ISOLATION AND SUBFRACTIONATION ON FICOLL GRADIENTS OF ADULT RAT HEPATO CYTES

Size, Morphology, and Biochemical Characteristics of Cell Fractions

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ABSTRACT

The recirculating perfusion of adult rat liver with a Ca++-free Hanks' solution produces a release of the adhesiveness of cells and a cleaving of the desmosomes. The addition of collagenase and hyaluronidase to the perfusion medium leads to complete dissociation of the liver tissue into a mixture of isolated cells and cell cords in which the hepatocytes remain connected with specific junctional differentiations, namely the gap and tight junctions. Individual cells are released by submitting the suspension of cell trabeculae to a gentle rolling. The gap junctions are ruptured at least in one of the two adjacent cells and remain generally attached to the other cell taking with them a small portion of cytoplasm. This technique of isolation of hepatocytes yields about 60–65% of the parenchymal cells contained in a liver; endothelial cells and other cells of the connective tissue are not recovered. The ultrastructural preservation of the isolated hepatocytes is excellent and the glucose-6-phosphatase activity, confined to the endoplasmic reticulum, appears unaltered in most cells. Protein, DNA and RNA recovery in the preparations of isolated hepatocytes is satisfactory, amounting to 70% of that found in liver homogenate; glycogen, the most labile component examined, is partly lost or degraded during the manipulations. Cell diameters measured by different methods confirm the preservation of the original volume of the in situ hepatocytes and the presence of more than one type of parenchymal cell. By submitting this heterogeneous cell population to an isopycnic density gradient centrifugation, two types of hepatocytes can be distinguished: the light hepatocytes, with a mean diameter of 20.5 μm and a mean density of 1.10, are characterized by an extended smooth-walled endoplasmic reticulum entrapping dispersed α-glycogen particles; the heavy hepatocytes, with a mean diameter of 19.0 μm and a mean density of 1.14, present a relatively reduced compartment of smooth endoplasmic reticulum, but large accumulations of glycogen. It is suggested that the cell fraction of low density is enriched in centrolobular cells and the high density fraction in perilobular hepatocytes.
Much effort has been devoted to the preparation of isolated hepatocytes from liver tissue. One of the major technical advances was achieved by Howard et al. (12) who proposed the use of a combined enzyme and mechanical treatment. Berry and Friend (3) improved the yield of isolated cells by introducing the continuous recirculating perfusion of the rat liver in situ. The perfusion media consisted of a calcium-free Hanks' solution containing the two enzymes, collagenase and hyaluronidase, recommended by Howard et al. More recently, these authors (13) have made a detailed report of new modifications proposed to improve the isolation of intact cells. A variant of these enzymatic tissue-dispersion methods was achieved by Seglen (22) using a two-step procedure which consists of perfusing the rat liver with a Ca"-free buffer followed by enzymatic treatment. Some aspects of the mechanism of tissue dissociation were described by Berry and Friend (3), in particular the different behavior of desmosomes which are cleaved, and tight and gap junctions which resist the enzyme action and are retained intact on the isolated cells.

In the present paper we describe the method of isolating hepatocytes from livers of adult rats. The continuous enzyme recirculating perfusion produces liver cell cords which are further dissociated by means of a mild mechanical action consisting of rolling the cell aggregates in a siliconized flask. The degree of preservation of the isolated hepatocytes was evaluated by biochemical determinations and ultrastructural and cytochemical studies. The suspension of isolated cells is subfractionated by isopycnic gradient ultracentrifugation into two cell fractions which differ in their density, size, morphology, and glycogen content.

