ISOLATION OF PERIPORTAL AND CENTRILOBULAR HEPATOCYTES BY ISOPYCNIC CENTRIFUGATION

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Modern views on the heterogeneity of hepatocytes are based on the results of morphological and histochemical analyses of the liver parenchyma [9]. Further progress in the study of this phenomenon will probably be linked with the use of biochemical methods. For this purpose microanalysis of zones of the acinus isolated from lyophilized liver tissue has been used [6, 7]. However, this method is laborious and does not enable separation of parenchymatous from nonparenchymatous cells, and this makes interpretation of the results difficult. One promising approach is by fractionating isolated hepatocytes into centrolobular and periportal cells. Hepatocytes can be fractionated by high-speed sedimentation, affinity chromatography, and isopycnic centrifugation. Only in the last case, however, have the greatest differences been obtained between the morphological and biochemical characteristics of the cells [5, 8]. Meanwhile the problem of which zones of the acinus correspond to "light" and "heavy" hepatocytes still remains unsolved [4, 5, 12].

In connection with the facts described above an attempt was made to isolate subpopulations of hepatocytes rich in centrilobular and periportal hepatocytes, respectively.

EXPERIMENTAL METHOD

Female Wistar rats weighing 200-250 g were used. Isolated cells were obtained by the method in [3]. The liver was perfused initially in situ for 5-10 min with Ca^{++}-free Hanke's solution. Recirculating perfusion in vitro was then carried out through the hepatic vein for 25 min with Krebs–Ringer solution (pH 7.4, 37°C) containing 0.03% collagenase (type I, from Sigma, USA). The subsequent procedures were carried out at 4°C. The tissue was dissociated with a spatula. Hepatocytes and nonparenchymatous liver cells were isolated from the resulting suspension by differential centrifugation [13]. The residue of hepatocytes was suspended in a 10% solution of Ficoll-400 (Pharmacia, Sweden) to a concentration of $13 \cdot 10^6$ cells/ml and 8 ml of the suspension was layered above a stepwise density gradient. The gradient was made up in a test tube (29 × 103 mm), using Ficoll solutions with the following densities: 1.126, 1.073, 1.054, and 1.044 g/ml. Centrifugation was carried out in the J-13 bucket rotor of the J2-21 centrifuge (Beckman, USA) for 40 min at 11,000 rpm [2]. The fractions thus obtained were removed by means of a syringe. The cells were counted in a Goryaev's chamber. Glucokinase and hexokinase activity was determined in 0.1 M Tris-HCl buffer, pH 7.4, using 100 and 0.5 mM glucose, respectively [14]. Phosphoenolpyruvate carboxykinase (PEPCK) activity was determined by the method in [1]. All enzyme were determined in the supernatant (50,000g) at 30°C. The protein concentration was measured by Lowry's method [11].

EXPERIMENTAL RESULTS

The yield of cells from 1 g tissue was $(40 \pm 3) \cdot 10^6$ hepatocytes and $(19 \pm 3) \cdot 10^6$ nonparenchymatous cells. During estimation of the viability of the cells by the trypan blue test [3] 88% of the hepatocytes and 95% of the nonparenchymatous cells eliminated trypan blue. Contamination of the cell preparations of one type by the other did not exceed 1%. During isopycnic centrifugation the original hepatocyte suspension was divided into four fractions (F1-F4) and the cell residue at the bottom of the tube (FS) (Fig. 1). The yield of cells from the

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TABLE 1. Protein Content and Viability of Cells in Hepatocyte Fractions and in Initial Suspension (M ± m, n = 10)

Fraction	Density, g/ml	Protein		Viability, %
		homogenate	supernatant	
		mg/10^6 cells		
F-1	<1,044	1,761±0,208	0,671±0,081	88±2
F-2	1,044—1,054	1,627±0,247	0,676±0,103	90±4
F-3	1,054—1,073	1,526±0,145	0,581±0,042	87±2
F-4	1,073—1,126	1,682±0,240	0,661±0,087	84±2
F5 (bottom)	>1,126	—	—	43±6
Original suspension		1,641±0,146	0,626±0,041	88±2

