12
KIO_4 → EtBr-SO_2 (90 min)	198±15
KIO_4 → Au-SO_2 (40 min)	159±8
KIO_4 → EtBr-SO_2 (40 min)	147±11
10% TCA → KIO_4 → Au-SO_2 (90 min)	66±5
10% TCA → KIO_4 → EtBr-SO_2 (90 min)	54±4
30% KOH → KIO_4 → Au-SO_2 (90 min)	5.9±0.4
30% KOH → KIO_4 → EtBr-SO_2 (90 min)	5.3±0.3
H_2O → Au- SO_2 (90 min)	9.7±0.5
H_2O → EtBr-SO_2 (90 min)	8.4±0.5
α -amylase → KIO_4 → Au-SO_2 (90 min)	6.4±0.4
α -amylase → KIO_4 → EtBr-SO_2 (90 min)	4.8±0.2
Cell autofluorescence in the "green" part of the spectrum (λ_{max} = 530 nm)	7.0±0.4
Cell autofluorescence in the "red" part of the spectrum (λ_{max} = 610 nm)	5.1±0.3

Note: the duration of pre-oxidation of preparations with potassium periodate (KIO_4) was 90 min in all cases. Fluorescence intensity of cells stained by Au-SO_2 or EtBr-SO_2 PAS reaction was normalised by tetraploid rat hepatocytes, which were stained with the same reagents according to Feulgen to detect DNA (Rozanov, Kudryavtsev, 1967). Mononucleate tetraploid hepatocytes are a convenient object for normalisation because they contain a constant amount of DNA and constitute ~80% of the cell population of the liver parenchyma of adult rats (Kudryavtsev et al., 1979).

Thus, our study showed that: 1) treatment of preparations with Au-SO_2 or EtBr-SO_2 for 90 min reveals all glycogen in hepatocytes (224±12 and 198±15 arbitrary units, respectively); 2) staining of preparations with Au-SO_2 or EtBr-SO_2 for 40 min reveals the fraction of glycogen extractable by treatment of preparations with 10% TCA (159±8 and 147±11 arbitrary units, respectively), i.e. the glycogen fraction not associated with proteins, or macroglycogen (MG); 3) subsequent staining of preparations with Au-SO_2 or EtBr-SO_2 for 40–90 min reveals the glycogen fraction which can be completely extracted only by treatment with KOH (66±5 and 54±4, respectively), i.e. glycogen fraction associated with proteins, or proglycogen (PG) (Bezborodkina et al., 2021a).

It has been shown in many studies, conducted mainly on rat and human skeletal muscles, that PG plays a major role in the recovery of glycogen stores after their depletion during muscle contractions (Battram et al., 2004; Wilson 2009; Granlund et al., 2011). The contribution of MG to glycogen accumulation becomes apparent late in the muscle recovery

phase or after intense and repetitive exercise (Graham et al., 2001; Shearer et al., 2005). In other words, the recovery of glycogen stores in skeletal muscles in these mammalian species mainly occurs not by filling the outer tiers of glycogen molecules with glucose residues, i.e., by increasing their size, but by an intensive *de novo* synthesis of PG. However, the rate of recovery of glycogen stores after their depletion and the nature of PG and MG interaction during glycogen resynthesis may apparently differ greatly in different mammalian species. For instance, it has been shown that it takes three days to fully restore the initial glycogen stores in horse skeletal muscles after a strenuous and prolonged exercise, whereas in humans it takes only 24 h (Hyyppä, 2007). Another important difference in glycogen metabolism in equine skeletal muscle is that post-load glycogen synthesis is mainly due to MG, especially during the initial resting period (1–24 h) (Bröjer et al., 2006). Differences in the rates of PG and MG synthesis in skeletal muscles are associated with the level of glycogen depletion. Extreme degrees of depletion probably require the formation of new glycogen molecules rather than the addition of glucose to the already existing granules. This hypothesis is supported by the evidence that there are no changes in glycogenin levels during glycogenolysis unless glycogen levels are significantly decreased (Shearer et al., 2005).

As shown above, MG can be detected by PAS staining of cells for 0–40 min, while PG can be detected by PAS staining for 40–90 min. In order to detect and separately determine the content of these glycogen fractions in the same cell, we used Schiff's reagents with stains fluorescing in different colours.

As an example, Figure 3.30 shows hepatocytes in which the glycogen fraction free of proteins (MG) is stained with EtBr-SO_2, while the fraction associated with proteins (PG) is stained with Au-SO_2. Cytofluorometry was used to measure the content of these glycogen fractions separately in individual cells. Hepatocytes stained with EtBr-SO_2 fluoresce in the "red" part of the spectrum when excited with green light (λ_{max} = 546 nm), and those stained with Au-SO_2 fluoresce in the "green" part of the spectrum when excited with blue light (λ_{max} = 436 nm). The total glycogen (TG) content in the cells was determined as the sum of MG + PG.

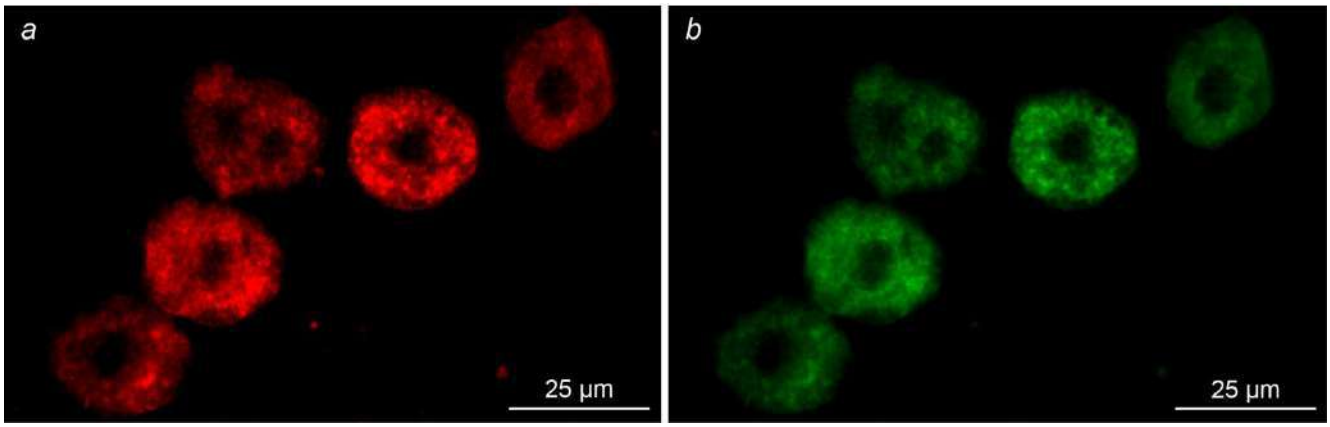


Figure 3.30 — Hepatocytes stained for 40 min with EtBr-SO_2 (*a*). The same cells subsequently stained with Au-SO_2 (*b*).

3.3.2. Structure of glycogen molecules in rat and human hepatocytes in the normal and the cirrhotic liver at different stages of the food cycle

The detection of PG and MG in the same cell using stains with different spectral characteristics made it possible to study changes in their content in rat hepatocytes during glycogenesis. The results presented in Figure 3.31, *a,* indicate that the MG content in the cells of control rats at all time points after glucose administration (except for 120 min) exceeded the MG content in the cells of rats with LC. To note, at different stages of refeeding the ratio of MG in some hepatocytes of the control and the cirrhotic rats could reach 90% (Chestnova et al., 2015).

PG content in hepatocytes of rats of the control group increased by 12.7% ($p < 0.01$) within 20 min after glucose administration to starved animals and did not change after that. In contrast, PG content during LC changed weakly during the first 60 min of glycogenesis but exceeded the initial level by 2.3 times at the end of the experiment ($p < 0.001$) (Fig. 3.31, *b*).

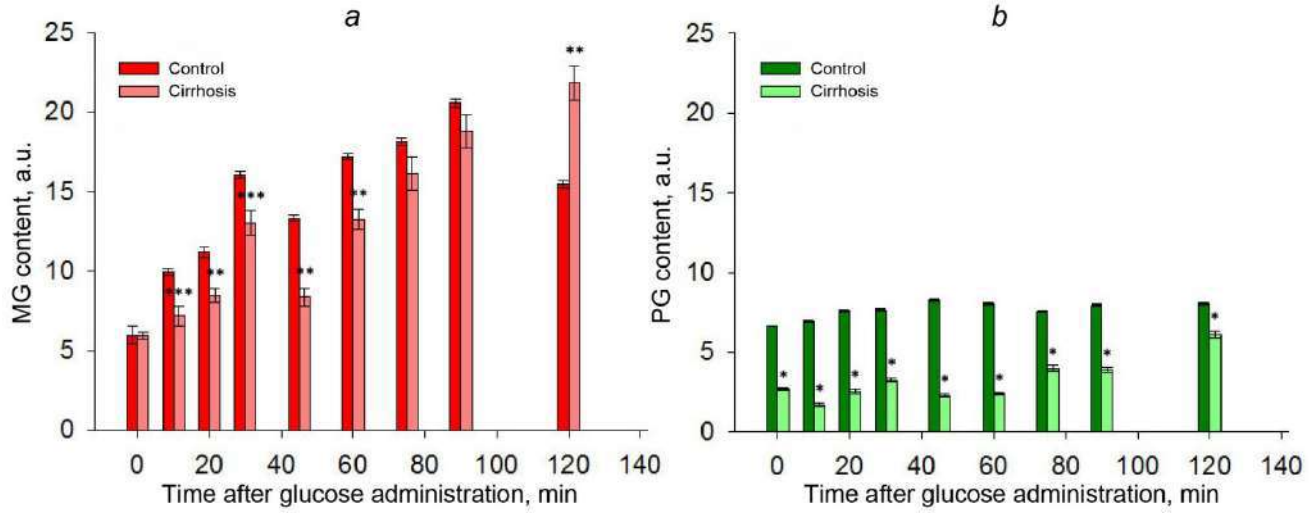


Figure 3.31 — Content of macroglycogen (MG) (*a*) and proglycogen (PG) (*b)* in hepatocytes of the control (Control) and the cirrhotic (Cirrhosis) rat liver at different time intervals after glucose administration to starved animals. Notes: 1) vertical sections — error of the mean; 2) mean values during LC are significantly different from the values in the norm (* $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$).

The analysis showed that glycogen accumulation in the liver and fluctuations in its levels in the cells of the control and the experimental rats at different stages of glycogenesis were mainly due to the changes in MG content (Fig. 3.32).

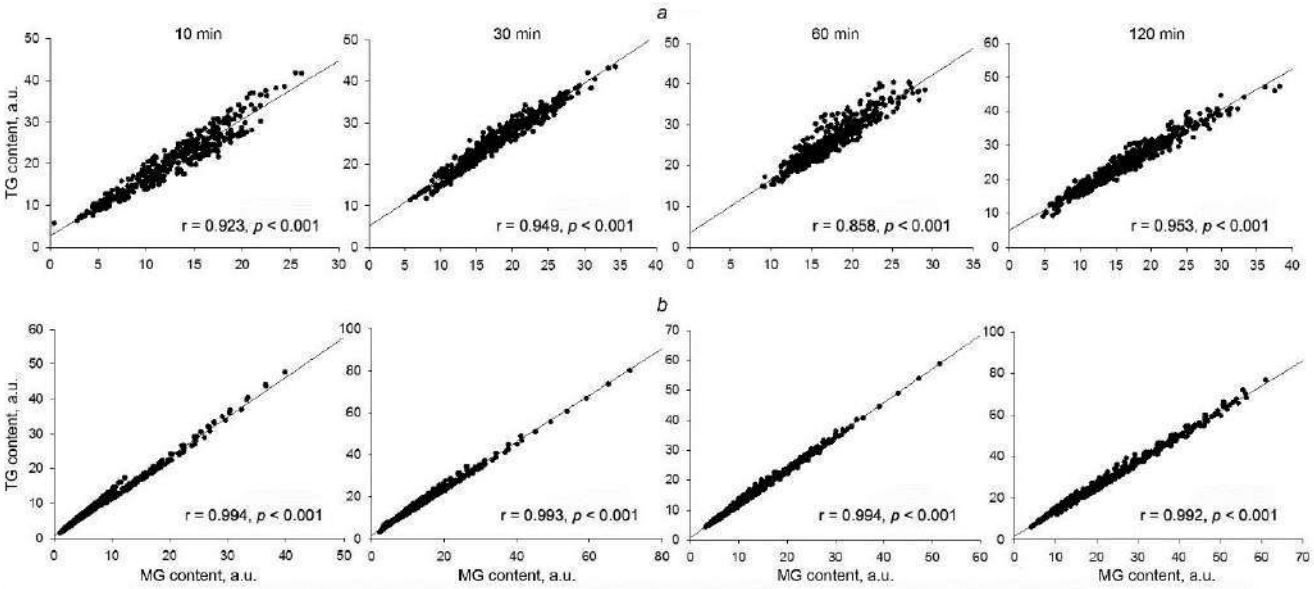


Figure 3.32 — Dependence of total glycogen (TG) content on macroglycogen (MG) content in the hepatocytes in the normal (*a)* and the cirrhotic (*b*) rat liver at different stages of glycogenesis (10, 30, 60 and 120th min). Note: each point on the graphs corresponds to one cell.

After 48 h of starvation and during 120 min of glycogenesis, the proportion of PG in the hepatocytes of the normal rat liver was higher than in the hepatocytes of the cirrhotic rat liver.

The proportion of PG in the hepatocytes of the normal and the cirrhotic liver slightly decreased during glycogenesis, while the proportion of MG increased (Chestnova et al., 2015) (Fig. 3.33, *a*, *b*).

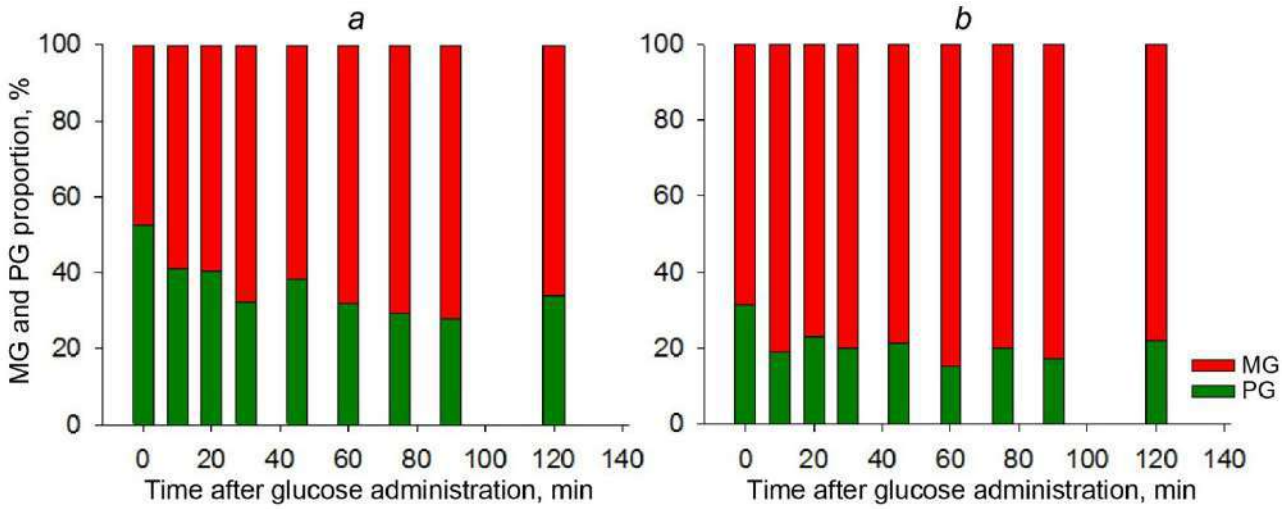


Figure 3.33 — Proportion (%) of macroglycogen (MG) and proglycogen (PG) in the hepatocytes in the normal (*a)* and the cirrhotic (*b*) rat liver at different time intervals after glucose administration to starved animals.

In the postabsorptive period (12–16 h after the last meal), the ratio of MG and PG in the hepatocytes of the normal and the cirrhotic rat liver differed from that at the early stages of glycogenesis. During this period of the food cycle, the proportion of MG significantly prevailed over PG in the normal liver hepatocytes, while the relative content of MG in the cirrhotic liver hepatocytes was only slightly higher than that of PG (Fig. 3.34). Comparison of the structure of glycogen molecules in hepatocytes in the normal liver of rats and humans in the postabsorptive period showed that it was very similar. The relative content of MG was 83–85%. As the pathological process in the liver develops, the proportion of MG decreases, and that of PG increases accordingly (Fig. 3.34) (Bezborodkina et al., 2021b).

These data confirm the results of earlier studies of the structure of glycogen in the hepatocytes in the normal and the pathological liver of rats and humans (Kudryavtseva, 1987; Kudryavtseva et al., 1988, 1992).

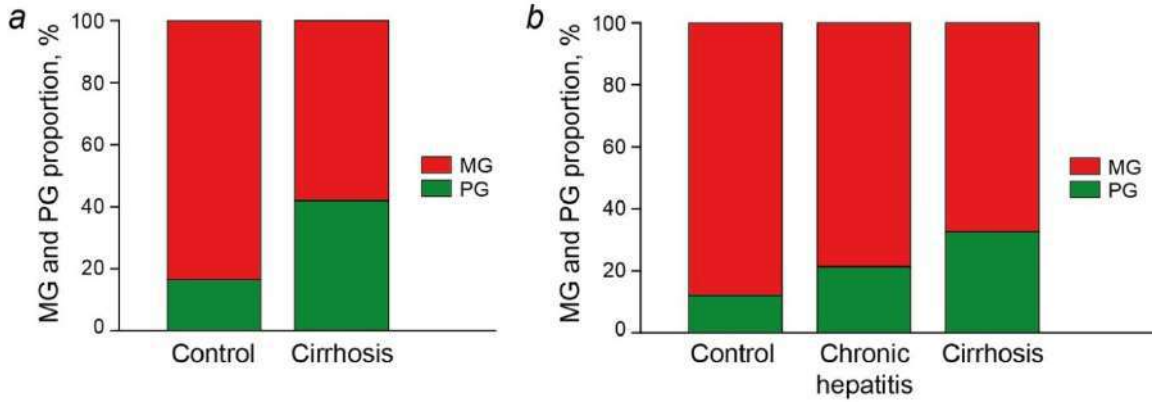


Figure 3.34 — Proportion of MG and PG (%) in glycogen molecules in rat (*a*) and human (*b*) hepatocytes in the postabsorptive period.

Thus, MG makes the greatest contribution to the volume of glycogen in hepatocytes and plays a leading role in the changes in its content. In addition, our data indicate that, in contrast to skeletal muscles, glycogenesis in rat hepatocytes after glucose administration to starved animals occurs mainly by filling the outer tiers of glycogen particles already present in the cells rather than by formation of new ones.

The analysis of PG and MG content in one and the same cell made it possible to study their dynamics under different physiological and pathological conditions. Even more importantly, it allowed us to determine the number of tiers in the glycogen molecules of an individual hepatocyte and the number of glucose residues on each of the tiers.

Based on the hypothesis that a **complete** glycogen molecule consists of 8 inner (PG) and 4 outer (MG) tiers of glucose residues (Lomako et al., 1993; Alonso et al., 1995; Melendez et al., 1997), and the total number of glucose residues in PG is 3315 (Roach et al., 2012), information about the absolute and the relative number of glucose residues in the outer tiers of the glycogen molecule can be obtained.

The degree of filling of the outer tiers of the glycogen molecule (F_n) with glucose residues depending on the experimentally determined MG/PG ratio (k) was calculated according to the formula:

$$\mathbf{F_9 = k \times P / t_9}\text{ , at } k \leq 1 \qquad \textbf{(13)}$$

$$\mathbf{F_{10} = (k \times P - t_9) / t_{10}}\text{ , at } 1 < k \leq 3 \qquad \textbf{(14)}$$

$$\mathbf{F_{11} = (k \times P - t_9 - t_{10}) / t_{11}}\text{ , at } 3 < k \leq 7 \qquad \textbf{(15)}$$

$$\mathbf{F_{12} = (k \times P - t_9 - t_{10} - t_{11}) / t_{12}}\text{ , at } 7 < k \leq 15 \qquad \textbf{(16)}$$

where: **P** is the total number of residues on the first 8 tiers (proglycogen), $\mathbf{t_n}$ is the maximum possible number of residues on tier **n**.