MATERIAL AND METHODS

Preparation of Isolated Rat Hepatocytes

The technique of preparation of isolated rat hepatocytes consists of three successive manipulations which necessitate much practice and care: (a) the cannulation of the liver vessels, portal vein, and inferior vena cava; (b) the continuous recirculating perfusion in the presence of the enzymes collagenase and hyaluronidase; (c) the mechanical dissociation of the hepatic cell cords into isolated cells. The first manipulation was carried out as recommended by Hems et al. (10). Adult Sprague-Dawley rats, weighing 200 g ± 25 g were anesthetized by intraperitoneal injection of Nembutal (Abbott Laboratories, North Chicago, Ill.), 0.1 ml per 100 g body weight. The abdomen was opened, heparin (Liquemine, S. A. Roche, Belgium) was injected into the inferior vena cava, 100 IU per 100 g body weight, and a ligature was prepared on this vessel, above the renal veins, which was tightened before starting the perfusion. The portal vein was cannulated with a sterile angiocath (Longdwell catheter, no. 16-gauge 2.5 inch), and a second cannula for the outlet was introduced into the vena cava through the atrium after opening the chest and sectioning the phrenic nerve. Only a negligible quantity of blood was lost during perfusion when carefully performed. This cannulated liver, remaining in the body, was then connected to the perfusion apparatus described by Hems et al. (10). The apparatus is composed of a multibulb glass oxygenator equipped with a thermometer, a pH-meter, and a mixing reservoir, housed in a Plexiglas cabinet and maintained at constant temperature, 37°C. The perfusion medium, a calcium-free Hanks' solution, constantly oxygenated with carbogen (O₂ and CO₂, in the proportion of 95:5) was pumped from the mixing reservoir by means of a type MHRE 200 Roller pump (Watson-Marlow Ltd., Falmouth, England) connected to the portal vein cannula. From the inferior vena cava the perfusate was returned to the mixing reservoir. At the start of the perfusion, the first 50 ml of perfusate containing the blood present in the liver were discarded. The perfusion was continued with 300 ml of perfusate to which 0.05% collagenase and 0.1% hyaluronidase were added as recommended by Berry and Friend (3). After 5 min, the perfusion liquid started to ooze freely from the surface of the liver and was collected in the peritoneal cavity and discarded. The perfusion was maintained for about 30 min, until the perfusate was about exhausted. The following parameters were rigorously controlled during perfusion: (a) the temperature of the cabinet and of the circulating medium was maintained at 37°C; (b) the pH of the perfusate was equilibrated at pH 7.4 after a preliminary 30-min carbogen bubbling and maintained within acceptable limits (pH 7.4 to 7.1) during perfusion; (c) the P_O₂ and P_CO₂ of the influent and effluent media were measured by means of a blood gas analyzer (Instrumentation Laboratory, Inc., Lexington, Mass.). These tests allowed us to control rat liver oxygen consumption by the perfused liver: the P_O₂ remained constant at 500 mm Hg for inflowing samples but fell to 275 mm Hg in samples taken at the liver outflow (mean values for eight measurements). In parallel, the P_CO₂ rose from 27 mm Hg to 31.5 mm Hg; (d) the flow rate was 20 ml/min at the beginning of the perfusion and increased to 30 ml/min by adjusting the oxygenator-animals level; (e) the osmotic pressure of the medium measured in each experiment was about 300 mosmol. At the end of the 30-min perfusion, the consistency of the liver was drastically decreased, and as a result of the digestion of the connective tissue, the parenchymatous part of the liver was transformed into a kind of pasty substance contained by the Grisson's capsule.

The main vessels having been sectioned, the liver was carefully removed with a spoon, and by opening the
capsule, the tissue was gently dissociated with a spatula in 50 ml calcium-free Hanks' solution to which 2 mM EDTA, and 2% crystalline bovine serum albumin (Pentex Biochemical, Kankakee, III.) were added. Addition of EDTA served to inhibit collagenase, and by adding albumin the cell surfaces were protected against further enzymatic digestion. Examined in the phase-contrast microscope, the cell suspension still contained many cell cords in which cells remained attached to one another by their surface differentiations adjacent to the bile canaliculi.

Further dissociation by mechanical means appeared to be essential to obtaining unicellular suspensions. Excessive dissociation results in an important loss of viable cells, while a too mild action fails to dissociate the remaining cell cords. A comparative study of different methods, i.e. drawing the suspension in and out of a Pasteur pipette, use of a loose Potter homogenizer, passage through capillaries of decreasing diameters, and finally rolling the cell suspension in a rotary evaporator, directed our choice to the last solution. The cell suspension was, therefore, transferred to a rotary evaporator (Quickfit 10 RE, Quickfit and Quartz Ltd., Stone, Staffordshire, England) equipped with a siliconized 50-ml glass round-bottom flask and with a thermostatic bath maintaining the temperature at 37°C. During the 3-min rolling, carboxygen was continuously flushed into the bulb and the pH remained between 7.0 and 7.2. Samples examined in the phase-contrast microscope allowed us to control the quality of the dissociation. Finally, the cell suspension was filtered through layers of Perlon (polyamide PA 6) of decreasing meshes of 100 μm, 68 μm, and 28 μm. Debris retained on the filters, which accounts for 10% of the total material poured on the nylon, was discarded.