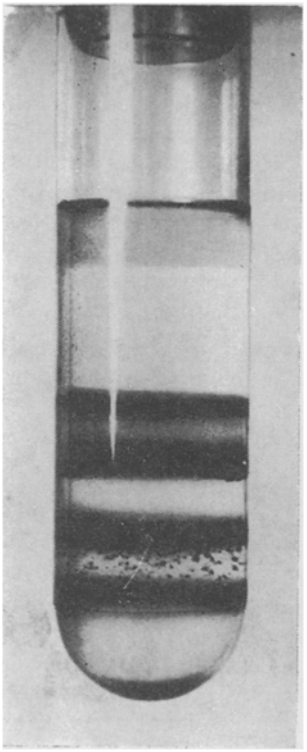

Fig. 1. Fractions of hepatocytes obtained by isopycnic centrifugation of hepatocyte suspension in Ficoll density gradient.

gradient was 74 ± 3%. Of that number 16% was accounted for by F1, 20% by F2, 24% by F3, 24% by F4, and 16% by F5. Some cells were probably damaged during centrifugation and were lost when the fractions were eluted from the Ficoll. The viability of the cells in fractions (F1-F4) was quite high and was the same as that of the original hepatocyte suspension (Table 1). No significant differences ($P > 0.05$) likewise were found between the cells with respect to their total and soluble protein content. Since F5 contained the largest number of stained cells (Table 1) no biochemical analysis was undertaken of this fraction.

The data obtained agrees with results of [15], the authors of which showed that in a gradient of Ficoll, 70%, of the hepatocytes have a sensity of 1.045 to 1.090 g/ml. However, during fractionation in metrizamide hepatocytes divided into 2 fractions with densities of 1.099 and 1.112 g/ml [12] or 1.10 and 1.12 g/ml [8]. Apparently on the surroundings used in preparation of the density gradient. It is notable that the difference between cell by gradient is expressed to a lesser degree in the metrizamide gradient [8, 12] than in the Ficoll gradient.

To determine which zones of the acinus correspond to "light" and "heavy" hepatocytes the distribution of marker enzymes between the fractions must be known. Activity of alcohol dehydrogenase, 5-nucleotidase, and glucose-6-phosphatase [4, 8] and the content of cytochrome P-450 [8] in the cells were determined for this purpose. In the present investigations, we used the key enzymes of glycolysis (glucokinase, hexokinase) and of glyconeogenesis (PEPCK) for the first time as markers of centrilobular and periportal hepatocytes. The specific PEPCK activity in light hepatocytes (F1) was found to be twice as high as in heavy (F3, Fig. 2).

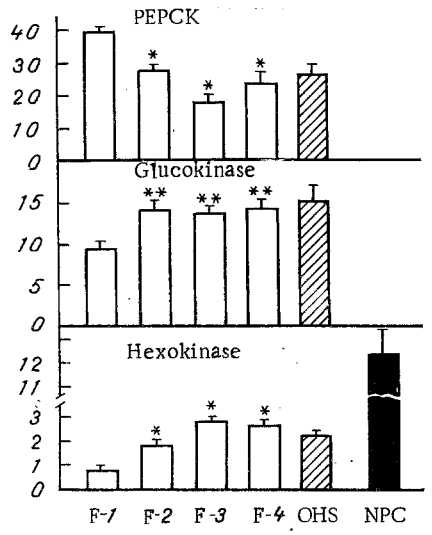

Fig. 2. PEPCK, glucokinase, and hexokinase activity in isolated hepatocyte subpopulations (F1-F4), original hepatocyte suspension (OHS), and nonparenchymatous liver cells (NPC). Abscissa, hepatocyte fractions, OHS, and NPC; ordinate, activity, nmoles/min/mg protein. Significance of differences shown relative to F1: *P < 0.001, **P < 0.01.

Conversely, hexokinase activity was three times higher in F3 than in F1. Differences between F3 and F4 were not significant (P > 0.05). Glucokinase activity, like hexokinase, was greater in the heavy cells, but the differences between the fractions were less marked (Fig. 2).