Calculations showed that glycogen in the hepatocytes of the normal liver of starved rats consists of granules containing PG, i.e., tiers 1–8 and tier 9 filled by 90% (Fig. 3.35, *a*), while in the cirrhotic liver it consists of particles containing tiers 1–9 and tier 10 filled by ~60% (Fig. 3.35, *b*). Analysis of the dynamics of the spatial structure of glycogen molecules during glucose refeeding in rats showed that in norm the maximum filling of the outer tiers of glycogen molecules at the refeeding stage is limited to the 10th tier, while during LC it is limited to the 11th tier (Fig. 3.35). No cells in which the entire glycogen consisted of 12-tiered particles were found.

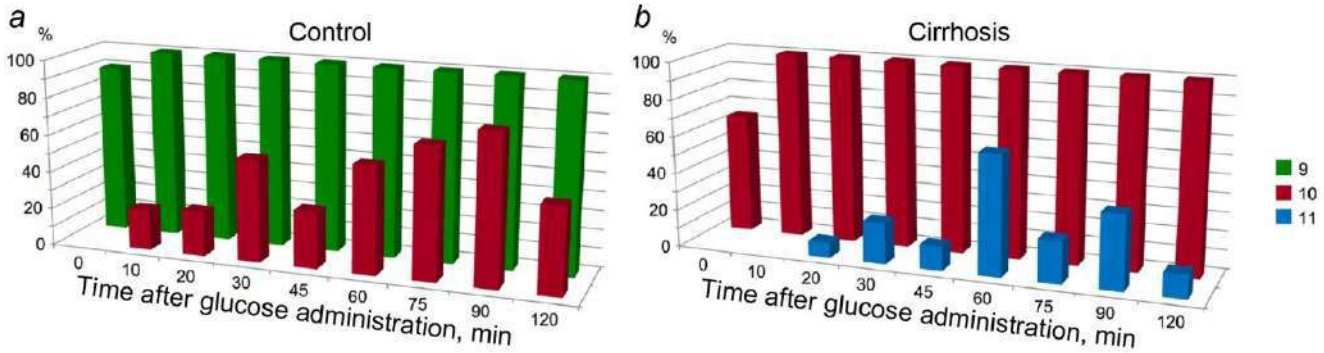


Figure 3.35 - Proportion of glucose residues on the outer tiers of the glycogen molecule in the normal (*a*) and the cirrhotic (*b*) rat liver, %.

Throughout the absorptive period, the amount of glucose residues on the outer tiers of glycogen molecules in hepatocytes of rats with LC exceeded that in the normal liver (Fig. 3.36).

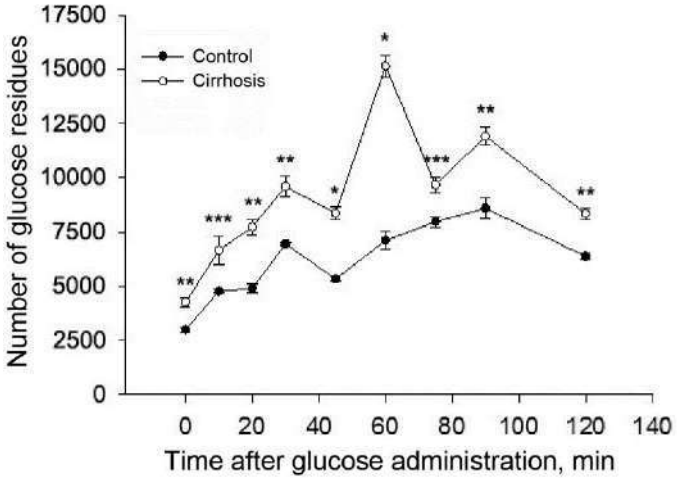


Figure 3.36 — Changes in the number of glucose residues on the outer tiers of glycogen molecules in the hepatocytes in the normal liver (Control) and the cirrhotic liver (Cirrhosis) of the rat. Notes: 1) vertical sections — error of the mean; 2) mean values during LC differ from the values in the norm at the significance level * — $p < 0.001$; ** — $p < 0.01$; *** — $p < 0.05$.

Despite the almost two-fold increase of glycogen content in hepatocytes of the control rats during 30 min of refeeding, the MG/PG ratio remained approximately at the same level (Fig. 3.37). This means that during the first stages of glycogenesis glycogen accumulation in cells occurs predominantly by increasing the number of molecules and only later (45, 60 and 90 min of refeeding), by filling their outer tiers. In contrast to the norm, during LC glycogen accumulation by filling the outer tiers is expressed at all stages of refeeding (Fig. 3.38).

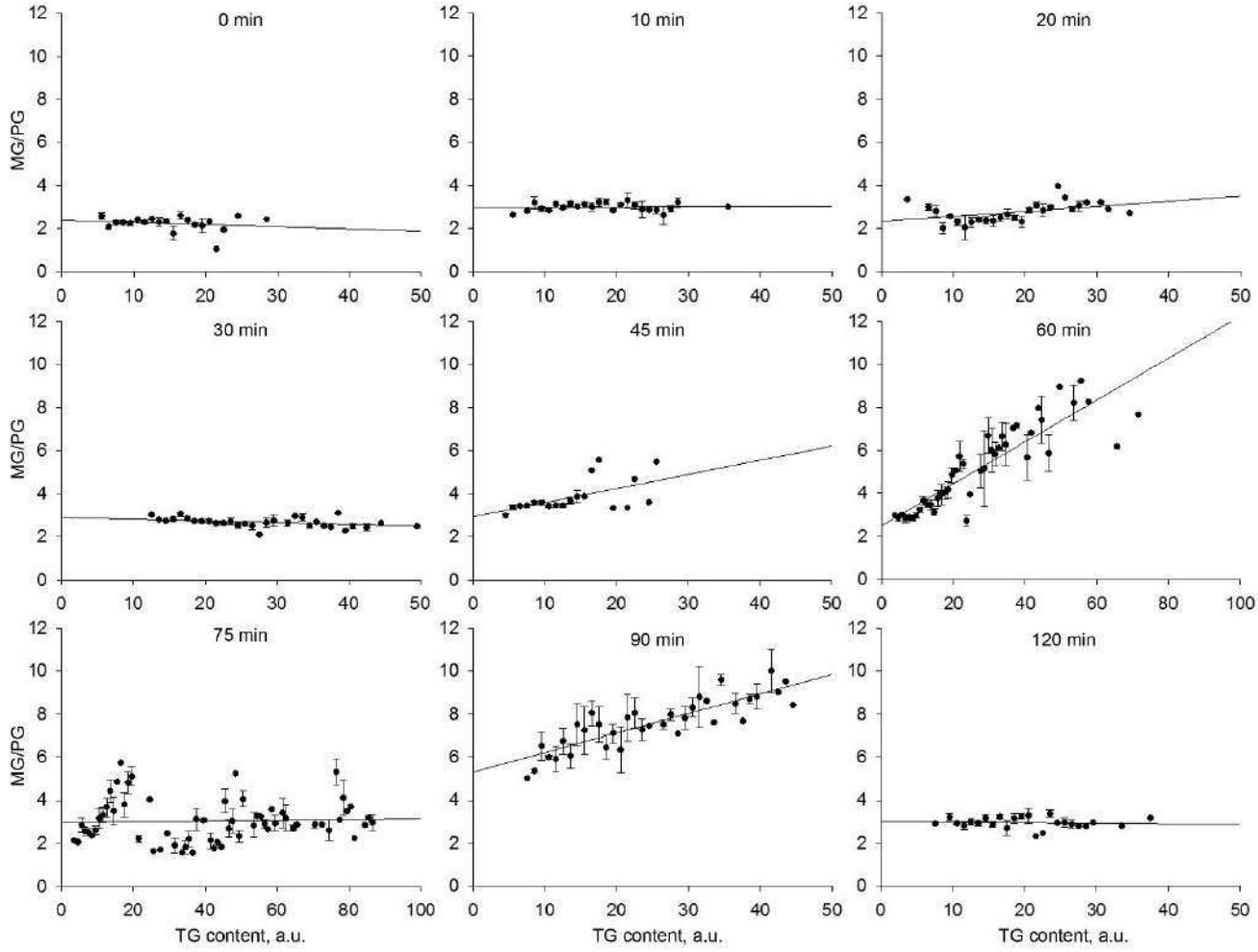


Figure 3.37 — Dependence of total glycogen (TG) content on the degree of filling (arbitrary units) of the outer tiers of glycogen molecules (MG/PG) in hepatocytes of the normal rat liver at different stages of glycogenesis. Note: vertical sections - error of the mean.

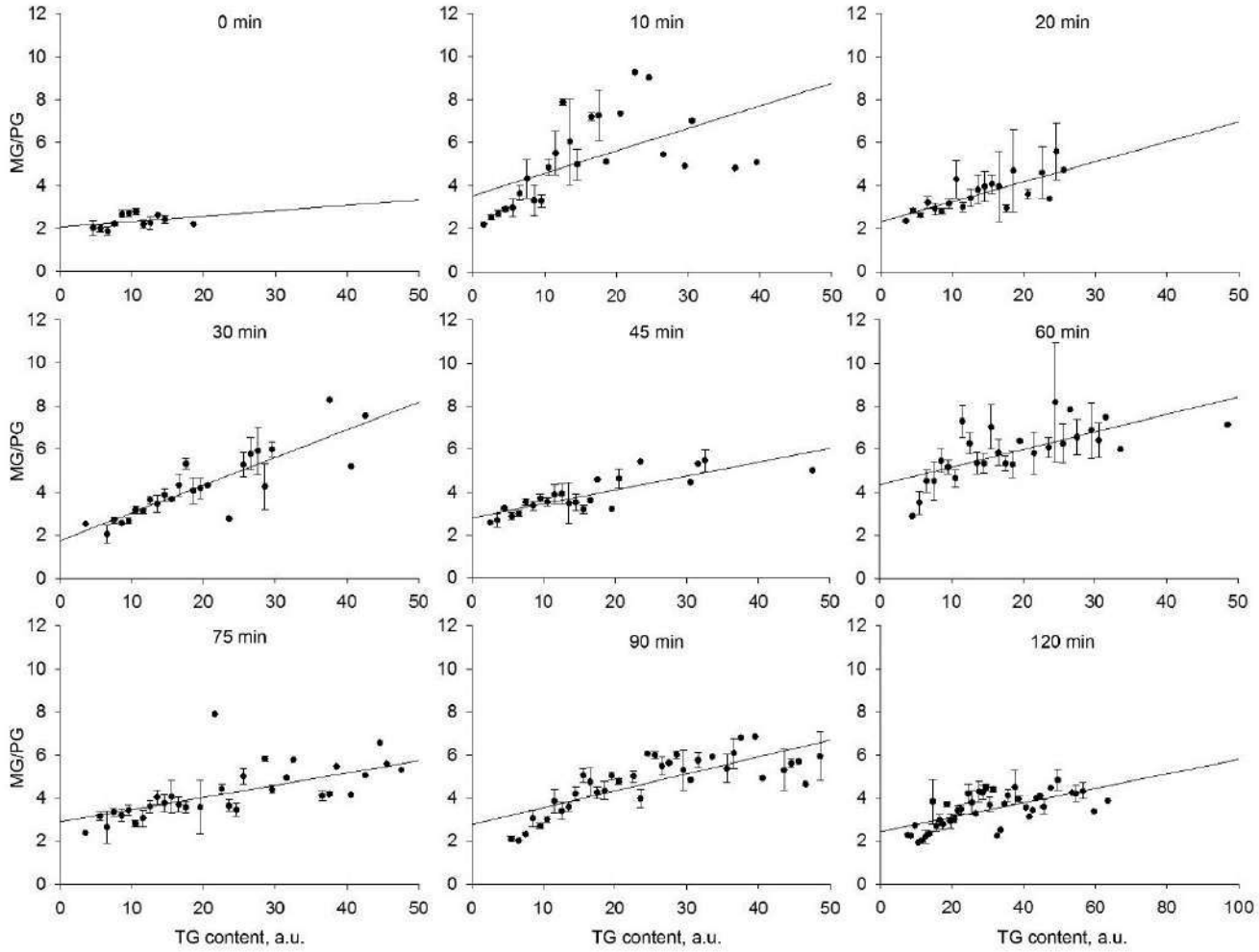


Figure 3.38 — Dependence of total glycogen (TG) content on the degree of filling (arbitrary units) of the outer tiers of glycogen molecules (MG/PG) in hepatocytes of the cirrhotic rat liver at different stages of glycogenesis. Note: vertical sections - error of the mean.

In the postabsorptive period, glycogen molecules in the hepatocytes of the normal liver of rats and humans contain, on average, 18810 and 24034 glucose residues, respectively. In rats, glycogen molecules, in addition to PG, have the 9th and the 10th tiers completely filled with glucose residues and the 11th tier filled with glucose residues by approximately 40%. In the normal liver of human hepatocytes, glycogen molecules have complete 9th and 10th tiers and the 11th tier filled by ~80%, indicating that they are, on average, slightly larger than in rats (Fig. 3.39).

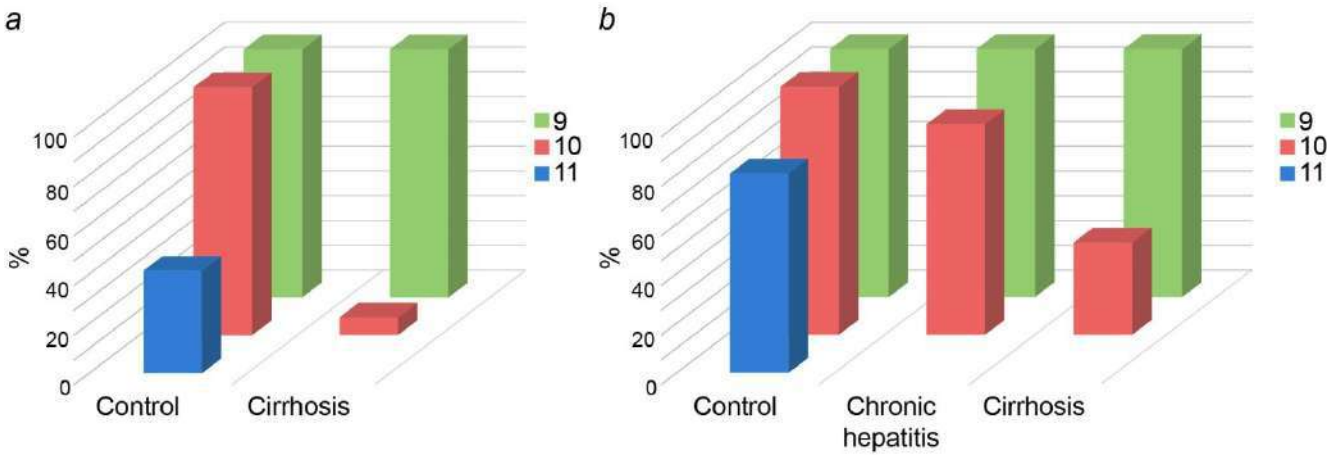


Figure 3.39 - Proportion of glucose residues on the outer tiers of glycogen molecules in the rat (*a*) and the human (*b*) liver in the postabsorptive period, %.

In hepatocytes of the cirrhotic liver, molecules in both species are smaller than in the normal liver. The structure of glycogen in the hepatocytes of CH patients occupies an intermediate position (Fig. 3.39, b).

Despite a lower, as compared to the norm, degree of filling of the outer tiers of glycogen during LC in humans, the glycogen content in hepatocytes is 160% ($p < 0.01$) higher than the norm (Fig. 3.25, *a*). Based on this, we can conclude that the number of β-particles in hepatocytes increases as the pathological process develops. Calculations have shown that if we take the average number of particles in the hepatocytes in the normal liver to be 1.0, it will be approximately 3.0 in case of CH and ~6.0 in case of LC.

Information on changes in the tier structure of glycogen granules is based mainly on the data on the size of glycogen particles in human skeletal muscle obtained using electron microscopy (EM) techniques. EM analysis of β-glycogen particles has shown that their diameter ranges from 10 to 44 nm and, on average, is 25 nm, which roughly corresponds to the diameter of a 7–8-tier glycogen particle (Marchand et al., 2002; Shearer, Graham, 2004; Graham et al., 2010). Granules with diameters greater than 44 nm were not detected, which is consistent with the theoretical ideas about the structure of glycogen molecules (Goldsmith et al., 1982). EM studies of cultured myotubules showed that the size of β-particles increased slightly during glycogenesis, from 24.9 to 28.1 nm (tiers 7→9th) (Elsner et al., 2002). The authors concluded that glycogen deposition in cells is associated with the formation of new 7–8-tier β-particles rather than the formation of additional tiers on existing particles. During glycogenolysis, the average diameter of particles decreased to 24.4 nm, and their destruction was observed (Elsner et al., 2002).

During recovery of human skeletal muscles after prolonged moderate exercise, the average diameter of glycogen particles doubles, with only a few having a diameter corresponding to MG. At the beginning of the recovery period (0–4 h), muscle glycogen content increased predominantly by increasing the number of particles, and in the following 44 h, by increasing their volume (Marchand et al., 2007). Studies of the size of glycogen molecules in mouse liver using exclusion chromatography showed that during the food cycle, α-particles break down into stable glycogen molecules with a radius of ~12 nm (Sullivan et al., 2014).

To sum up, the available data on the spatial structure of glycogen molecules in cells are extremely scarce and are based either on the study of PG and MG dynamics using cytochemical techniques or on the measurement of molecule size using EM or exclusion chromatography. Since the glycogen content of cells under different physiological and pathological conditions is mainly due to changes in MG, more detailed information on its spatial characteristics would be very desirable. For this purpose, we developed a method of determining the number of tiers in glycogen molecules and the number of glucose residues on each tier in individual hepatocytes.

3.3.3. Analysis of the structure of glycogen molecules in rat and human hepatocytes using FRET method

Förster Resonance Energy Transfer (FRET) method is one of the possible approaches to obtaining information about the spatial structure of MG in cells. The method allows one to determine the distance between fluorophore molecules (donor and acceptor) involved in energy transfer.

Förster distance ($\mathbf{R_0}$) for the pair of stains used in this work, Au-SO_2 (donor, Don) and EtBr-SO_2 (acceptor, Ac), is 3.2 nm, according to our data (Bezborodkina et al., 2011). Calculations show that the average distance between the tiers of the glycogen molecule, whose total radius is ~21 nm, is ~1.9 nm (Fig. 3.40) (Goldsmith et al., 1982; Shearer, Graham, 2004; Roach et al., 2012).

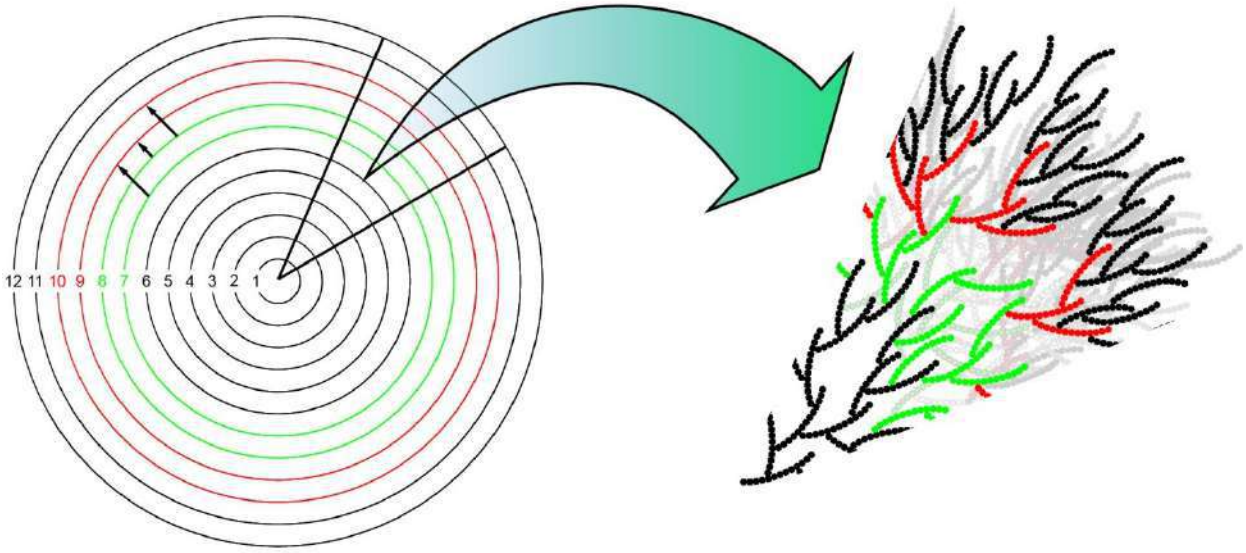


Figure 3.40 — Schematic representation of the glycogen molecule. The colours indicate the tiers between which resonant energy transfer (FRET) is possible. The distance between the tiers in a spherical β-particle with a radius of 21 nm is ~1.9 nm.