Criteria of Cell Preservation

The vital trypan blue staining, currently used by authors who separate hepatocytes, was only occasionally performed on our preparations. For reasons which will be discussed later, we preferred to judge the quality of the preservation and to evaluate the proportion of damaged cells on semithin sections of cell pellets examined in the phase-contrast microscope and on ultrathin sections for electron microscopy. The pellets were prepared by centrifuging an aliquot of the total cell suspension, as it is obtained after the last filtration on the 28-μm meshes of Perlon, in a microfuge (see next section) or on a millipore filter. The even pellet resting on the filter is easy to manipulate for further fixation and embedding and consists of a layer 10-20 cells thick. The semithin sections cut perpendicular to the surface of the filter were stained with alcian toluidine blue. The ultrathin sections of areas chosen at various depths were placed on a single-hole grid or on other types of grids convenient for observations at low magnification. The Philips EM 200 and 301 were used, following the recommendations of Wisse et al. (26) and our own experience (6).

Isopycnic Centrifugation on Ficoll

Density Gradients

The suspension of hepatocytes which passed through a Perlon mesh of 28-μm pore size was layered on a Ficoll density gradient and centrifuged at 25,000 rpm for 3 h (Spinco, model L2 65B centrifuge, rotor SW27, Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). The linear Ficoll gradient with high concentrations of 15% and 40% was obtained by dissolving the polysaccharide (Ficoll, Pharmacia Fine Chemicals A.B., Uppsala, Sweden) in a calcium-free Hanks' solution to which 2 mM EDTA and 2% serum albumin (fraction V, Calbiochem, San Diego, Calif.) were added. After centrifugation, the gradients were fractionated in 2.5-ml fractions from bottom to top. The density of each fraction was measured in an Abbé refractometer (Carl Zeiss, Oberkochen, Wurttemberg, West Germany).

The biochemical determinations were performed on the cell fractions after the cells were first washed in order to discard the Ficoll and the albumin which, at the concentration used in the gradient, interfere with practically all the reactions. Therefore, the cell fractions were carefully diluted with about 5 vol of Hanks' buffer to allow a rapid sedimentation of the cells at relatively low speed, 300 rpm for 3 min in the International PR 1 centrifuge. The cells were washed once more by resuspending and recentrifuging each fraction.

The preparations of cells for light and electron microscopy were carried out by resuspending the first cell pellet in chilled fixative as described below in the section Light and Electron Microscopy. Consequently, the influence of various high concentrations of Ficoll on the size of the cells is ruled out, and the use of the same fixative for all cell fractions to be measured ensures a unique osmolality.

Biochemical Determinations

Proteins were determined by the Folin-phenol colorimetric method (16) with crystallized bovine serum albumin as standard. Glycogen was estimated by the method of Krisman (14) after digestion of proteins by concentrated 33% boiling potassium hydroxide solution and precipitation of the polysaccharide with alcohol. RNA was determined by the orcinol procedure and DNA by the diphenylamine method according to Schneider (21), after precipitating with 10% TCA, washing twice with ethanol, and extracting with 5% TCA at 90°C for 15 min. Glucose-6-phosphatase activity was measured by the method of Hers and de Duve (11) at pH 6.5. After 30-min incubation at 37°C, the release of inorganic phosphate from 0.05 M glucose-6-phosphate (BDH Chemicals Ltd., Poole, England) was estimated by the colorimetric method of Fiske and Subbarow (8) using a Gilford spectrophotometer (model 300 N, Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The enzymatic activity was expressed in micromoles of phosphorus liberated per minute per milligram of proteins. All these
enzyme determinations were performed on isolated hepatocytes resuspended in hypotonic solution and homogenized by treatment with a Potter Elvejem homogenizer.

**Light and Electron Microscopy:**

**Cytochemistry and Morphometry**

The cells and the cell pellets were fixed in fresh 2.5% chilled, distilled glutaraldehyde buffered with 0.1 M cacodylate, pH 7.2, for very short times (1-2 min).