It has recently been shown by microbiochemical analysis of zones of the acinus isolated from lyophilized liver tissue that glucokinase [6] is located mainly in the centrilobular zone, and PEPCK [7] and hexokinase [6] mainly in the periportal zone. We showed in the case of isolated cells that glucokinase and PEPCK are present only in hepatocytes, whereas hexokinase is present in both hepatocytes and nonparenchymatous cells. Hexokinase activity in the nonparenchymatous cells was six times higher than in hepatocytes (Fig. 2). The number of nonparenchymatous cells was significantly greater in the periportal zone of the acinus [10]. Consequently, it can be postulated that the character of distribution of hexokinase determined by microanalysis of the tissue reflects the localization of nonparenchymatous cells, and not the distribution of the enzyme among the hepatocytes.

During isopycnic fractionation of a hepatocyte suspension in a Ficoll density gradient the periportal cells are thus found in the light fractions and centrilobular cells in the heavy fractions of the gradient. A similar conclusion was reached previously by Castagna and Chauveau [4]. Conversely, most workers in recent years have considered that the centrilobular hepatocytes are "lighter" cells [5, 8]. Data are now available to explain these contradictions.

The results are evidence of an uneven distribution of the key enzymes of carbohydrate metabolism among hepatocytes in accordance with the "metabolic zones" hypothesis [9]. Glycolysis probably predominates in the heavy (centrilobular) hepatocytes whereas glyconeogenesis predominates in the light (periportal) hepatocytes.

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SYMPATHETIC INFLUENCES ON SKELETAL MUSCLE STUDIED BY RECORDING MITOGENETIC RADIATION

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Sympathetic stimulation is known to restore the working capacity of the fatigued neuromuscular apparatus (the Orbeli–Ginetsinskii effect). The sympathetic nervous system exerts its influence through several points of application, with the aid of mechanisms located in pre- and postsynaptic regions [6]. Each stage of neuromuscular transmission is coupled with conformational and energetic reorganizations of pre- and postsynaptic structures [1].

We know from the character of mitogenetic radiation that changes in the functional state of the neuromuscular synapse also are connected with reorganizations of nonequilibrium molecular assemblies (constellations) of the sarcoplasm [3].

The aim of this investigation was to study radiation of the frog gastrocnemius muscle when achieved by sciatic nerve stimulation and restoration of ability to contract by simultaneous stimulation of the sympathetic chain [5].

EXPERIMENTAL METHOD

Experiments were carried out on male frogs (*Rana temporaria* L.) in the fall and winter. The frog nerve-muscle preparation was fatigued by the method described previously [5]. To reproduce the Otbeli–Ginetsinskii effect in the frogs the lateral sympathetic trunk was dissected and the seventh and eighth ganglia of the sympathetic chain were laid on electrodes. Electrical stimuli were applied to the sympathetic nerve with a frequency of 15-20 Hz, duration 20-50 msec, and amplitude 0.7-1 V. The gastrocnemius muscle was brought into a state of incomplete fatigue by direct stimulation, for the sympathetic effect is manifested more clearly against this background [4].

A yeast culture, the technique of working with which was described previously [2], was used as a radiation detector. The intensity of ultraviolet chemiluminescence was judged by the increase in the number of young buds in the experimental specimen compared with the control ($P = 0.99$). Mitogenetic radiation was recorded in the early phases of development of the sympathetic effect, with exposures of 3, 5, and 8 sec: during the first seconds of sympathetic stimulation, in the maximal phase of development of the sympathetic effect, and after discontinuation of sympathetic stimulation.

EXPERIMENTAL RESULTS

The results of experiments to stimulate the sympathetic nervous system against the background of prolonged motor stimulation are given in Table 1. During the first 3 sec of sympathetic stimulation the number of budding yeast cells differed significantly from the control. With an increase in exposure (5 and 8 sec) no significant differences were observed compared with the control. The intensity of radiation fell after discontinuation of sympathetic stimulation.

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