It was found (see Section 3.3.1) that PAS staining of cells for 40 min with EtBr-SO_2 reveals MG, i.e. glucose residues on the outer (9th to 12th) tiers of glycogen molecules. Subsequent staining of Au-SO_2 cells reveals PG, i.e. glucose residues located on the inner (1st to 8th) tiers of glycogen molecules. Only Au molecules bound to glucose residues on the 7th and 8th tiers of PG can participate in Förster interaction. Au on glucose residues located on these tiers transfers energy to EtBr bound to glucose residues on the 9th and 10th tiers of MG, which receive the radiation energy (Bezborodkina et al., 2011; Bezborodkina et al., 2018).

Glucose residues stained with Au-SO_2 and located below the 7th tier, as well as those stained with EtBr-SO_2 and located above the 10th tier in the glycogen molecule, cannot participate in the Förster interaction due to the excess $\mathbf{R_0}$ between the donor and the acceptor. Thus, the efficiency of FRET ($\mathbf{E_{FRET}}$) strongly depends on the degree of occupancy of tiers 9 and 10 in the glycogen molecule. If they are not filled or barely filled, the radiation energy of Au cannot be transferred to EtBr. Therefore, the presence in cells of a large number of β-particles consisting only of PG (i.e., glucose residues in tiers 1 to 8) will reduce the level of $\mathbf{E_{FRET}}$. On the other hand, even complete filling of the 11th and 12th tiers would not lead to an increase in $\mathbf{E_{FRET}}$, as the distance between the 8th and 11th tiers (~ 5.7 nm) would be much greater than $\mathbf{R_0}$ and radiation energy would not be transferred from the donor to the acceptor (Bezborodkina et al., 2011; Bezborodkina et al., 2018).

Measurement of $\mathbf{E_{FRET}}$ in individual rat hepatocytes in the normal and the cirrhotic liver at various time intervals after administration of glucose to starved rats showed that this index is, on average, ~3.3-fold higher in the norm than during LC (Fig. 3.41).

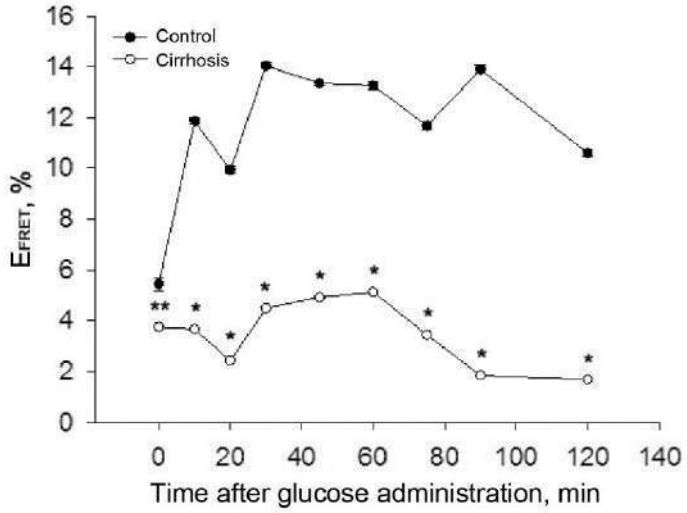


Figure 3.41 - FRET efficiency ($\mathbf{E_{FRET}}$) in hepatocytes of the normal (Control) and the cirrhotic (Cirrhosis) rat liver at different stages of glycogenesis. Notes: 1) vertical sections are error of the mean; 2) mean values during LC differ from the values in the norm at the significance level * — $p < 0.001$; ** — $p < 0.01$.

At the beginning of refeeding (0–30th min), $\mathbf{E_{FRET}}$ in the hepatocytes of the normal liver more than doubled, and after that remained at about the same level. In contrast, in hepatocytes of the cirrhotic liver, $\mathbf{E_{FRET}}$ was relatively stable until 75 min and then decreased approximately twofold. Since in the cells of the normal liver the 9th and especially the 10th tier of the glycogen molecules remained incompletely filled (Fig. 3.35, *a*), one would expect $\mathbf{E_{FRET}}$ to increase as they were filled (Fig. 3.35, *a*). This assumption is supported by the results presented in Figure 3.42 and Figure 3.43, which show that in the cells of the normal liver, in contrast to the cells of the cirrhotic liver, $\mathbf{E_{FRET}}$ at many stages of glycogenesis strongly depends on the increase in the number of glucose residues in glycogen molecules (Bezborodkina et al., 2011).

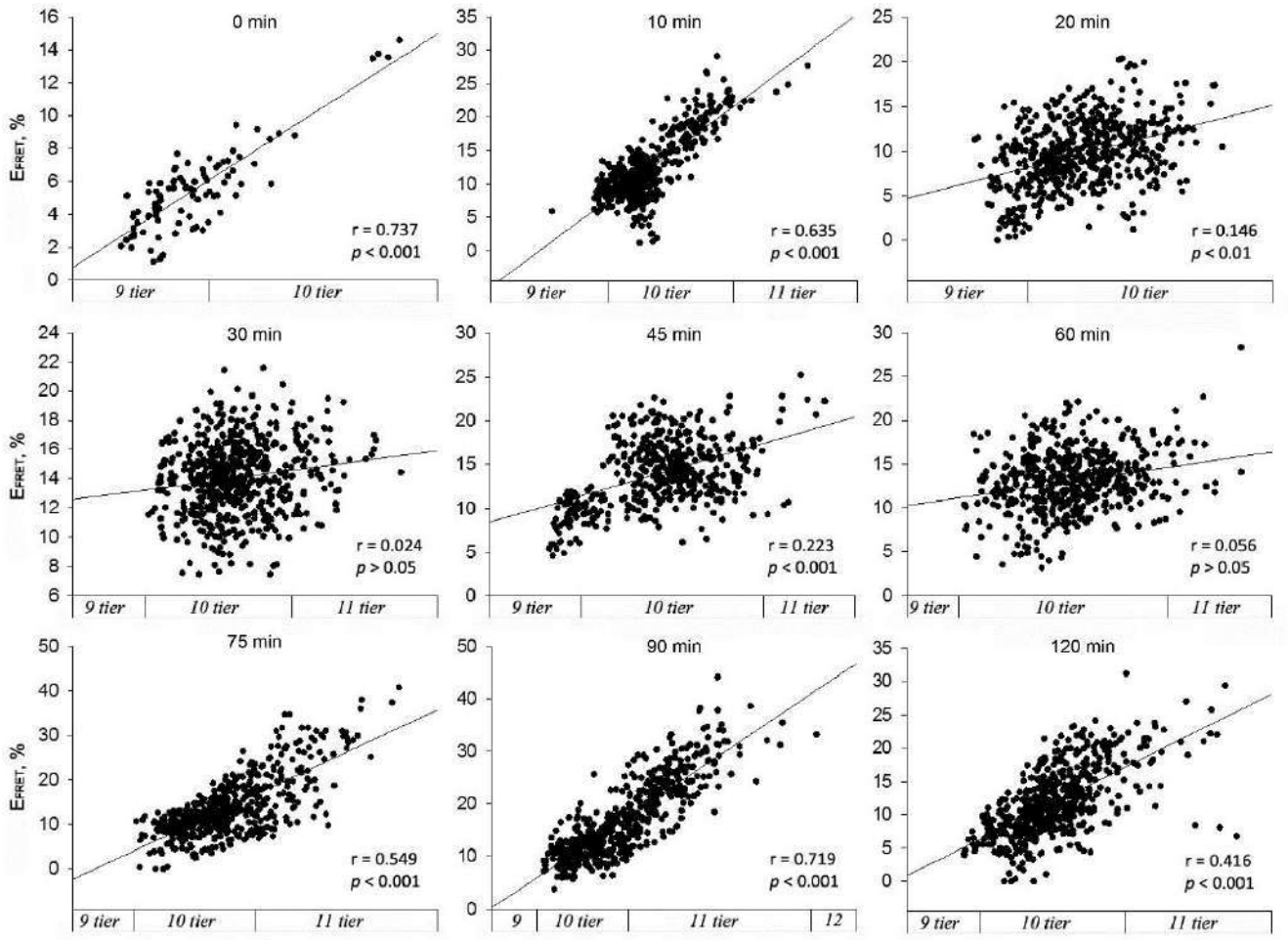


Figure 3.42 — Dependence of FRET efficiency ($\mathbf{E_{FRET}}$) on the degree of filling of the outer tiers (9–12th) of glycogen molecules in hepatocytes of the normal rat liver at different stages of glycogenesis. Note: each point on the graphs corresponds to one cell.

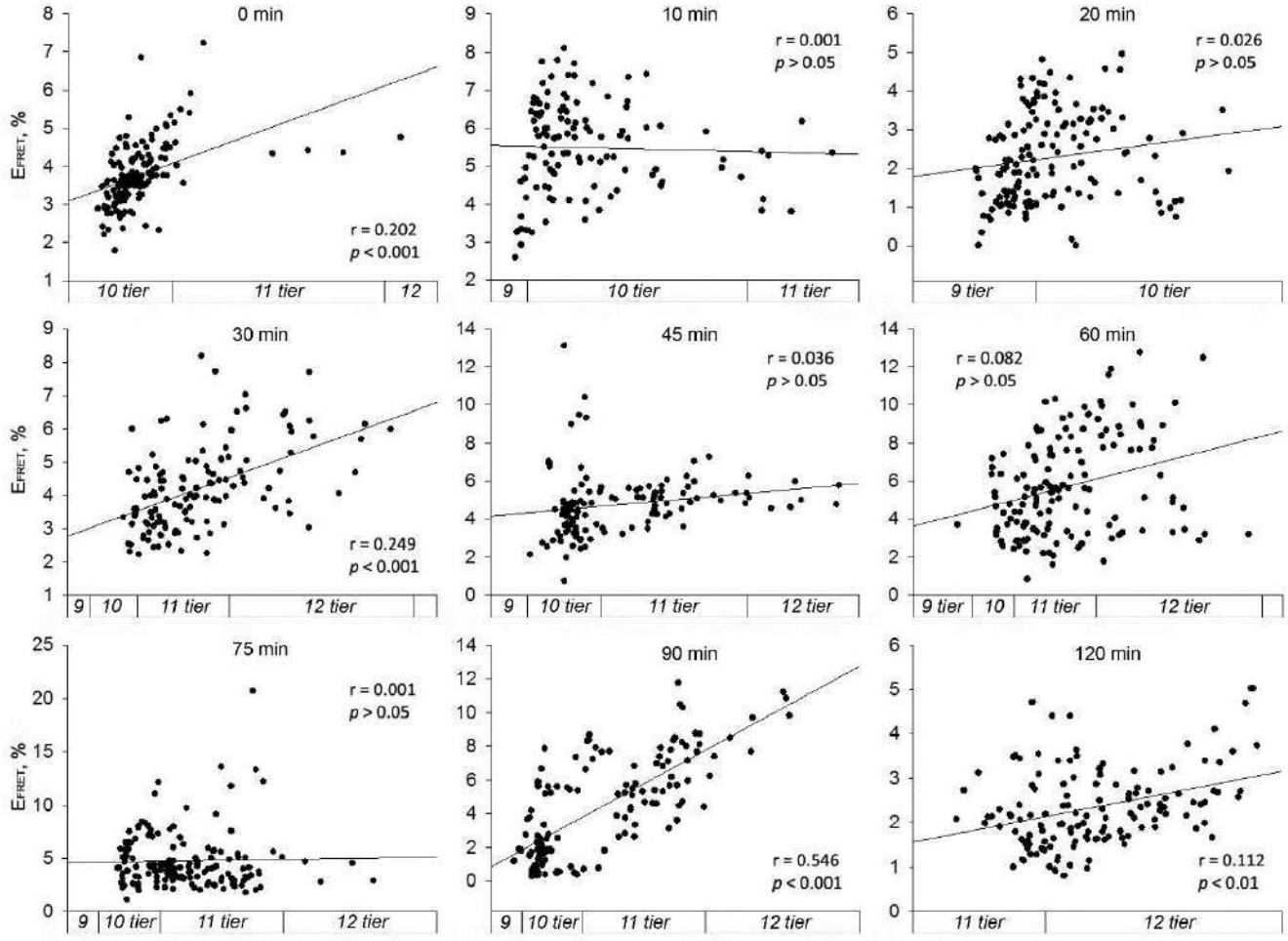


Figure 3.43 — Dependence of FRET efficiency ($\mathbf{E_{FRET}}$) on the degree of filling of the outer tiers (9–12th) of glycogen molecules in hepatocytes of the cirrhotic rat liver at different stages of glycogenesis. Note: each point on the graphs corresponds to one cell.

An important advantage of FRET method is its high sensitivity to small changes of the distance between Don and Ac molecules (**r**). When **r** increases above $\mathbf{R_0}$ for a given pair of stains, $\mathbf{E_{FRET}}$ decreases sharply. The distance between Don and Ac in hepatocytes of the normal liver during rat refeeding averaged 4.401±0.004 nm, while hepatocytes of the cirrhotic cells it averaged 5.447±0.009 nm (Fig. 3.44). Therefore, $\mathbf{E_{FRET}}$ was higher in the norm than during LC (Fig. 3.41).

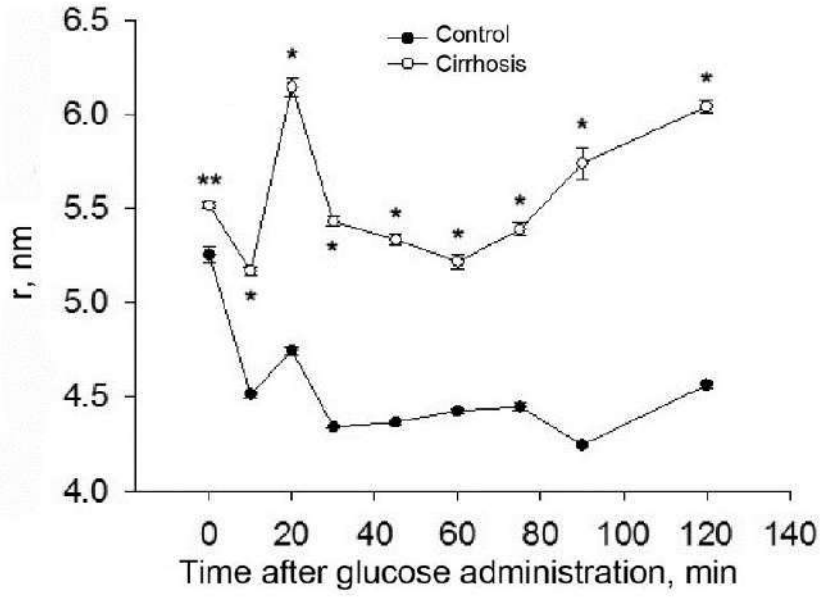


Figure 3.44 - Distance between Don and Ac (**r**) in glycogen molecules in hepatocytes of the normal (Control) and the cirrhotic (Cirrhosis) rat liver at different stages of glycogenesis. Notes: 1) vertical sections - error of the mean; 2) mean values during LC differ from the values in the norm at the significance level: * — $p < 0.001$; ** — $p < 0.01$.

Determination of the distance between Don and Ac during glycogenesis showed that as the outer tiers of glycogen are filled with glucose residues, the value of **r** decreases. In the hepatocytes of the normal liver, **r** decreases by 1.08±0.18 nm during refeeding of rats with glucose and by 0.64±0.21 nm in the hepatocytes of rats with LC ($p < 0.05$). The sharpest decrease in **r** was found at 0, 75, 90 and 120 min after glucose administration to starved animals (**Δr** = 1.48; 1.4; 1.7 and 1.59 nm, respectively) (Fig. 3.45), while during LC it was found at only one time point (90th min), at which **Δr** was 2.23 nm (Fig. 3.46).

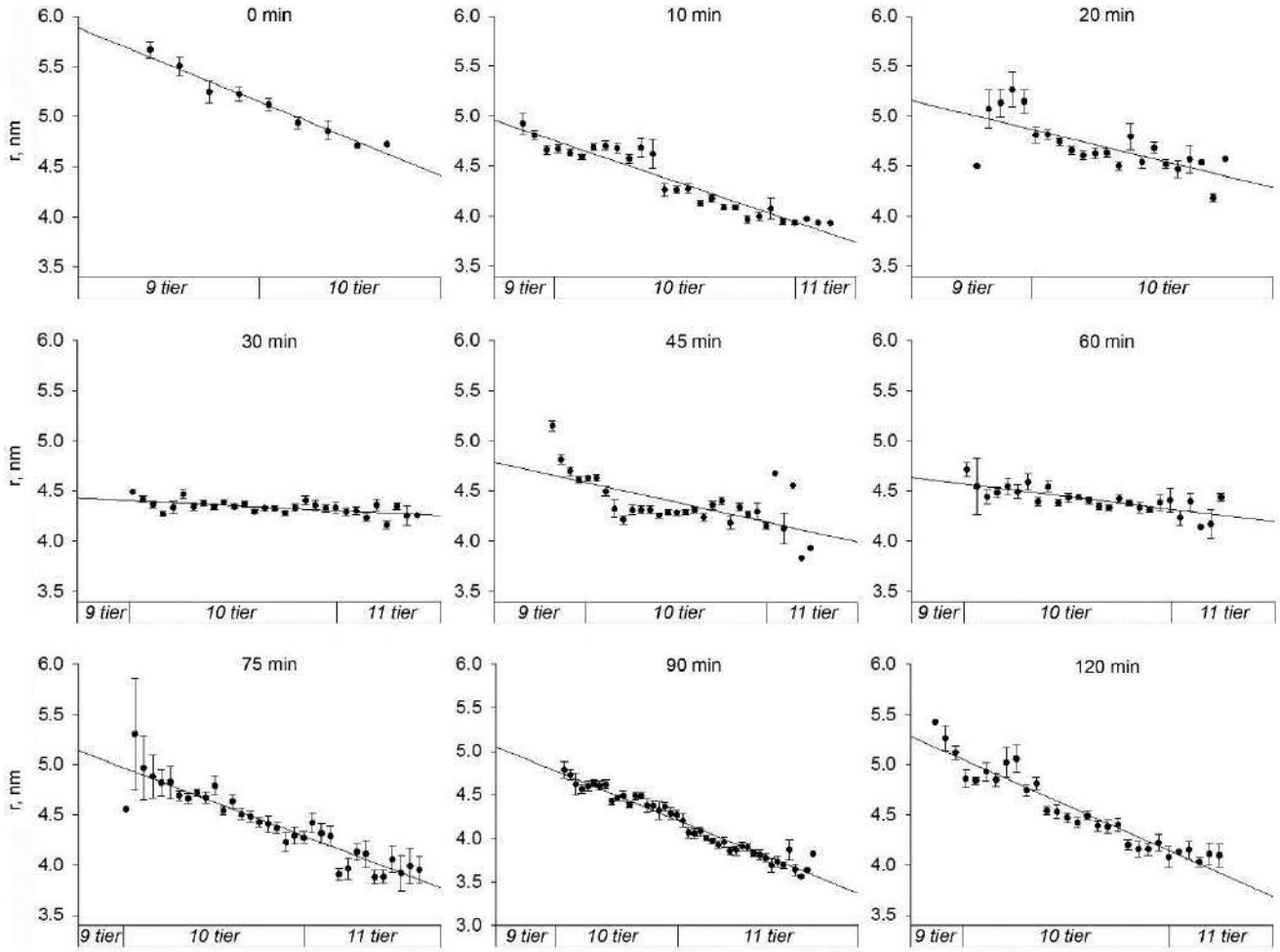


Figure 3.45 — Dependence of the distance between Don and Ac (**r**) on the degree of filling of the outer (9–11th) tiers of glycogen molecules in the hepatocytes of the normal rat liver at different stages of glycogenesis. Note: vertical sections - error of the mean.