Fixation of the cell suspensions was stopped by a centrifugation at 15,000 rpm (about 10,000 g) for 30 s by means of the Microfuge (Beckman Instruments Inc., Spincro Div., Palo Alto, Calif.). The cell sediments, about 1-2 mm thick, were then cut into small precisely oriented blocks. After several short washings in 0.15 M cacodylate buffer and 1-h postfixation with 2% osmium tetroxide, buffered at pH 7.2 with 0.1 M sodium cacodylate, the blocks were rapidly dehydrated in graded ethanol, and embedded in Epon 812. The blocks were cut with an LKB Ultrotome (LKB Produkter, Stockholm, Sweden). Thin sections (~400 Å) were stained with lead citrate and distilled glutaraldehyde, buffered with 0.1 M cacodylate buffer, pH 7.4, containing 7.5% sucrose; (d) incubation of the hepatocytes for 30 min at 37°C in the Wachstein-Meisel (23) medium; (e) short immersion of the cells into 0.4% ammonium sulfide solution in order to reveal the reaction, followed by postfixation in cacodylate-buffered 2% osmium tetroxide for 60 min, rapid dehydration in graded ethanol and propylene oxide, and embedding into Epon 812. The size measurements of the cell profiles were converted into cell diameters of hepatocytes, considered as spherical bodies. The mean diameter, surface, and volume of the cells were derived. In order to assess the degree of asymmetry of the cells, 100 major and minor axes of cell profiles were measured and their ratio was calculated. Electronic computer measurements were carried out in collaboration with Dr. Baudhuin (Laboratoire de Chimie Physiologique, University of Louvain, Belgium).

**RESULTS**

**Preparation of Isolated Hepatocytes**

Partial dissociation of liver tissue after perfusion with a Ca++-free medium: After 20-min perfusion with Ca++-free Hanks’ solution, a partial dissociation of the liver tissue was apparent in most of the blocks fixed and processed for electron microscopy: the control livers perfused for an equivalent time with Hanks’ solution containing Ca++ and serum showed no signs of tissue dissociation.

The dislocation observed with Ca++-free perfusion consists of the following ultrastructural changes: (A) The endothelial cells and Kupffer cells which normally line the sinusoids appear loosened (Fig. 1, en) from their normal adhesion to the surface of the hepatocytes. They undergo profound alterations and seem to disappear in the course of the perfusion. We were unable to recover them in the perfusion medium. This confirms the different behavior of hepatocytes and endothelial cells. Similar observations have been mentioned before, when chelating agents were used (18). (B) The surface differentiations which make the hepatocytes adhere and form cell cords and plates within the liver lobule show different degrees of resistance to the dissociation. Complete dissociation occurs where, under normal conditions, adjoining cells are closely aligned without presenting apparent morphological membrane differentiations (Figs. 1 and 2, arrows). The multiple sites where cells remain attached are clearly illustrated in Fig. 1 (ad) and at higher magnification in Fig. 2. In the latter micrograph, alternating regions of
dissociation and of remaining contacts may be distinguished. Moving from the Disse space (Fig. 2, upper left) to the biliary canaliculus (bc, lower right of Fig. 2), one observes successively a first segment (a) of complete dissociation, a gap junction (b) which generally remains entirely intact, this being particularly evident in transverse sections in which the structure of the adjoining unit membranes are distinguished, a short segment of adhesion (c) where no membrane differentiation is observed except the presence of a paramembranous smooth cisterna, another segment of complete dissociation (d), and finally the tight junction which delineates the bile canaliculus (ti). A symmetrical and similar structure (ti±) exists at the opposite side of the bile canaliculus. It is prolonged by the two cell membranes which in this gapping segment include a cleaved macula adherens appearing as a pair of hemidesmosomes, each one attached to a membrane (arrowheads).

By removing the liver at the end of the Ca²⁺-free perfusion, there is no tendency for the tissue to separate into individual cells or cell cords because the partially dissociated cells are trapped in the abundant connective tissue which sustains the lobular structure of the liver.

**COMPLETE DISSOCIATION OF LIVER TISSUE AFTER ENZYME PERFUSION:** The dissociation of liver tissue leading to a complete separation of cells is a stepwise process during which the framework of connective tissue is partly digested, the cell contacts are progressively impaired, and the final sites of intimate junctions are mechanically broken.

At the end of the 30-min enzyme perfusion, as mentioned in Material and Methods, a cell suspension was obtained consisting mainly of cell cords (Fig. 3). Further dissociation of these cell cords was achieved by gentle mechanical treatment consisting of rolling the cell suspension in a round-bottom flask. After filtration through Perlon of decreasing mesh sizes, a suspension of free cells was obtained (Fig. 4).