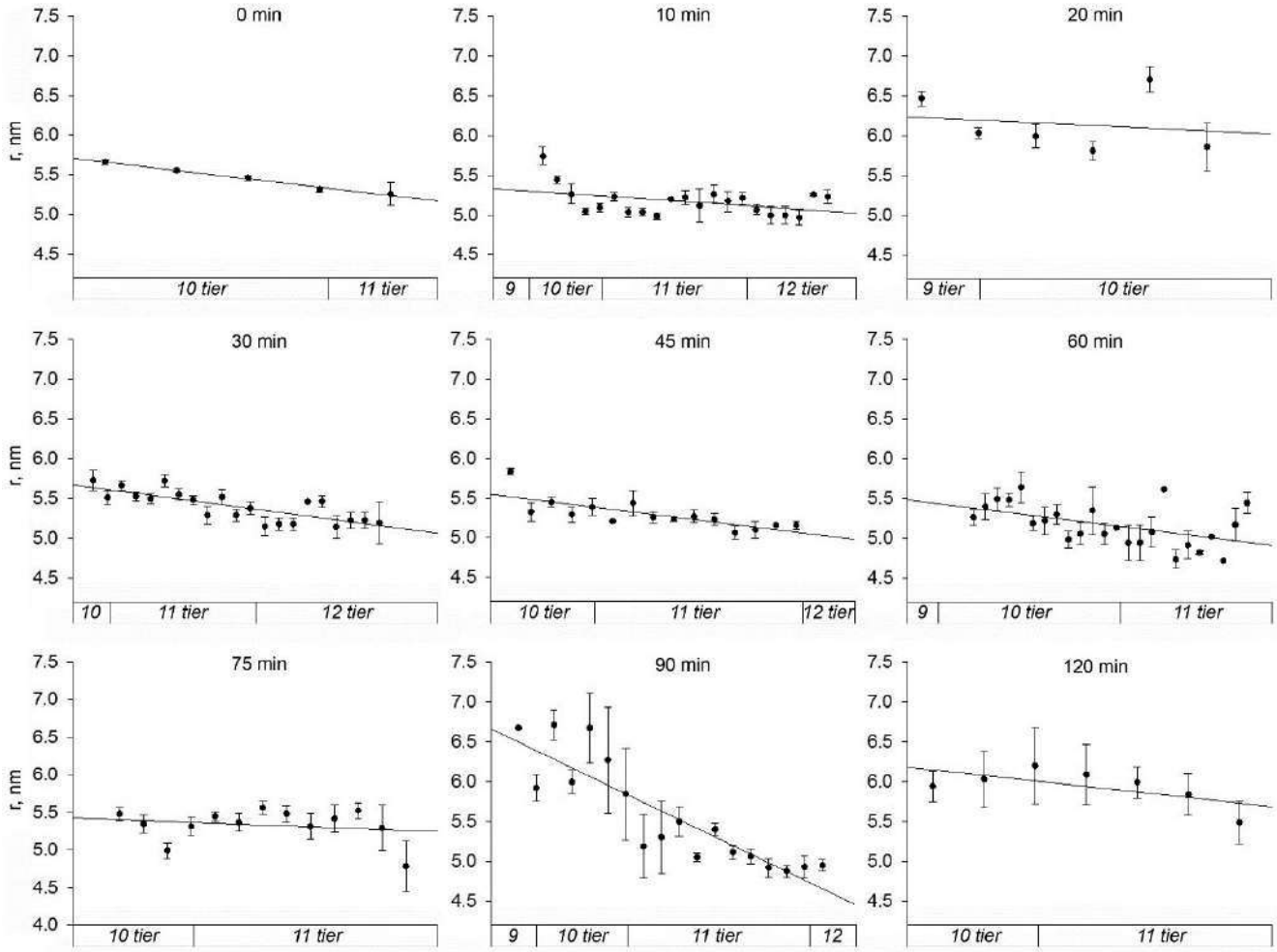


Figure 3.46 — Dependence of the distance between Don and Ac (**r**) on the degree of filling of the outer (9–12th) tiers of glycogen molecules in hepatocytes of the cirrhotic rat liver at different stages of glycogenesis. Note: vertical sections — error of the mean.

Thus, the increase in $\mathbf{E_{FRET}}$ in hepatocytes of the normal rat liver at the initial stages (0–30 min) of glycogenesis (Fig. 3.41) as compared with those of the cirrhotic liver is apparently due to the smaller distance between the glucose residues located at tiers 7, 8 and 9, and 10 (Fig. 3.44, 3.45) and the increased number of glucose residues at tier 10 (Fig. 3.35).

Earlier, in a study of the localisation of glycogen synthesis in hepatocytes isolated from the liver of starved rats, it was shown that stimulation of glycogen synthesis by glucose causes the relocation of GS to the periphery of cells and that the amount of the enzyme concentrated near the plasma membrane increased with time (Garcia-Rocha et al., 2001). In addition, it was found using incubation of isolated hepatocytes with ^{14}C-glucose that glycogen synthesis always starts near the plasma membrane of the cells. After that, however, as glycogenesis increases, the synthesised glycogen gradually moves to the centre of cells giving way to new synthesised molecules (Fernandez-Novell et al., 2002).

Acceptor Bleaching (AB) procedure makes it possible to obtain information about the structure of glycogen particles and to express morphologically the energy transfer from donor to acceptor in the form of images of cell regions showing the distribution of FRET signal in the cytoplasm of hepatocytes. In a series of images of hepatocytes (Fig. 3.47), it can be seen that the level of $\mathbf{E_{FRET}}$, marked with different colours reflecting the amplification of the signal, quite clearly reflects the localisation of glycogen particles with different degrees of filling of the outer tiers.

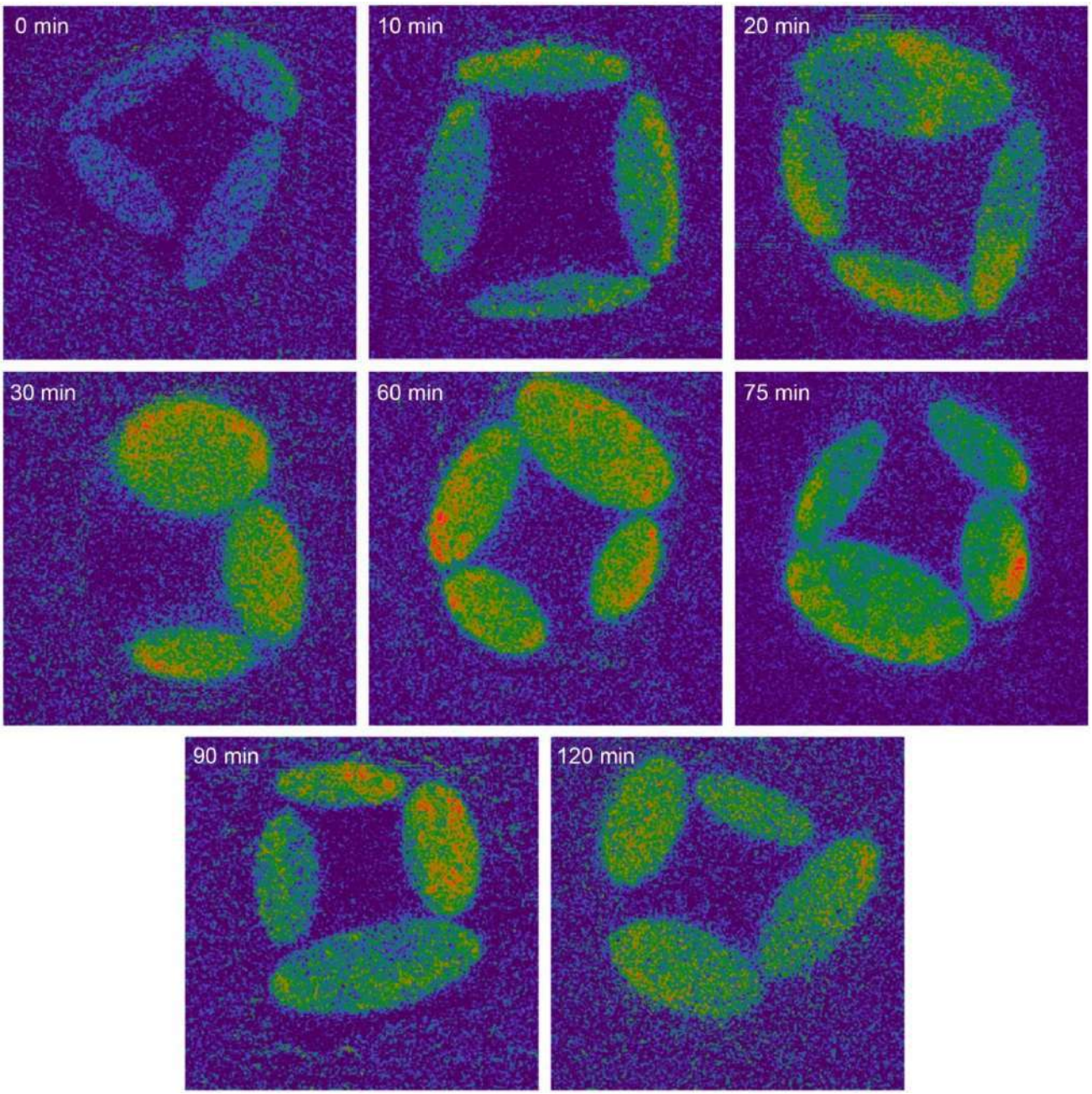


Figure 3.47 — Distribution of FRET signal in the cytoplasm of rat hepatocytes at different stages of glycogenesis. Note: as FRET efficiency ($\mathbf{E_{FRET}}$) increases, the colour of loci in which energy transfer from donor to acceptor takes place changes as follows: blue→green→orange→red.

In hepatocytes of starving rats energy transfer from the donor to the acceptor is weak. This is expressed in the fact that almost the entire cell is coloured blue. Green colour, indicating a higher level of FRET signal, is noticeable only at the periphery of hepatocytes. As early as 10 min after glucose administration, orange dots appear at the periphery of the cell, where the FRET signal is higher than in the green dots. As glycogenesis progresses, red dots, reflecting the highest FRET signal, appear at the cell periphery and are then distributed throughout the cytoplasm. Towards the end of the experiment (120 min), when glycogen breakdown begins to predominate over its synthesis, the number of red dots decreased and they disappeared completely in some cells.

Measurement of $\mathbf{E_{FRET}}$ in human hepatocytes in the postprandial period showed that in the normal liver cells it was 3%. As the severity of liver damage progressed, this index increased 1.3-fold in patients with CH ($p < 0.001$) and 3-fold in patients with LC ($p < 0.001$) as compared to the norm (Fig. 3.48).

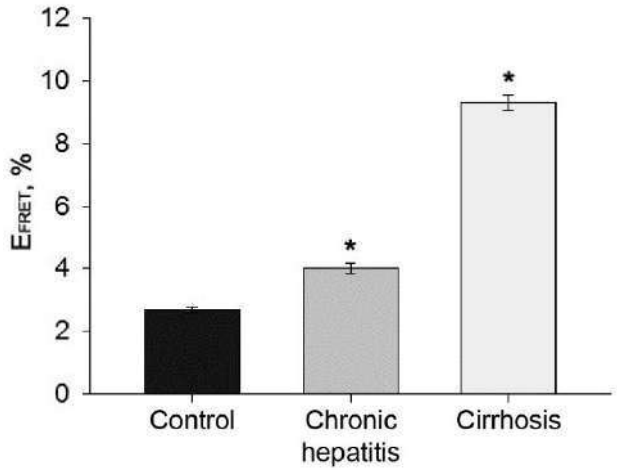


Figure 3.48 — FRET efficiency ($\mathbf{E_{FRET}}$) in hepatocytes of healthy humans (Control), patients with chronic hepatitis (Chronic hepatitis) and cirrhotic patients (Cirrhosis). Notes: 1) vertical sections - error of the mean; 2) mean values during chronic hepatitis and cirrhosis differ from the control at the significance level: * — $p < 0.001$.

Analysis of FRET signal distribution in the cytoplasm of human hepatocytes (Fig. 3.49) showed that the energy transfer from the donor to the acceptor is fairly efficient, since most of the zones in the cytoplasm of hepatocytes are “green”. Only the areas closest to the nucleus are “blue”. During CH, all areas in which energy transfer occurs, except “green” loci, contain numerous orange dots. In the cytoplasm of hepatocytes of the cirrhotic liver, a few “green” areas are observed, but much more space is occupied by “orange” and even “crimson” areas.

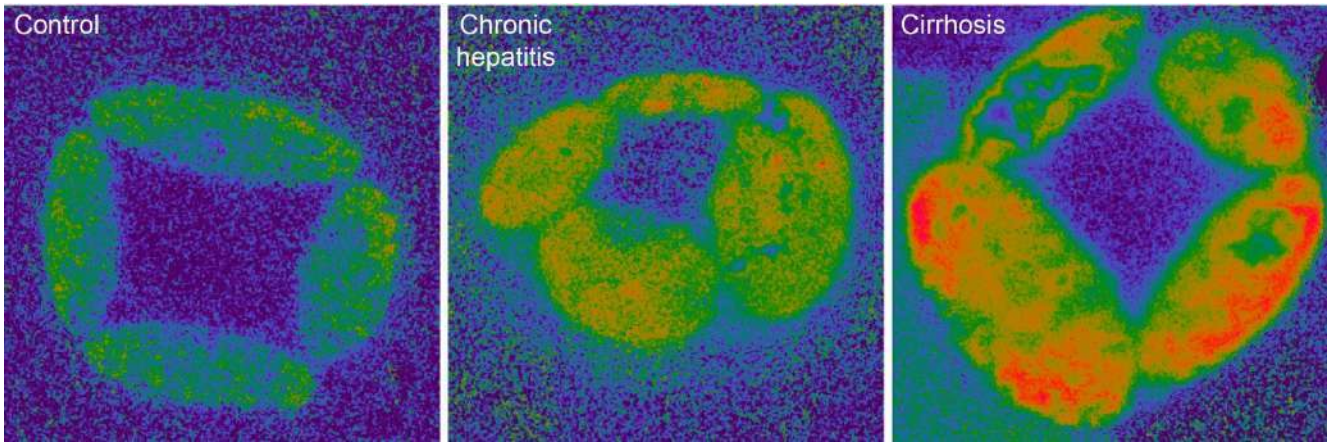


Figure 3.49 - Distribution of FRET signal in the cytoplasm of human hepatocytes in the postabsorptive period. Note: as the FRET efficiency ($\mathbf{E_{FRET}}$) increases, the colour of the loci where energy transfer from donor to acceptor takes place changes as follows: blue→green→orange→red.

Determining the distance between Don and Ac (**r**) in glycogen particles, we established that in hepatocytes of the normal human liver it is, on average, 6.14±0.05 nm, in hepatocytes during CH, 5.69±0.04 nm, and in hepatocytes during LC, 4.88±0.02 nm ($p < 0.001$) (Fig. 3.50).

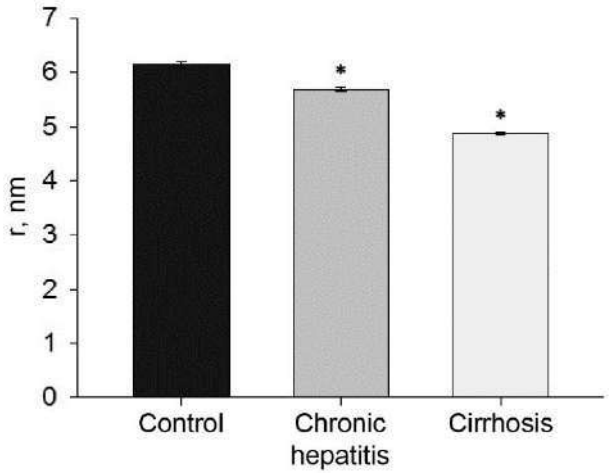


Figure 3.50 — Distance between Don and Ac (**r**) in glycogen molecules in human hepatocytes in the normal liver (Control), in the liver during chronic hepatitis (Chronic hepatitis) and in the cirrhotic liver (Cirrhosis). Notes: 1) vertical sections — error of the mean; 2) mean values during chronic hepatitis and cirrhosis differ from the control at the significance level: * — $p < 0.001$.

The analysis of cell distribution by **r** in the hepatocyte population showed that the proportion of cells with glycogen molecules in which the distance between Don and Ac (**r**) does not exceed the Förster interaction increases with increasing severity of the disease. During LC, the proportion of such cells increases approximately 50-fold as compared to the norm (Fig. 3.51).

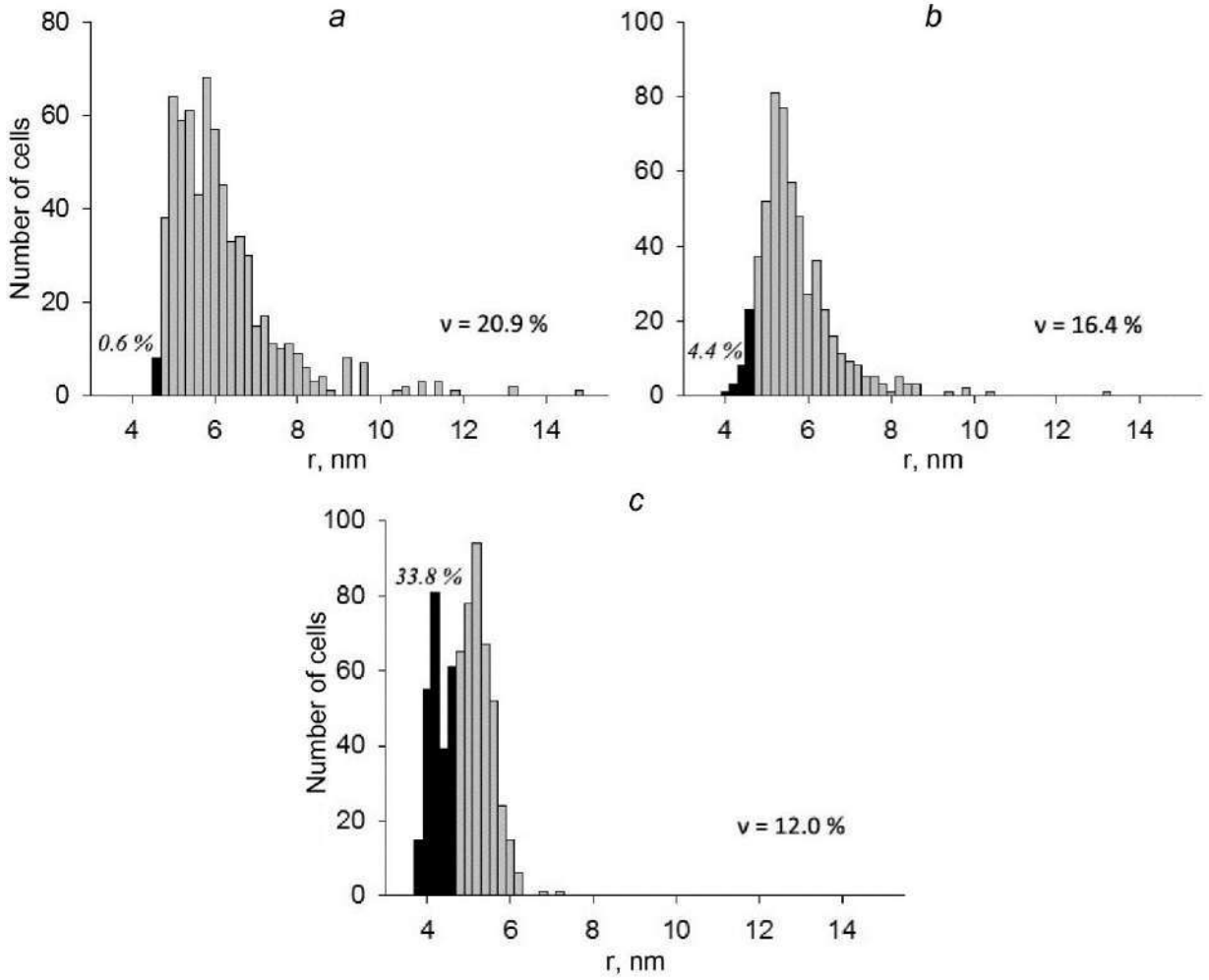


Figure 3.51 — Histograms of cell distribution in the human hepatocyte population according to the distance between Don and Ac (**r)** in glycogen molecules in the normal liver (*a)*, during chronic hepatitis (*b)* and in the cirrhotic liver (*c*). Note: black colour indicates the proportion of cells with glycogen particles in which the distance between Don and Ac (**r**) does not exceed the Förster interaction. ν — correlation coefficient.

It has been previously established (Fig. 3.39) that glycogen molecules in the normal liver of human hepatocytes have complete 9th and 10th tiers and the 11th tier of glucose residues filled by ~ 80%. During LC, the outer tiers of glycogen molecules are practically represented only by the 9th tier. As shown in Figure 4.40, energy transfer in glycogen molecules stained with auramine-SO_2 and ethidium bromide-SO_2, can only occur from the 7th and 8th tiers (Don) to the 9th and 10th tiers (Ac). The 7th and the 8th tiers of glycogen molecules together contain 2496 glucose residues. The 9th and the 10th tiers of glycogen molecules in hepatocytes of the normal liver together contain 9984 glucose residues (Roach et al., 2012). Since both outer tiers (9th + 5% of the 10th) of glycogen molecules in hepatocytes of the cirrhotic liver contain only 3661 glucose residues, it can be assumed that energy transfer

from Don to Ac during LC would be less efficient. Therefore, it seems that the main reasons why FRET efficiency in hepatocytes during LC is much higher than the norm are a shorter distance between Don and Ac in glycogen molecules and a much greater relative number of hepatocytes containing glycogen molecules in which the distance between Don and Ac (**r**) is within the Förster interaction interval.

The complete 12-tier glycogen molecule is known to consist of two parts (fractions): the internal (proglycogen) part consisting of the first eight tiers of glucose residues and the external (macroglycogen) part consisting of the four outer tiers of glucose residues (Fig. 1.18, Section 1.7). Only macroglycogen is involved in the synthesis and degradation of glycogen. The glucose residues located on the four outer tiers of the molecule are used as potential energy sources for various cellular processes. The remaining 4–5% of glucose residues in the proglycogen part of the glycogen molecule serve as a "platform" during further formation of the complete molecule. The glucose residues of proglycogen can be used for energy production only in exceptional cases (Melendez et al., 1998).

It is believed that once the outer, 12th tier is filled, the growth of glycogen molecules stops. The mechanism that stops the biosynthesis of glycogen molecules once they reach their full size is still unknown. The only hypothesis explaining this phenomenon is that of Madsen and Cori (1958), who suggested that the size of glycogen molecules is controlled by the structure of the molecules themselves.

Madsen and Cori (1958) suggested that since the degree and the principle of branching in glycogen molecules of their organisation remains constant at all levels and since the number of chains of glucose residues increases as the molecules grow, the density of residues on the tiers will increase as they approach the surface of the molecules. Accordingly, glucose residues will be more closely packed together on the outer tiers than on the inner tiers, which are more distant from the surface. An important role in the mechanism of controlling the size of glycogen molecules is played by the size of the enzymes of its synthesis. The authors believe that when the density of glucose residues on the surface of glycogen molecules becomes too high, there is not enough space for glycogen synthase to work and the growth of molecules stops.

The results of determining the distance between Don and Ac at different tiers of glycogen molecules in human hepatocytes indicate that it decreases with the increasing number of tiers in the molecules (Fig. 3.52). As the tiers of glycogen molecules in human hepatocytes

fill up, **r**, on average, decreases by 2.94 nm in the norm, by 2.03 nm during CH, and by 0.55 nm during LC (Fig. 3.52). During LC, the distance between Don and Ac is, on average, significantly smaller than in the normal liver cells.

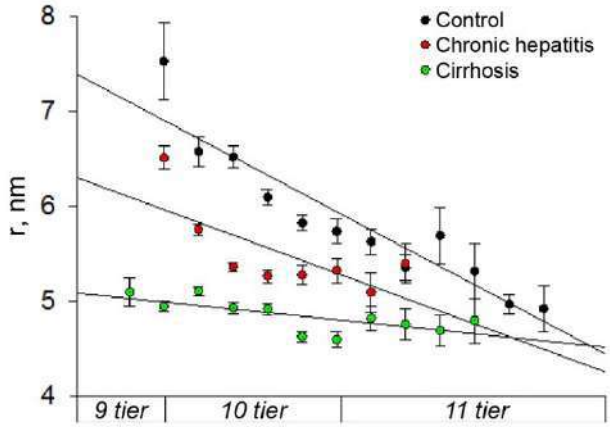


Figure 3.52 — Dependence of the distance between Don and Ac (**r**) on the degree of filling of the outer tiers (9–11th) of glycogen molecules in human hepatocytes: in the normal liver (Control), during chronic hepatitis (Chronic hepatitis) and in the cirrhotic liver (Cirrhosis). Note: vertical sections are the error of the mean.

The data presented in Figure 3.52 indicate that in the normal hepatocytes the distance between Don and Ac (**r**) decreases as glycogen molecules grow. These data suggest that as the size of the glycogen molecules increases, the glucose residues become more and more tightly packed. Unlike glycogen molecules in the hepatocytes in the normal liver, glycogen molecules during LC seem to have a denser structure at all stages of their formation. This means that our data confirm the hypothesis of Madsen and Cori (1958) that the size of glycogen molecules is regulated by its own structure.

However, another question arises: does the growth of glycogen molecules stop only after reaching the 12th tier? Or can it stop much earlier? To answer this question, we need to consider the data on the size of glycogen molecules obtained by electron microscopy (Wanson, Drochmans, 1968; Elsner et al., 2002; Marchand et al., 2002; 2007; Graham et al., 2010; Obel et al., 2012; Brewer et al., 2019).

EM studies of glycogen particle size, conducted mainly on skeletal muscles, have shown that the diameter of molecules can vary within a wide range. The limiting diameter of glycogen molecules is 42–44 nm (Marchand et al., 2002), which is consistent with the theory (Goldsmith et al., 1982; Melendez-Hevia et al.,1993; Melendez et al., 1997). However, it

should be emphasised that particles with a diameter of 42–44 nm, which corresponded to a 12-tier glycogen particle (molecule), were extremely rare. According to many authors, the average diameter of glycogen molecules is ~25 nm, which corresponds to glycogen molecules with glucose residues on 7–8 tiers (Shearer, Graham, 2004). In addition, the results presented in Figure 3.46 and Figure 3.47 suggest that a decrease in the distance between glucose residues with their increasing number in glycogen molecules is observed even at the earliest stages of glycogenesis in hepatocytes. These data suggest that in addition to steric mechanisms related to the glucose concentration of glucose residues on the surface of molecules and the size of enzymes involved in glycogen synthesis-degradation, there are some other mechanisms limiting the growth of glycogen molecules and their number in cells.

To sum up, using a specially developed cytofluorimetric method for the study of the spatial structure of glycogen molecules in hepatocytes we established that in the absorptive period the number of glucose residues in β-particles in the cirrhotic liver is higher than in the normal liver. During LC, glucose residues in β-particles are packed in denser structures than in the normal liver. It was also shown that glycogen synthesis in hepatocytes after glucose administration to starved rats starts at the periphery of cells and gradually moves to their central part, confirming the data obtained earlier on isolated hepatocytes using ^{14}C-glucose (Garcia-Rocha et al., 2001; Fernandez-Novell et al., 2002).

3.4. Morphofunctional state of the cirrhotic liver in rats and humans after different treatments

Liver cirrhosis (LC) is a diffuse process characterised by fibrosis and transformation of the normal liver structure with nodule formation. It is the final stage of most chronic diffuse liver diseases (Ivashkin et al., 2021).

Cirrhosis is a widespread liver disease with a high mortality rate and high economic losses for the state. Its causes may be different but its essence is the same: the development of fibrosis and a gradual progression of the disease, leading to the reorganisation of liver architectonics and metabolic shifts not only in the target organ but also in the whole organism.

The search for ways and means of alleviating the suffering of cirrhotic patients and, even more importantly, eliminating the structural and metabolic abnormalities of the liver resulting from long-term cirrhotic process required great efforts of scientists in different

countries for many years. As a rule, the therapeutic measures developed during these studies targeted particular symptoms (reduction of hypertension, elimination of ascites, improvement of microcirculation, etc.) and did not results in a complete recovery (Naumov, 1997; Sorinson, 1998). This may be the reason why hepatologists generally believe that LC is an irreversible and eventually lethal pathology (Friedman, Bansal, 2006; Gieling et al., 2008, Bravo et al., 2012). There are currently no therapeutic methods for the radical cure of LC, the treatments being mainly aimed at relieving its symptoms (Cui et al., 2021; Kronborg et al., 2021).

It is generally accepted that liver transplantation is the only effective treatment of cirrhosis (van der Helm et al., 2019). However, this operation is practically inaccessible to most patients due to the lack of suitable donors, high costs and numerous other factors (Jadlowiec et al., 2016). At the same time, the number of successful liver transplantations is steadily increasing. For example, this number made up ~6000 in Europe in 2010 (Zarrinpar, Busuttil, 2013), 3.4 per 1 million population in Russia in 2018, and 30 in the Moscow region in the same year (Moisyuk et al., 2018; Gautier, Khomyakov, 2019). The life expectancy of the patients is also increasing. According to statistical data, more than 90% of patients survive for one year after this surgery; after 5 years, the survival rate is 70%, and after 20 years, 53%. Moreover, the quality of life of patients after liver transplantation increases, too (Onghena et al., 2016). However, despite all these achievements, the number of cirrhotic patients who need liver transplantation to survive is not decreasing, and the waiting time for the operation remains long. For example, it makes up 5.5 months in the Moscow region (Moisyuk et al., 2018).

An acute shortage of donor livers for transplantation to patients with terminal cirrhosis has stimulated the search for new approaches to the treatment of this disease. These approaches include gene and cell therapy. It has been shown, e.g., that the transfer of the IGF-I (insulin-like growth factor I) gene into the cirrhotic liver triggers a process that upregulates the expression of cytoprotective and antifibrogenic molecules, while downregulating the expression of profibrogenic factors (Sobrevals et al., 2010). It is also believed that in principle transplantation of cells capable of differentiating into hepatocytes and cholangiocytes can, under certain conditions, compensate for the loss of functional liver cells during the pathological process (Burra et al., 2004; Alison et al., 2007; Conigliaro et al., 2010). Among these cells there are embryonic stem cells, extrahepatic stem cells (mesenchymal cells from bone marrow or other locations, particularly adipose tissue), and intrahepatic progenitor cells

(Galvao et al., 2006; Aquino et al., 2010; Darwiche, Petersen, 2010; Katoonizadeh et al., 2014; Yovchev et al., 2014). Theoretically, LC therapy can indeed greatly benefit from cell transplantation and genetic engineering. However, the introduction of these techniques into clinical practice as routine operations requires considerable effort (Laurson et al., 2005; Muraca et al., 2006; Lysy et al., 2008; Piscaglia et al., 2010; Tirnitz-Parker et al., 2013).

Therefore, in parallel with the development of new cell therapies, research is being conducted aimed at enhancing the ability of the pathologically altered liver itself to recover its original normal level. This research is needed to avoid organ transplantation and prevent mortality due to immune incompatibility. Therefore, the identification of cell populations capable of participating in liver regeneration and the establishment of cellular mechanisms for restoring the mass and function of the damaged organ are important tasks on the way to increasing its endogenous regenerative potential.

Reparative regeneration of the pathologically altered liver parenchyma, which can occur either by increasing the volume of functioning parenchyma or by resorption of excess collagen, is aimed at normalisation of stromal-parenchyma relationships. Insufficient regeneration may contribute to the chronic nature of the pathological process in the liver and increase its severity (LaBrecque, 1994). It is known that normal and reparative growth of any organ can proceed by increasing the number of cells (proliferation), by increasing the number of genomes in cells (polyploidisation) and by increasing the mass of cell cytoplasm unrelated to their polyploidisation (hypertrophy proper) (Goss, 1966).

It is generally accepted that polyploidization of liver parenchyma occurs by alternating acytokinetic and polyploidising restitution mitosis according to the scheme: 2c → 2c×2 → 4c → 4c×2 → 8c, etc. The details of this process may differ in different mammalian species (Nadal, Zaidela, 1966; Brodsky, Uryvaeva, 1985; Kudryavtsev et al., 1993; Duncan et al., 2010; Donne et al., 2020), but its starting point is a pool of mononucleate diploid hepatocytes. It is believed that it is the diploid (2c) hepatocytes that largely determine the regenerative potential of the liver (Sakuta, Kudryavtsev, 1996, 2005; Patterson, Swift, 2019).

Tables 3.3 and 3.4 (Section 3.1.4) present our data on the relative number of hepatocytes of different ploidy classes in the normal and the cirrhotic liver of rats and humans. Based on these data, we can conclude that despite considerable differences in the levels of polyploidization in the normal liver parenchyma of rats and humans, the dynamics of the hepatocyte population in rats and humans during LC development are similar. The similarity is

expressed, first of all, in the increase of the average ploidy level of hepatocytes during LC as compared to the norm. In addition, the results presented in Tables 3.3 and 3.4 suggest that the diploid hepatocyte population, which increases during LC in rats and makes up a considerable part of the human liver parenchyma cell population, plays a significant role in the repairs of the damaged liver parenchyma. This idea is supported by the results of other authors. Firstly, it was shown that the proportion of 2c-hepatocytes during LC in rats gradually increases after cessation of CCl_4 poisoning (Sakuta, Kudryavtsev, 1996). Secondly, it was found that diploid hepatocytes enter the mitotic cycle much more actively than polyploid ones (Uryvaeva, Marshak, 1969; Watanabe, 1970; Watanabe et al., 1984; Gerlyng et al., 1993; Gorla et al., 2001).

It is known that along with proliferation and polyploidization, cell hypertrophy plays an important role in normal and reparative growth of various organs (Goss, 1966; Brodsky, Uryvaeva, 1981). It is usually associated with an increase in the number of genomes in a cell (polyploidy), which inevitably leads to an increase in cell size. Hypertrophy that is not associated with an increase in cell ploidy or pre-mitotic syntheses is studied much less often. At the same time, it may be of great importance for understanding the mechanisms of normal and reparative growth of an organ. The degree of cell hypertrophy in this case is associated with the size of their cytoplasm, since the change in the size of nuclei practically does not affect the total size of cells. Accurate estimation of the cell hypertrophy unrelated to ploidy can be achieved by parallel determination of cell ploidy level and objective indicators of its size. Possible indicators are the dry mass (DM) of the cell, more than 80% of which is composed of proteins (Giese, 1959; Brodsky, 1966). Measurement of DM of hepatocytes showed that before the beginning of poisoning of rats with CCl_4, DM of cells was 812±17 pg. In 6 months after the beginning of the experiment, DM values of hepatocytes in rats of the control group (not exposed to CCl_4) were 943±21 pg, while those of rats with LC were 1184±56 pg. DM of hepatocytes in the normal liver of adult humans averaged 546.0±29.6 pg, and in cirrhotic patients, 675.4±41.3 pg. The increase in hepatocyte mass during cirrhosis development, which was 25.6% ($p < 0.001$) in rats and 23.7% ($p < 0.05$) in humans as compared to the norm, suggests that cell hypertrophy plays a prominent role in the reparative liver growth (Bezborodkina et al., 2021b).

Data on the changes in the liver parenchyma mass during LC development, DM of hepatocytes and their ploidy in the normal and the cirrhotic liver of rats and humans make it

possible to quantify the contribution of proliferation (**Q_1**), polyploidization (**Q_2**) and hypertrophy (**Q_3**) of cells to normal and reparative liver growth.

The development of CCl_4-induced cirrhosis in rats is associated with a 19% ($p < 0.05$) increase in the liver mass (Bezborodkina et al., 2021b). In contrast to rats, human liver mass decreases during LC (Patel et al., 2019; Simon et al., 2020; Hagen et al., 2022), which is accompanied by a significant decrease in the proportion of the liver parenchyma. While in the normal human liver it makes up 90.6±0.9%, during LC it is only 54.0±2.5% (Table 3.2) (Bezborodkina et al., 2021), indicating that hepatocyte death prevails over their replenishment.

The data presented in Table 3.11 indicate that in rats cell hypertrophy (about 18 %) plays a significant role in the increase of liver mass during LC development. However, the main contribution to the reparative growth of the liver is made by cellular processes associated with DNA synthesis, accompanied by an increase in the number of cells. Proliferation associated with normal mitotic cell divisions accounts for 66% and polyploidization, for 16%. Similar data were obtained earlier in studies of cellular mechanisms of normal and reparative liver growth in rats (Sakuta, Kudryavtsev, 1996). A somewhat smaller contribution of proliferation (57%) and an increased contribution of polyploidization (30%) during LC formation found by the authors is apparently related to the age differences of the animals used in the experiment.

Table 3.11 — Relative contribution (%) of proliferation (Q_1), polyploidization (Q_2) and hypertrophy (Q_3) of hepatocytes to changes in rat and human liver mass during the development of liver cirrhosis (LC)

	Q_1	Q_2	Q_3
Rats	66	16	18
Humans	111.2	–7.3	–3.9

Note: In calculating the contribution(s) of cell proliferation, polyploidy and hypertrophy, it was assumed that the ratio of the liver parenchyma mass of the cirrhotic liver to that of the normal liver (M) is 4.0 in rats and 0.37 in humans, taking into account cell renewal during LC development.

Determination of the contribution(s) of hepatocyte proliferation, polyploidy and hypertrophy to reparative liver growth in humans showed that reparative growth of the human liver during the development of cirrhosis was solely due to mitotic divisions of small diploid hepatocytes (Table 3.11).

Although many researchers believe that liver cirrhosis is irreversible, several authors hold an opposite viewpoint (Sarkisov, Rubetskoy, 1965; Powell et al., 1970; Solopaev, 1985; Dufour et al., 1997; Kaplan et al., 1997; Lau et al., 1999; Benyon, Iredale, 2000; Wanless, 2001; Kweon et al., 2001; Hammel et al., 2001). The key questions in the studies of LC reversal are the degree of reversal of the pathological changes in the liver to the normal state after application of appropriate therapeutic measures and the level of damaging effect of the therapy itself on the liver of cirrhotic patients.

Understanding the mechanisms involved in liver fibrosis is a fundamental challenge for researchers working on the development of LC therapy. Fibrosis and cirrhosis are dynamic processes, and can progress as well as regress over time depending on whether their cause is permanently present or not (Arthur, 2002). Therefore, the elimination of the underlying cause of LC has a paramount importance for the reversal of the structure and function of the cirrhotic liver to those of the liver in its initial normal state. Our results indicate that cessation of CCl_4 poisoning of rats results, already after 1 month, in a noticeable improvement of the liver structure, which is mainly expressed in the reduction of the proportion of the connective tissue. However, even 6 months[*)] after the end of the damaging effect, there was no complete recovery of the organ's lobular structure (Fig. 3.53) (Kudryavtseva et al., 1998, 1999, 2001; Kudryavtseva et al., 2000).

In experiments on rats, we tested a number of treatments that, in principle, could increase the degree and speed of recovery of the structure and function of the liver disturbed during the development of LC. With some reservations, one might distinguish several possible ways of reversing cirrhotic changes in the liver **if the damaging agent is eliminated**: enhancing the proliferative potential of hepatocytes, reducing the mass of connective tissue, reducing the load on the liver and enhancing the functional state of some body systems that have a direct influence on the level of the regenerative process.

[*)] In rats, the age of 11–13 months is thought to correspond roughly to the human age of 30 years, and the age 24–27 months, to the human age of 60–70 years (Weisfeld, 1998). This means that 6 month of life in rats roughly correspond to 12–15 years of human life.

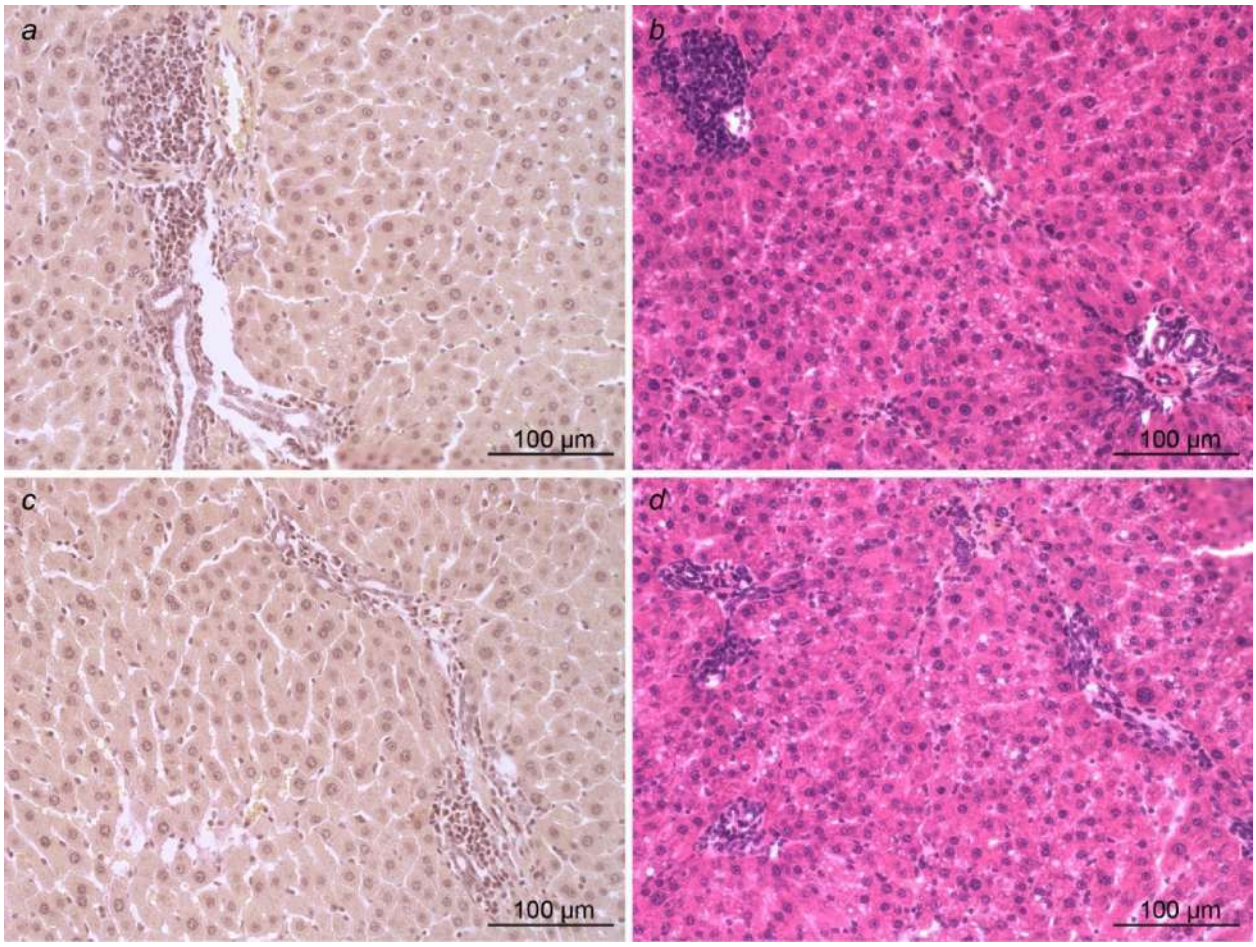


Figure 3.53 — Rat liver sections: *a, b* — during liver cirrhosis; *c, d* — 6 months after cessation of CCl_4 exposure*; a, c* — van Gieson staining, *b, d* — hematoxylin-eosin staining.