**Properties of Isolated Hepatocytes**

**MORPHOLOGY AND GLUCOSE-6-PHOSPHATE CYTOCHEMISTRY OF THE ISOLATED HEPATOCYTES:** Semithin sections of the cell pellet obtained after centrifugation of the cell suspension show a thick cell layer in which whole cells are predominant and, on top of this, a thin layer of cell debris mixed with a few dislocated hepatocytes. Among the whole cells which sediment at the bottom of the pellet, more than 80% appear morphologically well preserved, presenting a clear outline, no swelling of the cytoplasmic constituents, and a normal affinity for basic stains such as toluidine blue. The whole cells which do not fulfill these criteria and which are not counted as well preserved appear weakly stained with toluidine blue and abnormally swollen. The debris which occupies the top of the pellet represents 10–15% of its total volume. This evaluation is approximative and varies with the quality of the preparation, and a more precise quantitative appreciation cannot be proposed on these morphological bases. The proportion of cells other than the parenchymal cells is very low, less than 4%.

The use of ultrathin sections prepared for low magnification electron microscopy allows us to examine large areas of cell pellets which are representative of the total population. This method appeared very convenient and extremely critical for evaluating the degree of cell preservation. Cells which were classified as well preserved present a continuous plasma membrane, no vesiculation of the smooth endoplasmic reticulum, and mitochondria with a normal density to the electrons. These ultrastructural criteria are superimposable in some respects to those used in light microscopy, taking into account the stain affinity and the absence of swelling. Fig. 5 gives an example of a low magnification electron micrograph which may be analyzed in more detail with standard magnifications as illustrated in Figs. 6 and 7. Besides the properties chosen as criteria of good preservation, some other characteristics may be seen in the isolated hepatocytes: some cells (Fig. 5, a) are relatively dense and contain well-preserved organelles distributed throughout the cytoplasm in the usual manner, but others of lower electron density (Fig. 5, b) contain mitochondria which contrast with the surrounding organelles and are more or less amassed in the perinuclear region of the cytoplasm. In all cells, short and irregular microvilli were observed on practically the entire cell contour. Fig. 6 is a higher magnification of a whole binucleated hepatocyte which, like all those examined, does not show surface differentiations. In some sections the classical Golgi complex consisting of curved, parallel cisternae of smooth membranes (Fig. 7, go) is located in the peripheral cytoplasm in an area where lysosomes (ly) are grouped with occasional autophagic vacuoles (av). The frequent occurrence of Golgi complexes...
and lysosomes at one side of the cell may be interpreted as being the only remnant of the biliary pole of the hepatocyte. It may be noticed on the same micrograph (Fig. 7) that other organelles such as the rough and smooth endoplasmic reticulum, mitochondria, and glycogen α-particles are well preserved.

Short glutaraldehyde fixations secure a good morphological preservation of isolated liver cells and also a satisfactory retention of some en-
zymatic activities. Isolated cells in suspension constitute a material of choice for enzyme cytochemistry because no problems of substrate penetration arise as in tissue slices. After incubation in the Wachstein-Meisel medium for 30 min, the cells remain intact. Mitochondria, endoplasmic reticulum (ER), Golgi complex, and glycogen particles are well preserved: there is no vacuolation of the ER, nuclear envelope, or mitochondria; glycogen deposits are very large and appear lightly stained with lead. A heavy lead phosphate deposit is detected in nearly all the cisternae of the ER and in the nuclear envelope (Fig. 8). At higher magnification (Fig. 9), the specificity of the reaction is well demonstrated. Cytochemical deposits are absent from the plasma membrane and from the cisternae of the Golgi complex (Fig. 9, go) whereas they appear heavy and uniformly distributed in the endoplasmic reticulum and in the nuclear envelope, where the nuclear pores are well delineated (Fig. 9, NP). 70–80% of the cells react positively. This constitutes another argument for the good preservation of the isolated hepatocytes.