Agents and procedures that may potentially enhance regeneration of the cirrhotic liver and normalise its structure and function include partial liver resection (Nakamura et al., 1984; Imai et al., 1990; Castells et al., 1994), high-carbohydrate diet (Stowell et al., 1951; Bernelli-Zazzera, Gaja, 1964; Jennische, 1983; Hinson, 1983; Frederiks, 1985; Kabadi et al., 1985), human chorionic gonadotropin (Solopaeva, 1969; Solopaeva, Solopaev, 1991), and lysine-glutamine dipeptide (Kiseleva et al., 1999).

Although agents of different nature were used to increase the regenerative ability of the cirrhotic rat liver, they action had similar results. The summary of our experimental studies (Kudryavtseva et al., 1998, 1999, 2001a; Kudryavtseva et al., 2000) is as follows:

1) The lobular structure of the organ is not restored even 6 months after the end of exposure of the animals to CCl_4, despite a certain normalisation of the histological structure of the liver expressed in the reduction of the proportion of the connective tissue, the disappearance of the inflammation foci and the decrease of the number of Kupffer cells (Fig. 3.54).

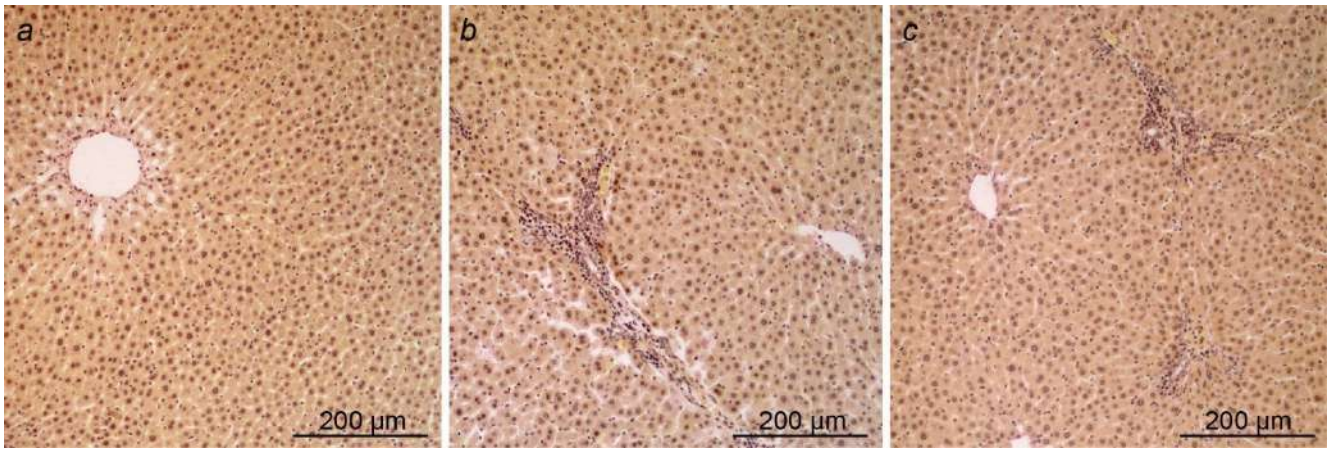


Figure 3.54 — Rat liver sections: *a* — normal liver; *b* — cirrhotic liver; *c* — liver 6 months after partial hepatectomy. Staining after van Gieson.

2) Functional indices of the liver after cessation of exposure of animals to CCl_4 recover faster and more completely than its histological structure. First of all, this is expressed in a significant decrease of glycogen content in hepatocytes, an increase of G6Pase activity and increase of GP*a* activity. At the same time, unlike GP*a,* glycogen content and G6Pase activity did not reach normal values even long after the cessation of toxic exposure.
3) An inverse relationship ($r = –0.84$, $p < 0.001$) was found between the decrease of glycogen content in hepatocytes and G6Pase activity in rat liver (Figure 3.55).

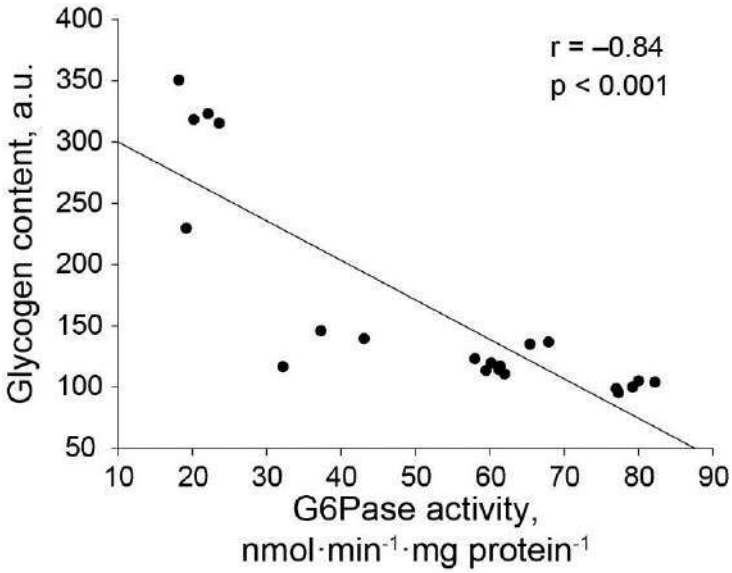


Figure 3.55 — Dependence of glycogen content in hepatocytes on glucose-6-phosphatase (G6Pase) activity in the rat liver.

Thus, our study with the use of different methods of LC therapy in rats showed that, despite a certain improvement of the liver architectonics, complete restoration of its original lobular structure does not occur even after long-term observation. The analysis of our data shows that the cessation of the pathogenic influence is the leading factor of the morphological improvement of the cirrhotic liver. The methods of LC treatment in rats used in this work had only a weak effect on the course of recovery of the affected organ (Kudryavtseva et al., 1998, 1999, 2001a; Kudryavtseva et al., 2000).

Large-scale studies using experimental models of LC (Iimuro et al., 2003; Parsons et al., 2004; Siller-Lopez et al., 2004; Roderfeld et al., 2006; Atta et al., 2014) and clinical data obtained on cirrhotic patients (Wanless et al., 2000; Poynard et al., 2002; Dienstag et al., 2003) have shown that exclusion of the aetiological source of chronic liver injury leads to decreased levels of anti-inflammatory and fibrogenic cytokines, decreased extracellular matrix production and apoptosis of stellate cell (Iredale et al., 1998; Iredale, 2007; Krizhanovsky et al., 2008; Wright et al., 2001). In addition, many studies have demonstrated a significant reduction in the fibrotic tissue volume after treatment of LC with various agents (Lewis et al., 1983; Weinbren et al., 1985; Dufour et al., 1997; Sobesky et al., 1999; Guerret, 1999; Poynard et al., 2002; Liu et al., 2013; Sun et al., 2020). However, despite all these positive results, most researchers currently believe that only fibrosis, but not mature cirrhosis, can be reversed (Desmet, Roskams, 2004; Malekzadeh et al., 2004; Bravo et al., 2012). Complete reversal of cirrhotic changes in the liver is thought to be possible only in rare instances, and reports of such reversal are often considered controversial (Benyon, Iredale, 2000; Di Vinicius et al., 2000; Di Vinicius et al., 2005; Ramachandran, Iredale, 2009; Metwally et al., 2017). Therefore, as yet, none of the various anti-cirrhotic treatments can be expected to reverse the structural changes in the liver to the level characteristic of the normal liver. This claim is confirmed by our data obtained in the study of CCl_4-cirrhosis in rats during a long recovery period after cessation of poisoning of animals with hepatotropic poison (Kudryavtseva et al., 1998, 1999, 2001a; Kudryavtseva et al., 2000).

Fibrosis and deep reorganisation of the vascular bed during LC result in hypoxia and deterioration of the supply of necessary substrates to the liver parenchyma cells. In addition, the development of LC is accompanied by ongoing processes of inflammation, death and proliferation of various cell types, as well as neutralisation of damaging agents, which require significant energy expenditure. Therefore, in principle, the cessation of the damaging effect on

the liver, which leads to a significant decrease in the intensity of all these processes, should release much energy, which can be redirected to restore the structure and multiple functions of the damaged liver. However, there are currently no data on the level of hypoxia in the liver and the energy status of the organ after cessation of damaging effects and LC therapy.

One of the pharmacological agents used during LC treatment is 2-ethylthiobenzimidazole hydrobromide (BM). It has been shown to accelerate reparative regeneration of the liver, improve the function of the pathologically altered organ, and exhibit immunomodulatory and antioxidant effects (Plotnikov et al., 1989; Smirnov, 1993; Smirnov, 1994; Gaivoronskaya, 2000). It has also been established that BM possesses antimutagenic properties (Durnev, Seredenin, 1995; Zinovieva et al., 1995; Seredenin et al., 1986) and has a favourable effect on carbohydrate metabolism of the cirrhotic rat liver. It is believed that the main mechanism of BM effect on carbohydrate metabolism in the liver is mediated by the enhancement of transcription and translation processes (Okovity, 1995).

The most common experimental model of LC is chronic exposure of animals to hepatotropic poison. Carbon tetrachloride (CCl_4) is used particularly often. LC developing in animals is believed to be similar to that in humans (Zimmerman, 1978; Zakusov, 1985; Sarkisov, 1987; Kohno et al., 1991; Skobeleva, 1994). At the same time, evaluation of the proportion of the connective tissue, determination of enzyme activity and glycogen content showed that the pathological process in the human liver leads to much deeper structural and functional rearrangements than in animal liver (Owen et al., 1981, 1983; Kudryavtseva et al., 1992). It is obvious that the data on pathogenesis and reversibility of LC in animals require considerable adjustment when applied to humans. Therefore, we conducted a comparative study of the effects of BM on the rehabilitation of pathologically altered liver in rats and humans (Kudryavtseva et al., 2002a, 2002b; Kudryavtseva et al., 2003; Kudryavtseva et al., 2004; Okovity et al., 2006). The effects of BM on the liver of patients with chronic viral hepatitis were studied both before and during LC development (Kudryavtseva et al., 2002a).

The study of the effects of BM on glycogen metabolism in the cirrhotic rat liver showed that the use of this drug caused a significant decrease in glycogen content in hepatocytes and an increase in G6Pase activity in the liver of animals (Table 3.12). However, similarly to other agents used in our work, BM does not result in a complete normalisation of these parameters. Glycogen levels in hepatocytes after BM therapy remain 23% higher than in the control ($p < 0.001$), while G6Pase activity is 23% lower than in the control ($p < 0.02$) (Kudryavtseva et al.,

2002b; Kudryavtseva et al., 2003). The decrease of the glycogen level in rat liver after BM therapy undoubtedly indicates a favourable effect of this drug on the pathologically altered organ (Kudryavtseva et al., 2002b; Kudryavtseva et al., 2004). It is necessary, however, to once again emphasize that the results obtained from studying the reversibility of cirrhosis in animals can be used in humans only with great caution.

Table 3.12 — Content of total glycogen and its fractions in hepatocytes (arbitrary units), glucose-6-phosphatase (G6Pase) activity (nmol/min/mg protein) in the normal and the cirrhotic rat liver (LC) and during treatment of cirrhosis with 2-ethylthiobenzimidazole hydrobromide (LC+BM), arbitrary units ($X \pm S_x$, n = 5).

Experimental variant	Total glycogen	Labile fraction of glycogen	Stable fraction of glycogen	G6Pase activity
Control	2.39±0.06	1.93±0.05	0.38±0.03	0.77±0.03
LC	7.73±0.07[1]	4.53±0.07[1]	3.05±0.02[1]	0.20±0.08[1]
LC + BM	2.94±0.05[1]	1.99±0.06	0.62±0.02[1]	0.59±0.05[2]

[1,2] Significantly different from the value in the control ([1] $p < 0.001$,[2] $p < 0.02$).

Our data indicate that the basic therapy of patients with CVH and LC did not actually affect the glycogen content in cells. At the same time, its content in hepatocytes decreased after treatment with BM, the decrease being more noticeable in patients with hepatitis than in cirrhotic patients (Table 3.13) (Kudryavtseva et al., 2002a). It should also be noted that therapy of cirrhotic patients with BM caused only a 9% drop in glycogen levels in hepatocytes ($p < 0.01$), while in rats BM reduced glycogen content in hepatocytes by 77 % (Table 3.12). The glycogen content in hepatocytes after the end of treatment of cirrhotic patients with the drug remained approximately 2.38 times higher than the norm ($p < 0.001$) (Table 3.13). In rats, the glycogen content in hepatocytes after a similar treatment scheme was only 1.23 times higher than the norm ($p < 0.001$) (Table 3.12) (Kudryavtseva et al., 2002b; Kudryavtseva et al., 2003). A weak effect of BM on the liver of human patients may be due to the persistence of the virus and/or deeper structural rearrangement of the liver parenchyma in humans compared to rats (Kudryavtseva et al., 2002a; Bezborodkina et al., 2021b).

Table 3.13 — Content of total glycogen and its fractions in hepatocytes of patients of different groups, arbitrary units ($X \pm S_x$).

Indicator	Patient groups						
	I (n = 10)	II (n = 12)	III (n = 3)	IV (n = 9)	V (n = 13)	VI (n = 4)	VII (n = 9)
Total glycogen	3.4±0.1	6.5±0.1	6.0±0.3	5.6±0.1	8.9±0.2	9.4±0.4	8.1±0.2
Labile fraction	2.9±0.1	5.2±0.1	4.4 ±0.1	4.3±0.1	6.0±0.1	6.7±0.1	6.5±0.1
Stable fraction	0.4±0.1	1.4±0.1	1.6±0.3	1.0±0.1	2.9±0.2	3.1±0.5	1.5±0.2

Note: I — control group, II — CVH without therapy, III — CVH after basic therapy, IV — CVH after 2-ethylthiobenzimidazole hydrobromide therapy, V — LC without therapy, VI — LC after basic therapy, VII — LC after 2-ethylthiobenzimidazole hydrobromide therapy. All values in patient groups II–VII were significantly different from the corresponding values in group I at $p < 0.001$.

Though considerable efforts were made to smooth over the differences between the groups of patients and make them uniform by a number of parameters, this proved impossible, and thus the problems associated with heterogeneity of cell populations in human liver parenchyma could not be eliminated. Therefore, to reduce the influence of heterogeneous composition of patients in the groups on the glycogen content in hepatocytes, we used serial liver biopsy samples obtained from the same patient at different time intervals after bemithyl therapy (Tables 3.14, 3.15) (Kudryavtseva et al., 2002a).

Table 3.14 — Content of total glycogen (TG) and its fractions in hepatocytes obtained from serial liver biopsy samples of CVH patients before (biopsy I) and after (biopsy II) therapy with 2-ethylthiobenzimidazole hydrobromide, arbitrary units ($X \pm S_x$).

Patient no.	Glycogen content					
	Biopsy I			Biopsy II		
	TG	labile fraction	stable fraction	TG	labile fraction	stable fraction
1	9.3±0.8	6.1±0.3	3.2±0.9	6.1±0.2	5.0±0.2	1.1±0.3
2	6.4±0.4	5.1±0.3	1.3±0.5	5.2±0.1	4.3±0.2	0.9±0.2
3	4.5±0.3	3.5±0.2	1.0±0.4	4.0±0.3	3.1±0.1	0.9±0.3
4	6.6±0.8	4.6±0.3	2.0±0.6	4.9±0.5	4.1±0.2	0.8±0.3
5	9.0±0.9	6.8±0.2	2.2±0.9	8.1±0.6	6.6±0.3	1.6±0.6
6	6.7±0.3	4.8±0.5	1.9±0.6	5.1±0.4	4.1±0.3	1.0±0.5
7	8.1±0.4	6.3±0.5	1.9±0.6	7.1±0.4	5.9±0.4	1.2±0.6
8	9.0±0.8	6.8±0.3	2.3±0.9	7.0±0.4	5.9±0.3	1.1±0.5
9	7.6±0.8	5.0±0.2	2.6±0.8	6.2±0.2	5.3±0.2	0.9±0.3

Table 4.15 — Content of total glycogen (TG), its labile fraction and stable fraction in hepatocytes obtained from serial liver biopsy samples of cirrhotic patients before (biopsy I) and after (biopsy II) therapy with 2-ethylthiobenzimidazole hydrobromide, arbitrary units ($X \pm S_x$).

Patient no.	Glycogen content					
	Biopsy I			Biopsy II		
	TG	labile fraction	stable fraction	TG	labile fraction	stable fraction
1	8.5±0.4	5.8±0.4	2.7±0.6	7.8±0.3	6.5±0.2	1.3±0.4
2	10.2±1.0	6.7±0.3	3.5±1.0	8.5±0.9	6.7±0.3	1.8±0.9
3	8.3±1.0	5.3±0.3	3.0±1.0	8.0±0.7	6.4±0.5	1.6±0.9
4	9.5±0.9	6.6±0.3	2.9±0.9	8.5±0.4	6.7±0.5	1.8±0.6
5	7.7±0.6	5.8±0.2	1.9±0.6	7.2±0.6	6.2±0.4	1.0±0.7
6	8.7±0.4	6.2±0.4	2.5±0.6	8.0±0.9	6.4±0.4	1.6±0.9
7	9.0±0.8	5.3±0.3	3.7±0.8	8.2±0.7	6.6±0.2	1.6±0.7
8	9.4±1.0	6.8±0.3	2.6±1.0	8.1±0.6	6.9±0.3	1.2±0.7
9	9.8±1.0	5.5±0.2	4.4±1.0	8.5±0.6	6.2±0.3	2.3±0.7

It has been established based on the material of more than 160 liver biopsies of CH patients that the glycogen content in hepatocytes does not depend on the aetiology of the disease and increases with the intensification of liver damage. In patients with severe liver damage the glycogen content in the cells could exceed the norm more than 4-fold (Kudryavtseva, 1987). Our study showed (Tables 3.13, 3.14, 3.15) that BM therapy, unlike basic therapy, leads to a decrease in glycogen content in both CVH patients and cirrhotic patients (Kudryavtseva et al., 2002a). In cirrhotic patients, an inverse correlation ($r = -0.87$; $p < 0.01$) was observed between the decrease of the glycogen content in hepatocytes and its initial level in the cells (Figure 3.56). This result means that the effect of BM on the glycogen content in cells is greater when liver damage is less severe (Kudryavtseva et al., 2002a).

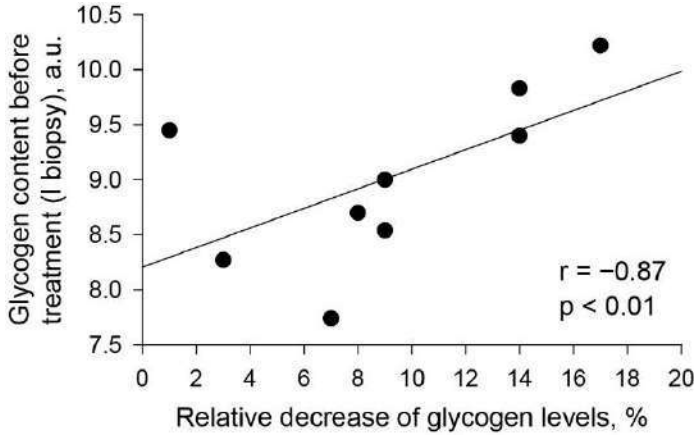


Figure 3.56 — Dependence of the decrease of glycogen content and its initial level in hepatocytes of cirrhotic patients after BM therapy. Based on the results of serial biopsies.