SIZE MEASUREMENTS OF ISOLATED HEPATOCYTES: The size measurements were determined by means of the Coulter Counter. Morphometric data are summarized in Table I: the mean diameters of unfixed and glutaraldehyde-fixed hepatocytes are, respectively, 22.8 μm and 21.4 μm. This reduction in size of the mean diameter is due to the hyperosmolarity of the fixative (~440 mosmol for 2.5% glutaraldehyde in 0.1 M cacodylate buffer). We chose this concentration of fixative, however, to obtain better preservation. Experiments in which the glutaraldehyde concentration was lowered from 2.5% to 0.5% reveal poor fixation of cells which present a vesiculation of their rough and smooth ER. Mean areas and mean volumes were calculated from the above data (Table I). Size distribution curves, as illustrated in Fig. 10, were drawn from experimental data, i.e., number of cells counted for each class of diameters. The size distribution histogram of unfixed cells (Fig. 10 a) presents two peaks: the first one, of low amplitude, corresponds to a mixture of free nuclei and cell debris having a mean diameter of 12.0 μm; a second one, of higher amplitude, representing the bulk of liver cells, is characterized by a symmetrical shape and a relative polydispersity, extending from diameters of 17–34 μm with a mode at 24.5 μm. After fixation of the cells, the size distribution curve (Fig. 10 b) is characterized by a shift of both peaks to the left, with a reduction of the polydispersity of the first peak. The second peak is bimodal, with one mode at about 22 μm and the other apparently at 26.5 μm.

BIOCHEMICAL DETERMINATIONS ON ISOLATED HEPATOCYTES: The entire cell fraction, obtained after filtration on Perlon tissue filter, was also subjected to protein, DNA, RNA, and glycogen determinations. The values reported in Table II express the recovery of material present in the filtrate, after elimination of the undissociated parenchymal cells and the connective tissue. It thus mainly consists of the hepatocytes contaminated with cell debris. 50–75 × 10⁶ hepatocytes were recovered per g of wet weight perfused liver tissue. 80% of the DNA present in the homogenate and a similar proportion of glycogen were recovered in the total filtered cell fraction. This indicates that not much glycogen was broken down or lost during that part of the preparation which follows the perfusion. It has to be mentioned here that about 25% glycogen is lost during the first part of the preparation, i.e., during the perfusion. In spite of this cumulative destruction of glycogen, which amounts to 40%, the recovery of this weak cell component is satisfactory; in our early tests on the mechanical treatment of the perfused liver, rough manipulations produced a loss of more than 80% of the original glycogen content.
Morphological and Biochemical Characteristics of Cells Separated on Ficoll Gradients

Cell Distribution Profiles and Subfractionation: The isolated hepatocytes at a concentration of 10 × 10⁶ cells per ml, layered on top of the Ficoll density gradient, and centrifuged at 25,000 rpm for 3 h in a Spinco SW 27 rotor maintained at 4°C were, in all experiments, distributed according to a similar pattern which consists of four main layers: the nonsedimented material on top of the centrifuge tube, then two distinct layers, one close to the top, the other located in the lower half of the tube, and finally a pellet at the bottom. Proteins, DNA, RNA, glycogen, and glucose-6-phosphatase activity were determined on the cell fractions collected from the density gradients. The profiles, obtained in an experiment cited as an example, are represented in the two graphs of Fig. 11. The four main layers observed in the centrifuge tube may be identified with corresponding peaks in the profiles. The series of tubes were pooled, dividing the gradient into four cell subfractions more or less equivalent to the layers observed in the gradient.

The nonsedimenting material (overlay and tubes 1–3) consisted mainly of cell debris, free organelles, and nuclei; the bottom fraction forming a compact pellet at the bottom of the tube was composed of agglutinated and partially damaged cells. The low density cell fraction (tubes 4–9) of a mean density of 1.10 consists of hepatocytes which were called light hepatocytes. These form 13–15% of the total amount of material layered on top of the gradient, when protein, DNA, and glucose-6-phosphatase activity were taken into account (Table III). Similarly, we call heavy hepatocytes those cells which sediment in tubes of lower levels (tubes 10–16) and which form a fraction with a mean density of 1.14. This fraction constitutes 22–23.5% of the total cell content in proteins, DNA, and glucose-6-phosphatase disposed on top of the gradient.

The total recovery of the constituents, ranging from 55% to 60% may be considered satisfactory, since these values concern only material which sediments at 100–200 g and which consists mainly of intact hepatocytes as specified by morphological examinations. The low values obtained for the recovery of RNA and glycogen may reflect a partial loss of these compounds during centrifugation and washings.

Morphology: Despite the high centrifugal force applied to the isolated cells, no severe ultrastructural damage was observed. A limited rearrangement of some organelles, otherwise well preserved, was noticed. The homogeneity of the cell preservation is striking in both light and heavy hepatocytes, more than 80% of the cells showing a normal appearance.

The low contamination of the low density fraction with debris and nuclei and the absence of free organelles in the high density cell fraction is due to the gradient centrifugation which retains most of them in the upper layers (tubes 1–3).

A light hepatocyte is illustrated in Fig. 12. The clear and constant characteristics of these cells are the relative abundance of the smooth ER (ser) and the scantiness of glycogen particles. When present, they appear dispersed as isolated α-particles within the network of the reticulum. As demonstrated in later experiments, the proportion of the smooth ER influences greatly the density of the cells.

The heavy hepatocytes, on the other hand, differ from the preceding type by a less developed smooth ER and by accumulations of glycogen, forming, in sections, large areas (Fig. 13, gO). These cytoplasmic regions of glycogen storage, examined at higher magnification, are devoid of...
smooth extensions of the endoplasmic reticulum, and it may be emphasized that the abundance of glycogen aggregates does not obscure the identification of the smooth walled structures. The rough ER generally consists of elongated cisternae which surround mitochondria.

This morphological classification into two types of cells, light and heavy, based essentially on quantitative differences in basic cytoplasmic constituents, namely membranous structures and glycogen, may also reflect particular metabolic behaviors. Furthermore, there are indications, which will be presented in the following paper (24), according to which the light cells are more often found in the centrolobular regions and the heavy cells are more frequently located at the periphery of the liver lobule.

SIZE MEASUREMENTS: We postulated, for
Figure 9 Glucose-6-phosphatase cytoenzymatic reaction. Higher magnification of a portion of isolated liver cell. Notice the uniform distribution of lead deposits in the cisternae of the ER and in the nuclear envelope where nuclear pores (NP) are nicely delineated. No lead deposits are detected in the Golgi apparatus (go). x 20,800.

TABLE 1
Coulter Counter Determinations

| Parameters                  | Unfixed isolated cells | Glutaraldehyde-fixed cells |
|-----------------------------|------------------------|---------------------------|
| Mean diameter, \( \mu m \)  | 22.78                  | 21.38                     |
| Standard deviation of diameter, \( \mu m \) | 5.78                  | 6.34                      |
| Mean area, \( \mu m^2 \)   | 1,629                  | 1,435                     |
| Mean volume, \( \mu m^3 \) | 6,186                  | 5,114                     |

Our calculations, that the isolated hepatocytes were spherical. This approximation is acceptable because the axial ratio calculated for 100 major axes and for an equivalent number of minor axes of cellular profiles measured on 1-\( \mu m \) thick sections was found to be less than 1.1.

The three histograms of the first column in Fig. 14 (continuous lines) illustrate the distribution of 1,500 diameters measured on sections of the original population of isolated hepatocytes, of the low density fraction (light hepatocytes) and of the high density fraction (heavy hepatocytes). Dotted lines represent the corrected cell distributions made primarily for the smaller diameters, i.e., for the near polar sections of cells which were not taken into consideration.

The above data, obtained by measuring the diameter of cell sections, were converted into diameters of cells, i.e., into equatorial diameters, according to the method of Wicksell (25). The distribution curves so obtained are shown in the second column of Fig. 14. By plotting the cumulative frequency values of these last curves on a probability scale with logarithmic abscissa (Fig. 14, third column), all points fell on a straight line of normality, except points 1-3 corresponding to the smallest diameters. The calculated mean diameters are reported on Table IV: 20 \( \mu m \) for the
FIGURE 10 Coulter counter determinations. Size distribution histograms of unfixed (a) and glutaraldehyde-fixed (b) isolated hepatocytes. Abscissa, diameters in micrometers; ordinate, number of cells counted per class of diameters. The distribution of unfixed hepatocytes is characterized by a symmetrical shape with a mode at 24.5 \( \mu m \) (a), whereas after fixation a bimodal distribution is observed with one mode at 22.0 \( \mu m \) and the other at 26.5 \( \mu m \) (b). Notice the presence of a peak of low amplitude in both distributions characterized by a mean diameter of 12 \( \mu m \) (a) and 9.5 \( \mu m \) after fixation (b).

total population of cells, 20.5 \( \mu m \) for the hepatocytes of the low density fraction, and 19 \( \mu m \) for the hepatocytes of high density; the mean surface area and the mean volume are derived from the diameter measurements. They show that the light hepatocytes are larger than the heavy ones.