Analyses of the dependence of the relative decrease of glycogen levels in hepatocytes on the duration of the interval between biopsy I and biopsy II revealed this dependence only in CVH patients (Fig. 3.57). This finding suggests that in case of CVH, when pathological changes in the liver are less pronounced than during LC, BM therapy leads to progressive and long-term improvement of glycogen metabolism in the liver (Kudryavtseva et al., 2002a).

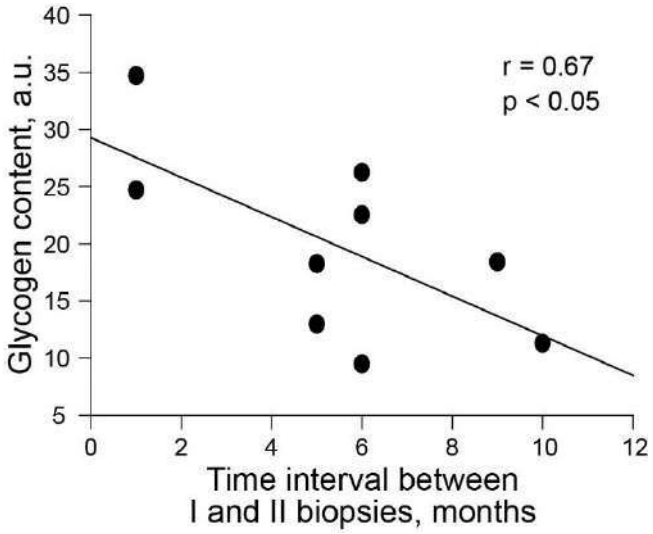


Figure 3.57 — Dependence of the decrease of glycogen content in hepatocytes of patients with CVH on the duration of the time interval between biopsy I and biopsy II after BM therapy.

The study of the dependence of glycogen content in hepatocytes of patients with CVH on the activity of G6Pase in the liver (Fig. 3.58) showed that, similarly to cirrhotic rats (Kudryavtseva et al., 2002b), the activity of this enzyme largely determines the level of glycogen in cells. The increase in G6Pase activity leads to a decrease in glycogen content in hepatocytes (Kudryavtseva et al., 2002a).

Based on the above, it can be concluded that glycogenosis in hepatocytes of rats and humans during LC is largely reversible after cessation of the damaging effect on the liver. Additional application of therapeutic methods after the removal of the pathogenic impact has only a small positive effect. It has also been established that the decrease of glycogen levels in hepatocytes of the cirrhotic liver after therapy is inversely proportional to the glycogen content in cells before the treatment and to the increase of G6Pase activity (Kudryavtseva et al., 1998, 1999, 2001a, 2003; Kudryavtseva et al., 2000; 2002a, 2002b, 2004).

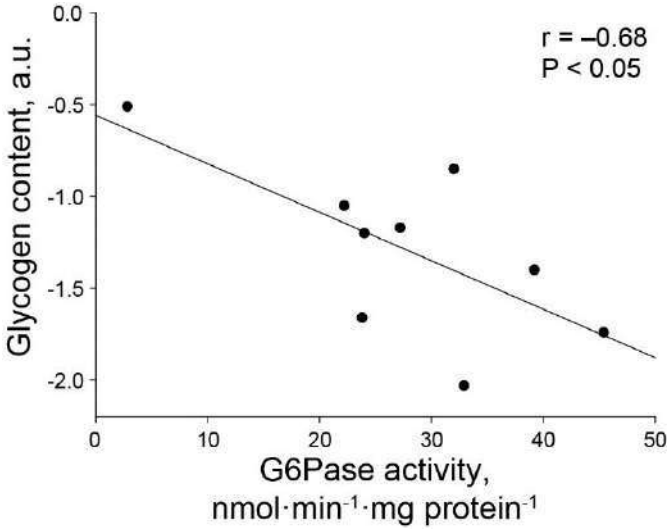


Figure 3.58 — Dependence of the decrease in glycogen content in hepatocytes of patients with CVH on the increase in glucose-6-phosphatase (G6Pase) activity in the liver after BM therapy. Based on the results of serial biopsies.

In addition to physiological requirements of the organism and metabolic factors (blood glucose concentration, insulin and glucagon levels, enzyme activity, etc.), the glycogen content in hepatocytes may depend on the number of glycogen molecules and their spatial structure. Theoretically, the number of glucose residues in the 4 outer tiers of glycogen molecules (~50,000) may exceed the number of residues contained in the 8 inner tiers (3315) 15-fold to 16-fold (Roach et al., 2012). Even taking into account that only 34.6% of α-1,4-glycosidic bonds in glycogen are subjected to the direct action of GF*a* (without the participation of the debranching enzyme) (Shearer, Graham, 2004), glycogen content in cells may vary approximately 5-fold due to changes in the spatial structure of its molecules alone. It can be assumed that the variability of glycogen content in hepatocytes associated with the different number of its molecules in cells is also significant.

The data shown in Figure 3.59 demonstrate that the total number of glucose residues in one molecule (β-particle) of glycogen averages 18810±734 in the control rats and is much lower in the cirrhotic rats, 7107±390.

Our data on the number of glucose residues in glycogen molecules indicate that in the normal rats the spatial structure of glycogen molecules includes ten tiers completely filled with glucose residues and the 11th tier filled by 41%, while in cirrhotic rats it includes nine complete tiers and the 10th tier filled only by 3.3% (Fig. 3.59). Based on the data on the dependence of the diameter of glycogen molecules on the number of glucose residues in them (Shearer, Graham, 2004; Roach et al., 2012), the average diameter of glycogen molecules in

hepatocytes is 36 nm in the normal rat liver and ~31 nm in the cirrhotic rat liver. Various rehabilitation methods may promote a certain reversal of the structure of glycogen molecules disturbed by long-term pathological process but cannot completely restore it (Fig. 3.59).

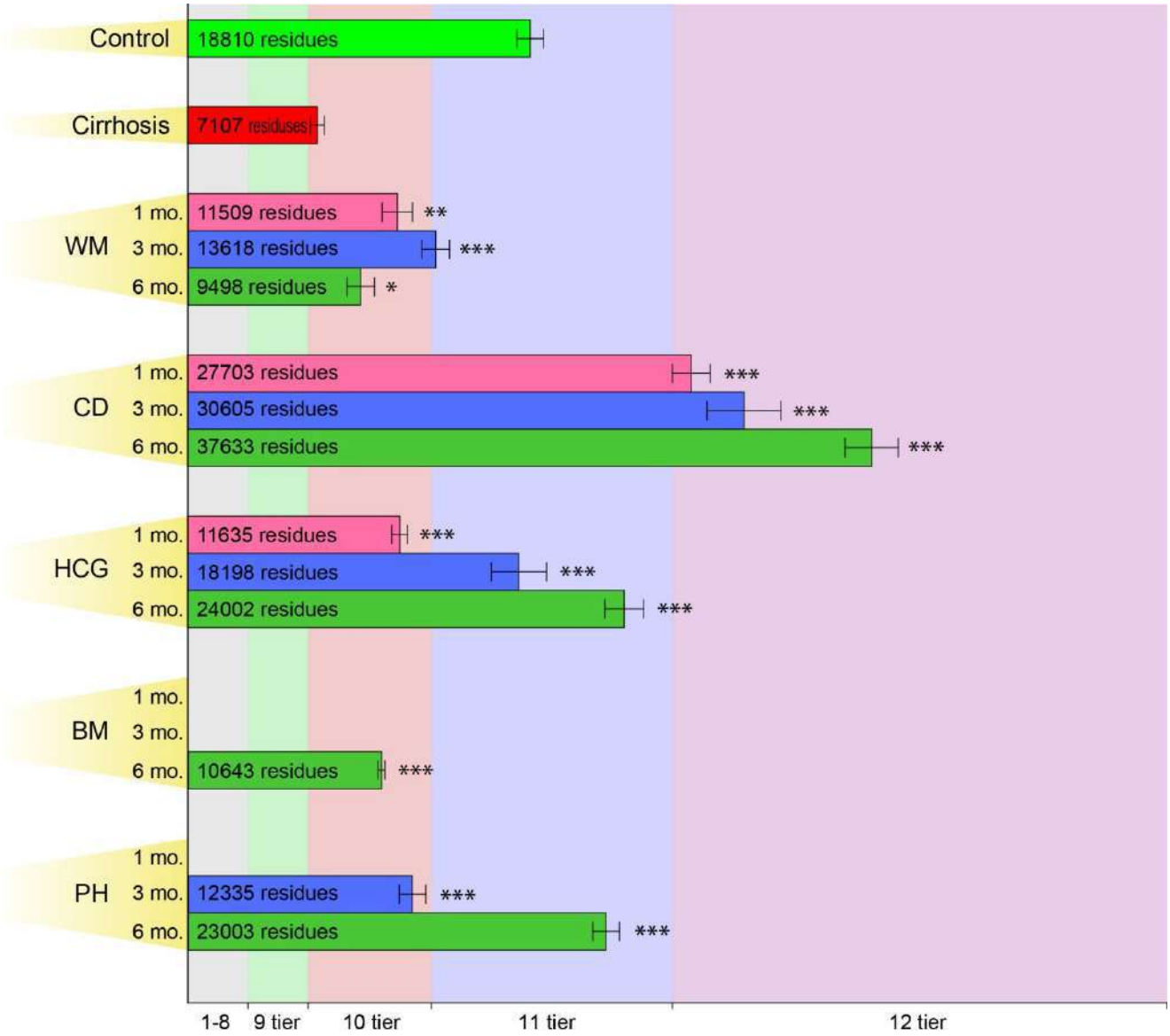


Figure 3.59 — Number of glucose residues in glycogen molecules in hepatocytes in the normal and the cirrhotic rat liver at different time intervals after cessation of CCl_4 poisoning and application of different methods of therapy of liver cirrhosis (cirrhosis). WM — without manipulations; CD — high-carbohydrate diet; HCG — human chorionic gonadotropin; BM — 2-ethylthiobenzimidazole hydrobromide; PH — partial hepatectomy.

Removal of the damaging agent and application of additional therapy leads to the same kind of changes in the spatial structure of glycogen. They are expressed in an increase in the number of glucose residues in its molecules. The greatest increase, as expected, was observed in the group of rats on a high-carbohydrate diet (Fig. 3.59). Already 1 month after the start of

the diet the amount of glucose residues in hepatocytes of rats of this group exceeded the normal level by 47% ($p < 0.001$), almost doubling after 6 months. The effect of the other treatments on rats was less pronounced but increased with the increasing duration of the treatment (Fig. 3.59).

The results of determining the relative amount of glycogen molecules in the hepatocytes in the normal and the cirrhotic rat liver are presented in Figure 3.60.

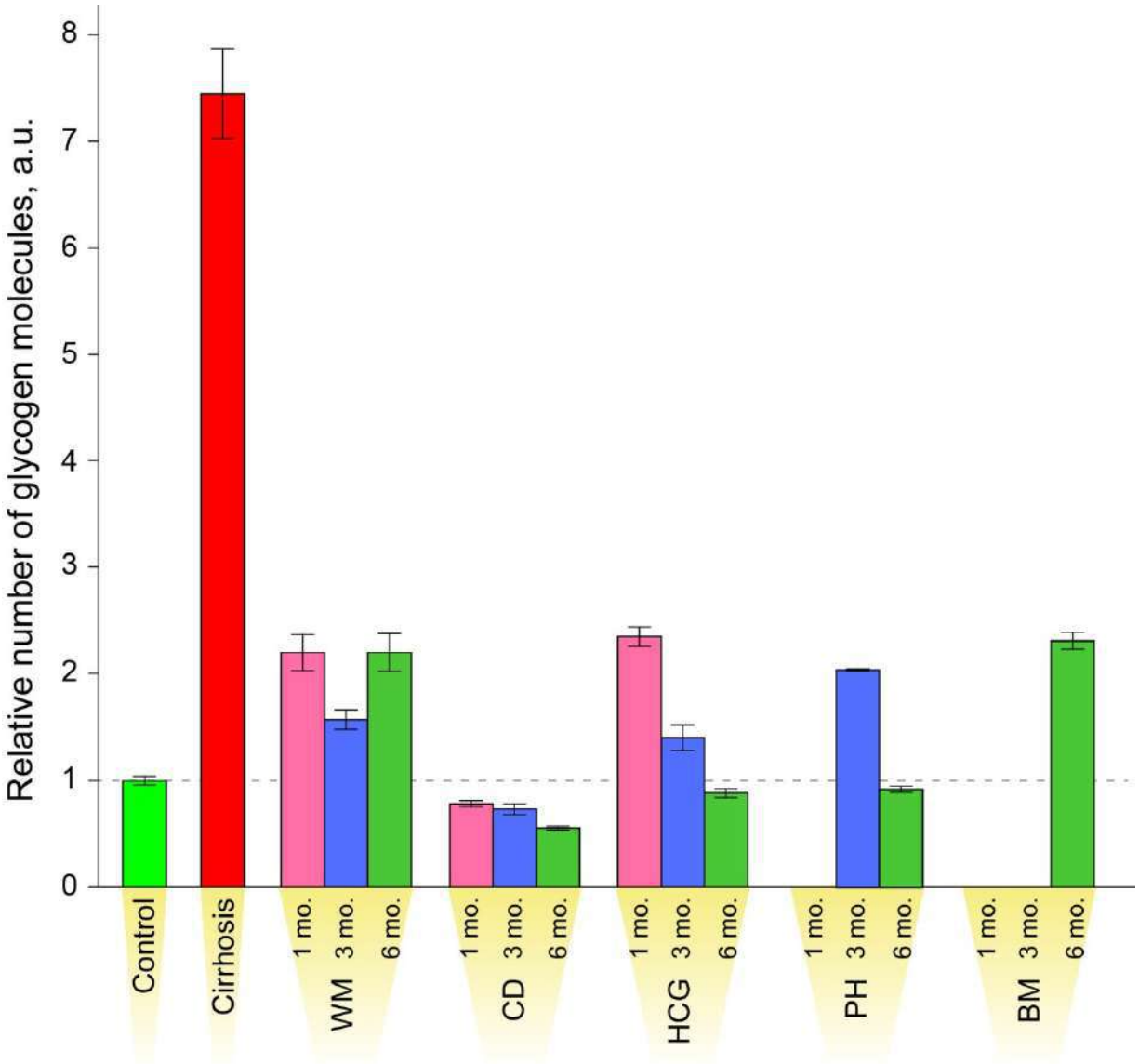


Figure 3.60 — Relative number of glycogen molecules in hepatocytes of the normal and the cirrhotic rat liver at different time intervals after cessation of poisoning of animals with CCl_4 and application of different methods of therapy of liver cirrhosis (LC). WM — without manipulations; CD — high-carbohydrate diet; HCG — human chorionic gonadotropin; PH — partial hepatectomy; BM — 2-ethylthiobenzimidazole hydrobromide.

Based on the data obtained in our study, we can conclude that the development of LC in rats is accompanied by a 2–3-fold decrease in the number of glucose residues in glycogen

molecules and a much larger (7–9-fold) increase in the number of its molecules (Fig. 3.59 and Fig. 3.60). Various methods of LC therapy lead not only to an increase in the number of residues in glycogen molecules and, consequently, to an increase in the size of the molecules themselves, but also to a decrease in the number of glycogen molecules accompanied by a decrease in its levels in hepatocytes. The relative contribution of the number of glycogen molecules and the number of glucose residues in them to the decrease in glycogen content in cells differs during different treatments of LC (Fig. 3.59 and Fig. 3.60). Nevertheless, we can conclude that the decrease in the number of glycogen molecules to the decrease of glycogen content in hepatocytes during LC rehabilitation makes the most significant contribution.

In the normal human liver, glycogen molecules are somewhat larger than in the normal rat liver, and contain, on average, 24034±707 glucose residues. The development of LC in humans is associated with a ~2.6-fold ($p < 0.01$) decrease in the number of glucose residues in glycogen molecules and an 8–10-fold increase in the number of glycogen molecules (Fig. 3.61). It is noteworthy that the spatial structure of glycogen β-particles in hepatocytes of the cirrhotic human liver is almost identical to that in rats: the molecules also consist of 9 complete tiers and the 10th tier partially filled with glucose residues (Fig. 3.59, Fig. 3.61).

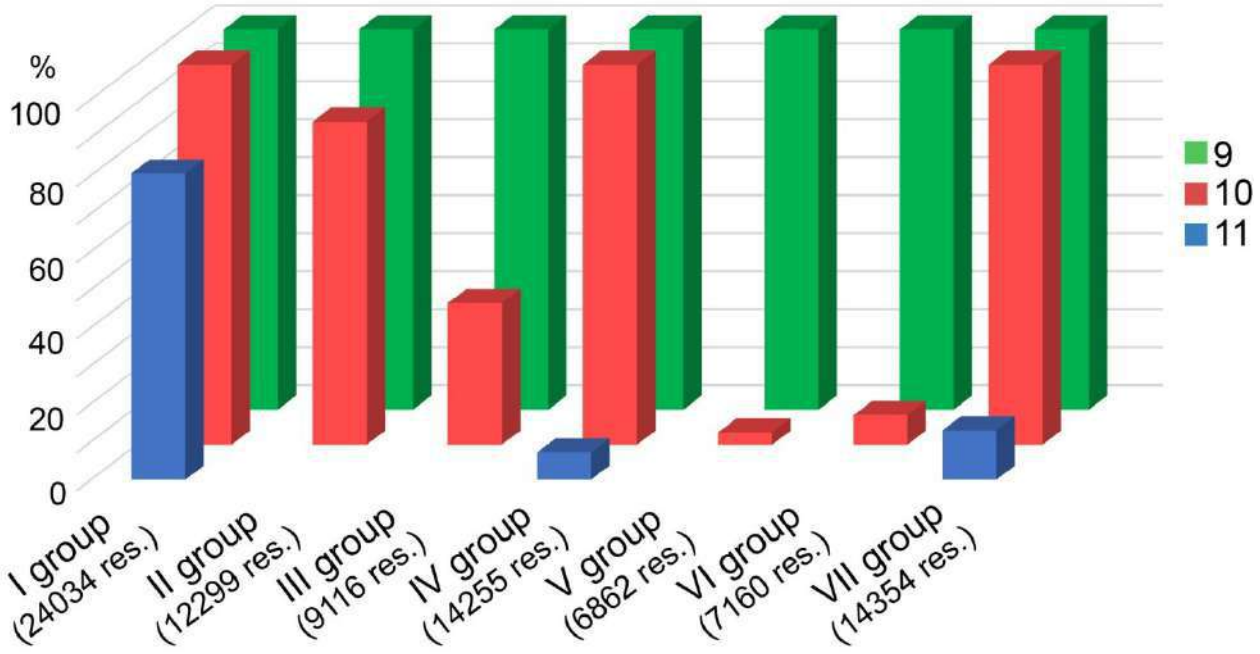


Figure 3.61 — Total number of glucose residues and their distribution on the outer tiers of glycogen molecules in hepatocytes of patients of groups I–VII. Note: Group I — control, II — CVH without therapy, III — CVH after basic therapy, IV — CVH after 2-ethylthiobenzimidazole hydrobromide therapy, V — LC without therapy, VI — LC after basic therapy, VII — LC after 2-ethylthiobenzimidazole hydrobromide therapy.

Therapy of cirrhotic patients with bemithyl (Fig. 3.61, group VII) resulted in a two-fold increase in the average number of glucose residues in glycogen molecules and approximately the same decrease in the number of glycogen molecules in hepatocytes. Similar data were obtained when determining the number of glucose residues in glycogen molecules in hepatocytes of patients with CVH during their treatment with BM (Fig. 3.61, group IV).

The reasons for the decrease in the size of glycogen molecules and increase in their number in hepatocytes during LC are unclear. It is known, however, that the glycogen degradation primarily affects relatively smaller β-particles and to a lesser extent, large α-particles (Sullivan et al., 2014). This conclusion is also supported by the fact that GP is more active in glycogen with a lower molecular mass (Stetten, Stetten, 1958). Data on the incorporation of labelled glucose into glycogen also indicate that the rate of metabolism of its larger molecules is lower than that of small molecules (Geddes et al., 1977). These results suggest that the surface area of glycogen molecules, which increases with decreasing size, may play an important role in controlling its degradation. Based on the above, it can be assumed that the decrease in the size of glycogen molecules during LC is an adaptive response that makes it possible to increase the rate of glycogen metabolism.

LC usually ends in liver failure and/or hepatocellular carcinoma (HCC) or malignant tumours in other organs (Kalaitzakis et al., 2011; Ganne-Carrié, Nahon, 2019). The risk of developing HCC is 26–60 times higher in cirrhotic patients than in the general population (Sorensen et al., 1998; Kalaitzakis et al., 2011). Therefore, many researchers, mainly based on the morphological analysis, consider LC as a precancerous condition (Anthony, 1978; Roncalli et al., 2011; Hytiroglu et al., 2012; Niu et al., 2017). HCC is one of the most lethal human cancers. Chronic infection with hepatitis B or C viruses, foods containing aflatoxins produced by micromycetes, metabolic disorders, diabetes, and excessive alcohol consumption are the main stimuli for chronic liver inflammation that leads to fibrosis (and) or cirrhosis, and ultimately to the development of HCC in humans (Figure 3.62).

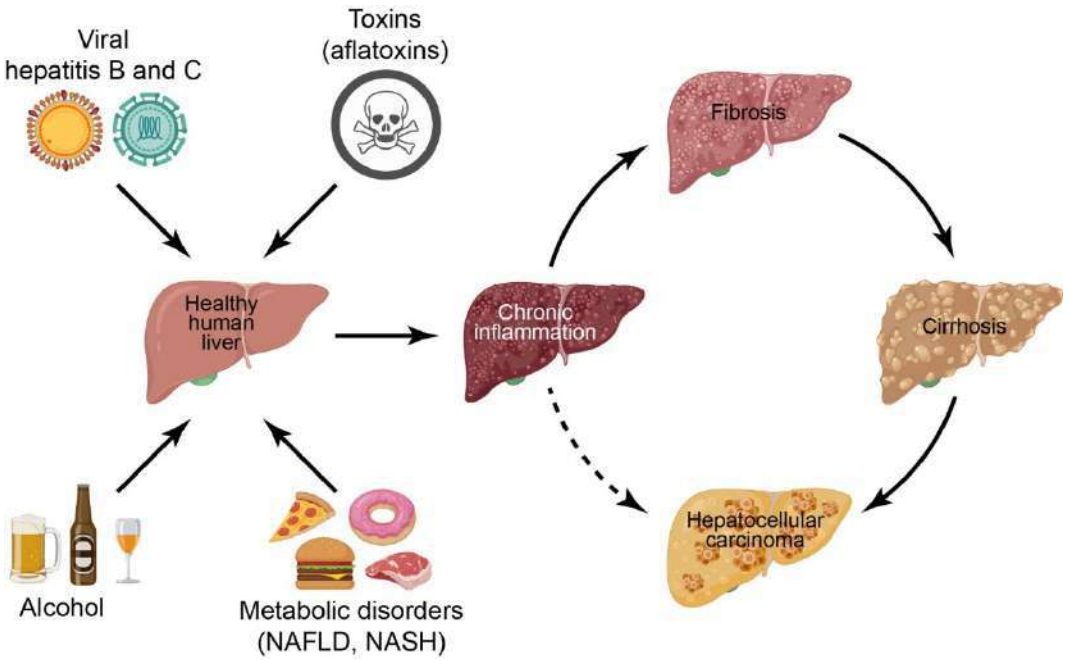


Figure 3.62 — Risk factors for the development of HCC in humans. NAFLD — non-alcoholic fatty liver disease; NASH — nonalcoholic steatohepatitis (Jilkova et al., 2019).

HCC is a tumour with a very high metabolic rate, consuming much more oxygen than the surrounding normal tissue (Guo et al., 2020). Therefore, despite strong vascularisation, the rate of O_2 diffusion in the tumour is often insufficient, resulting in a hypoxic cell microenvironment. The average oxygenation level in HCC has been shown to be ~0.8% (McKeown, 2014; Chen, Lou, 2017), whereas in surrounding normal tissue it ranges from 4.0–7.3% (Leary et al., 2002; Brooks et al., 2004). Hypoxia plays a key role in tumour neoangiogenesis, progression, invasion and metastasis (Hockel, Vaupel, 2001; Liu et al., 2017; Lin et al., 2018; Al Tameemi et al., 2019; Guo et al., 2020; Zhang et al., 2020; Emami Nejad et al., 2021; Cramer, Vaupel, 2022).

It is natural to assume that hepatocarcinogenesis developing under hypoxic conditions will lead to a profound rearrangement of carbohydrate metabolism in HCC and liver cells surrounding the tumour, which will be accompanied by marked changes in glycogen content and activity of enzymes of its metabolism. However, the data on glycogen content in tumour cells are contradictory. On the one hand, it has been shown that the glycogen content in malignant liver cells, for which glycogen accumulation is normally one of the main differentiated functions, is low or even absent (Su, Bannasch, 2003; Takegoshi et al., 2016; Berndt et al., 2021). It is believed that the decrease in glycogen stores in this case is associated with the transformation of preneoplastic hepatocytes into tumour cells. Malignant

transformation of hepatocytes during long-term hepatocarcinogenesis leads to a fundamental change in carbohydrate metabolism, the consequence of which is a gradual decrease of the initially high glycogen content in cells (Bannasch et al., 1984; Bannasch, 2010; Harjes, 2022). It has been shown that in mice with HCC induced by nitrosamine there is an inverse correlation between tumour size and glycogen content, i.e. smaller tumours have significantly greater glycogen stores, while larger tumours contain less glycogen. Similarly, in human liver sections, glycogen staining was often observed in small nodules but rarely detected in larger tumours. Despite the high variability between individuals, in most cases glycogen content was increased at stage I (early stage) but dramatically decreased at the later stages of human HCC development (stage II, stage III and stage IV) compared to neighbouring normal tissues. In addition, the location of glycogen-containing cell foci was found to correlate with groups of alpha-fetoprotein-positive cells in these liver sections (Liu et al., 2021). These data suggest that glycogen, as an energy reserve of glucose, is consumed for proliferation and growth of malignant cells during HCC progression.

High glycogen levels are thought to be a key event for the initiation of oncogenetic processes during malignant transformation of cells in the liver (Cui et al., 2021; Liu et al., 2021; Davidson et al., 2022). Glycogen accumulation in cancer cells favours their survival in a hypoxic environment with scarce nutrient supply and enables them to maintain high proliferative activity and enhance invasion and migration (Favaro et al., 2012; Zois, Harris, 2016; Althemus et al., 2019; Curtis et al., 2019). Inhibition of glycogen metabolism has been shown to cause cancer cell death, while increased glycogen accumulation accelerates oncogenesis (Dukhande et al., 2018; Barot et al., 2019; Liu et al., 2021).

Thus, the glycogen content in liver cells can serve as a marker of early diagnosis and prognosis of HCC. Therefore, the development of therapeutic measures for reducing glycogen levels in tumour cells may represent a basic strategic for HCC therapy. At the same time, the use of data on glycogen content in tumour liver to predict the course and outcome of HCC requires a more detailed development of the method of glycogen quantification in tumour cells and a thorough study of its metabolism.

At present, almost all studies of glycogen content in HCC are based on visual assessment of the intensity of staining of tissue or cell sections using PAS reaction. At the same time, in many works where PAS reaction was used, the time of oxidation of preparations in iodic acid was very short (5–10 min), and the duration of staining in Schiff's reagent was

only 10–15 min (Jiang et al., 2010; Favaro et al., 2012; Chen et al., 2019; Curtis et al., 2019; Chen et al., 2020; Tang et al., 2021; Liu et al., 2021). At the same time, it has long been known that for complete detection of glycogen in cells, oxidation of preparations in iodic acid should be longer, and the duration of their staining in Schiff's reagent should be ~90 min (Kudryavtseva et al., 1972; Gahrton et al., 1975). This is due to the fact that rapid PAS staining of preparations, used by many authors, determines only a small part of glycogen in cells (see Section 4.3) and does not allow one to study its structure in the tissue.

One of the key factors contributing to increased glycolysis in tumour and non-tumour liver cells is a low activity of G6Pase. Decreased activity of this enzyme attenuates endogenous glucose production and leads to accumulation of G6P in cells (Sun et al., 2009). Excessive G6P enhances glycolysis, increases the synthesis of fatty acids, uric acid and lactate, and, due to the accumulation of glycogen and lipids, causes hepatomegaly and eventually leads to tumour development (Gjorgjieva et al., 2016; Cho et al., 2018). This is particularly clear in glycogenosis type I (von Gierke disease). In this inherited disease associated with G6Pase deficiency, hepatocytes accumulate extremely large amounts of glycogen (Beaty et al., 2002).

G6Pase activity in human HCC has been shown to be ~47% of the control level (Taketa et al., 1976). However, an even lower G6Pase activity as compared to the norm was found in HCC of woodchucks. Using a natural model of HCC that develops during ontogenesis in the North American woodchucks *Marmota monax*, it was found that G6Pase activity in the tumour itself makes up 17 % and in the normal liver tissue surrounding HCC, 59.4% of the level of this enzyme’s activity in the liver of healthy animals (Kuang et al., 2006).

Carbon tetrachloride (CCl_4), which was used in our work to induce LC in rats, is not believed to be a genotoxic agent (Lee et al., 2013). Therefore, to induce HCC in animals CCl_4 is usually used in combination with some genotoxic agent, most commonly, with diethylnitrosamine (DEN) (Santos et al., 2017; Jilkova et al., 2019). However, although CCl_4 is not genotoxic, free radicals CCl_3^* and Cl^* formed by its microsomal oxidation (Britton and Bacon, 1994) lead to the destruction of cellular structures, which is accompanied by inflammation, fibrosis and increased cell proliferation in the liver. With prolonged exposure of the liver to CCl_4 all these processes significantly increase the risk of genetic errors and may lead to the formation of HCC. It was shown that intragastric administration of CCl_4 to rats for 30 weeks caused HCC formation in 30–40% of animals (Frezza et al., 1994). Fujii and co-authors observed numerous well-formed HCCs on the liver surface after 15–17 weeks of

exposure of mice to CCl_4 (Fujii et al., 2008). Repeated injections of CCl_4 to mice for two years resulted in HCC formation in 50% of mice (Zhao et al., 2015), whereas 100% tumour development was observed in mice after simultaneous administration of DEN and CCl_4 (Uehara et al., 2014).

The data obtained in our work suggest that, though CCl_4 is not genotoxic, a prolonged chronic exposure to this agent causes changes in liver structure and metabolism very similar to those observed during the development of HCC:

1) Nodular proliferation and atypic hepatocytes.

2) Increased LP and ROS production.

3) Depletion of antioxidant systems.

4) Much lower concentration of inner membranes of mitochondria than normal.

5) Intense glycolysis.

6) Extremely low glucokinase activity.

7) Low glucose-6-phosphatase activity.

8) Low glycogen phosphorylase activity.

9) High levels of glycogen in hepatocytes.

Thus, the results of the present work confirm the conclusion of many authors that LC is a pre-carcinogenic state and that cirrhosis is the main prerequisite for the formation of hepatocellular carcinoma (HCC). The identified changes in the structure and metabolism of hepatocytes during LC are a necessary condition for the start of HCC development and progression. Only changes in the genetic apparatus of cells are then needed to trigger these processes.

CONCLUSION

Annual deaths from liver diseases account for about 4% of all deaths worldwide (Devarbhavi et al., 2023). Liver cirrhosis (LC), along with hepatocellular carcinoma (HCC), are the final irreversible stage of chronic progressive liver diseases. Alcohol and hepatotropic viruses are not only the main but also the oldest aetiological factors of LC. Winemaking and immoderate alcohol consumption appeared as far back as the Neolithic era, 8–10 thousand years ago (Leibowitz, 1967; Nikolova et al., 2018; Hirst, 2019). Hepatotropic B virus was found in bone fragments and human mummies of an equally old age (Simmonds, 2001; Souza et al., 2014; Locarnini et al., 2021; Trovao, 2022). Recently, drug-induced liver damage (Leise et al., 2014; Wang et al., 2021) and non-alcoholic fatty liver disease, which affects a quarter of the world's adult population (Farrell et al., 2006; Zhai et al., 2021b; Noureddin, Harison, 2023), have started to play an increasingly more significant role in the formation of LC. Although the liver is not a major target organ for COVID-19 virus, population analysis has shown that LC mortality increased during the pandemic. It has been suggested, however, that this increase is due to an increased alcohol consumption (Ye et al., 2022; Huang et al., 2023).

LC does not only cause great suffering to patients themselves but also entails enormous economic expenses for their treatment and care. The prognosis of cirrhotic patients depends on a number of factors including aetiology, severity, type, complications and comorbidities. The average life expectancy of patients with compensated LC is 9–12 years, whereas during decompensated LC it does not exceed 3 years (Zipprich et al., 2012). Most cirrhotic patients have an age between 40 and 60 years (Sajja et al., 2014), but this disease has been “getting younger” in the last decades, affecting the most active, useful and productive part of the population in various countries.

Cirrhosis is very difficult to treat. There are currently only two treatments, and each has its own advantages and disadvantages. One of them is the replacement of the cirrhotic liver with a donor liver. Transplantation of liver or a part of it is the second most common organ replacement surgery. The average survival of patients 1 year and 5 years after surgery is 82–85% and 68–70%, respectively; in some patients it reaches 20 years or more (Roberts et al., 2004; Onghena et al., 2016; Shavelle et al., 2021). Despite tremendous advances in transplantation, its current tempo meets less than 10% of the world's demands. This situation is mainly due to the shortage of suitable donors and financial constraints. Another problem is the

long waiting time, which often leads to disease progression, malignancy and other life-threatening processes. The second treatment of LC is via identification of the damaged links of the structure and metabolism of the cirrhotic liver, elucidation of mechanisms of these damages and development of methods of eliminating the defects that have arisen during the pathological process. This way is not as radical and fast as liver transplantation but appears to be a more fundamental and large-scale one.

The mammalian liver fulfils a huge amount of vital functions. The maintenance of a stable high level of functional activity of the pathologically altered liver requires a large amount of energy, which poses a serious problem under conditions of hypoxia and deep structural rearrangements. Energy metabolism in hepatocytes during LC is characterised by insulin resistance and preferential oxidation of lipids instead of glucose and proteins, characteristic of prolonged starvation. As a result, the rearrangement of oxidative metabolism and hypoxia in hepatocytes leads to an almost twofold decrease in the functional activity of mitochondria, where most ATP is formed. This fact is consistent with the inverse correlation between the severity of liver damage and the level of energy expenditure in cirrhotic patients (Schneeweiss et al., 1990). On the other hand, glycogen stores, an important source of energy for hepatocytes, increase with increasing severity of the disease, and may become several times higher than in the norm.

Glycogen synthesis in hepatocytes of the cirrhotic liver is carried out mainly via the indirect pathway, by gluconeogenesis. Its initiation and further accumulation in cells occur against the background of a stably low activity of glycogen phosphorylase *a* (GP*a*). Glycogen begins to be synthesised at the periphery of hepatocytes, and in the process of glycogenesis its newly synthesised molecules move to the centre of cells, being replaced by new ones. Both in the norm and during LC, the glycogen content in hepatocytes correlates with the level of cell ploidy throughout glycogenesis. At the same time, if in the normal liver the glycogen content in hepatocytes is related to the cell size (mass), no such dependence is observed during LC. The development of LC is accompanied by a 7–9-fold increase of the number of glycogen molecules in hepatocytes and a 2–3-fold decrease of glucose residues in them. The number of glycogen molecules in hepatocytes increases as the severity of the disease increases, and the packing of glucose residues becomes more dense.

Almost all numerous functions of the liver are performed by hepatocytes, so their number and metabolic activity are extremely important for the normal functioning of the

organ. In the course of LC development, the damaging agent causes continuous hepatocyte death and their *de novo* formation. Although the rates of glycolysis and pentose phosphate pathway, the intermediate products of which are necessary for a high proliferative activity of hepatocytes, are significantly increased during LC, this is not sufficient to reduce cell death. The total number of new cells formed during LC development exceeds their initial number in rats ~4–5 times, and in humans, ~1.5 times. Nevertheless, despite the high turnover, hepatocyte death during LC development by far exceeds their replenishment. In the cirrhotic human liver, the replacement of lost cells occurs by proliferation of small diploid hepatocytes, whereas in rats, in addition to hepatocyte proliferation, polyploidization and hypertrophy make a noticeable contribution to the reparative growth of the liver. In contrast to the human liver, where hepatocyte loss during LC development means a significant loss of parenchyma, the mass of liver parenchyma in rats practically does not change due to an increased ploidy and hypertrophy of the cirrhotic liver cells.

The data obtained in our study allow us to conclude that the weakening of the glucostatic function of the liver is one of the key events in the formation of cirrhosis. Increased gluconeogenesis and glycolysis in the cirrhotic liver are accompanied by a decrease in glucose production, significant glycogenosis of hepatocytes and considerable changes in the structure of glycogen molecules.

SUMMARY OF RESULTS

1. During glycogenesis, hepatocyte dry mass and glycogen content in them change in proportion to gene dosage both in the normal and in the cirrhotic liver.
2. In the normal liver of humans and rats, glycogen content in hepatocytes is commensurate with cell mass, whereas in the cirrhotic liver there is no such relationship.
3. An original microfluorometric method for estimating the structure of glycogen β-particles (molecules) in individual hepatocytes was developed.
4. In hepatocytes of the normal rat liver, glycogen accumulation at early stages of glycogenesis is associated with the synthesis of new β-particles, while at later stages it is associated with the addition of glucose residues to the existing particles. In the cirrhotic liver, the change in glycogen content in hepatocytes during glycogenesis is due to the filling of the outer tiers of β-particles.
5. The increase in glycogen content in human hepatocytes during the development of liver cirrhosis is mainly due to *de novo* formation of β-particles.
6. In rat hepatocytes, as cirrhosis progresses, the distance between the chains of glucose residues in β-particles increases, while in human hepatocytes, the distance between chains of glucose residues in β-particles decreases.
7. The distance between the outer tiers of β-particles decreases as they are filled with glucose residues, which supports the hypothesis that particle size is self-regulated.
8. In the postabsorptive period of the food cycle, glycogen content in hepatocytes of patients with diabetes mellitus type 2 is increased as compared to the norm. Comorbidity of diabetes mellitus with liver cirrhosis does not increase glycogenosis.
9. In contrast to rats, in which the polyploidization and hypertrophy of hepatocytes play a significant role in the formation of cirrhosis, reparative regeneration of the human liver during the development of cirrhosis occurs exclusively at the expense of mitotic divisions of small diploid hepatocytes.
10. The leading factor of rehabilitation of glucostatic function of the cirrhotic liver is the removal of the pathogenic influence. Treatment of cirrhosis does not restore the original architectonics and lobular structure of the liver.

ABBREVIATIONS

AB — acceptor bleaching

Ac — acceptor

ADP — adenosine diphosphate

AlAT — alanine aminotransferase

AMP — adenosine monophosphate

AP — alkaline phosphatase

AsAT — aspartate aminotransferase

ATP — adenosine triphosphate

BE — branching enzyme

BM — bemithyl (2-ethylthiobenzimidazole hydrobromide)

cAMP — cyclic adenosine monophosphate

CD — high-carbohydrate diet

CH — chronic hepatitis

CVH — chronic viral hepatitis

CZ — central zone of the liver lobule

DBE — debranching enzyme

DC — diene conjugates

DEN — diethylnitrosamine

DM — diabetes mellitus

DM — dry mass

DNA — deoxyribonucleic acid

Don — donor

EM — electron microscopy

F1,6BPase — fructose-1,6-biphosphatase

FRET — Förster resonance energy transfer

G1P — glucose-1-phosphate

G6P — glucose-6-phosphate

G6Pase — glucose-6-phosphatase

GDP — guanine diphosphate

GK — glucokinase

GKRP — glucokinase regulatory protein

Gn — glycogenin

GP — glycogen phosphorylase

GP*a* — glycogen phosphorylase, active form

GS — glycogen synthase

GSD — glycogen storage diseases

GTP — guanine triphosphate

HCC — hepatocellular carcinoma

HCG — human chorionic gonadotropin

LC — liver cirrhosis

LF — labile glycogen fraction

LP — lipid peroxidation

MDA — malonic dialdehyde

MG — macroglycogen

MIMC — mitochondrial inner membranes concentration

mRNA — matrix ribonucleic acid

MVD — mitochondrial volume density

N — norm

NAFLD — non-alcoholic fatty liver disease

NMR — nuclear magnetic resonance

PAS — Periodic Acid-Schiff

PFK — phosphofructokinase

PG — proglycogen

PH — partial hepatectomy

Pi — inorganic phosphorus

RER — rough endoplasmic reticulum

ROS — reactive oxygen species

SF — stable glycogen fraction

SOD — superoxide dismutase

TB — total bilirubin

TCA — trichloroacetic acid

TG — total glycogen

UDP — uridine diphosphate

UDP-glucose — uridine diphosphate glucose

UTP — uridine triphosphate

VLDL — very low density lipoproteins

WT — wild type

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