**Biochemistry:** DNA, RNA, and glycogen content, and glucose-6-phosphatase activity were determined in the two purified fractions and expressed per milligram of protein (Table V). Light and heavy hepatocytes contain similar amounts of DNA and RNA. Glucose-6-phosphatase is equally distributed between the two cell fractions. The most striking difference between these cell types lay in their glycogen content, as already mentioned in the morphological observa-
TABLE II

Protein, DNA, RNA, and Glycogen Content and Glucose-6-Phosphatase Activity of Isolated Liver Cell Fractions Compared to the Homogenate of Perfused Liver

| Biochemical assays | Homogenate of perfused liver | Isolated hepatocytes (unwashed) |
|--------------------|-----------------------------|---------------------------------|
| Protein mg/g*      | 148.3 ± 6.4                 | 99.2 ± 8.0t                    |
| %                  | 100                         | 67.0                            |
| DNA mg/g*          | 2.6 ± 0.2                   | 2.0 ± 0.1                      |
| %                  | 100                         | 77.0                            |
| RNA mg/g*          | 6.1 ± 0.6                   | 4.2 ± 0.8                      |
| %                  | 100                         | 69.0                            |
| Glycogen mg/g*     | 27.6 ± 3.6                  | 22.0 ± 4.5                     |
| %                  | 100                         | 79.7                            |
| Glucose-6-phosphatase μmol/min/g* | —                  | 5.6 ± 1.0                      |

Mean values and standard deviations calculated for 6 experiments.

* The data are expressed per gram wet weight of perfused liver; this is done for both homogenate and isolated hepatocytes; the values are not corrected for the perfusion liquid retained by the liver tissues.

† The protein content of the isolated hepatocytes is corrected for the albumin added to the medium.

DISCUSSION

The isolation of liver parenchymal cells implies the removal of calcium ions, which undoubtedly play a role in cell adhesiveness. Anderson (1) established that the use of chelating agents releases cells from their adhesion forces. The sites at which calcium acts precisely on plasma membranes are not clear. Curtis (5) believes that calcium may decrease the negative surface charges of the cells and, as a consequence, may reduce the repulsive forces provided by the surface potential. Van der Waals-London attraction forces in equilibrium with repulsive forces would account for the constancy of the 100–200 Å gap existing between adhering cells. Hays et al. (9) observed that pairs of plasma membranes disposed into convoluted folds and juxtaluminal junctional complexes in the toad bladder are generally disrupted above and below the desmosomal region during cell dissociation in calcium-free medium. In our experience, the perfusion of liver with calcium-free Hanks’ solution alone provokes a dissociation of the tissue that consists of releasing the adhesion forces between cells and of cleaving some of the membrane differentiations, namely the desmosomes. These results confirm those obtained by Emmelot and Benedetti (7) using EDTA which loosens the desmosomal junctions of adjoining liver cells. According to Berry and Friend (3), the resulting hemidesmosomes are destined to be engulfed, probably by phagocytosis of these membrane segments into intracellular vacuoles. A similar finding has been reported by Overton (20) in trypsinized embryonic epithelial tissue. In our laboratory, Waëlbroeck (unpublished observations) was able to demonstrate the presence of hemidesmosomes on plasma membranes prepared from isolated hepatocytes. The gap junctions, on the contrary, resist the removal of Ca++ from the perfusion medium, also the hyaluronidase action, and even the mechanical treatment. These segments of the junctional complexes which belonged formerly to two adjacent cells are now entirely transferred to one of the two cells, the other cell having lost that portion of its cytoplasm which has been torn away. That is also the reason why we recovered intact gap junctions in the isolated plasma membranes. These observations lead to the conclusion that local disruptions of cell membranes take place during the isolation process, followed by an immediate healing.

As stated by Howard et al. (13), factors which play an important role in obtaining the best morphological preservation comprise: first, rigorous control during perfusion of parameters such as pH, Po2, temperature, osmotic pressure, and time of exposure to enzymes used at the adequate final concentration. In our experimental conditions, described under Material and Methods, all these parameters remained constant during the 30-min recirculating perfusion; second, mechanical treatment must be kept to a minimum to avoid important tearing of plasma membranes. Gentle rolling of the hepatocyte suspension in a siliconized flask improves the dissociation of the remaining cell cords and the reproducibility of the results, because time, speed of rotation, and all other factors are controlled. Finally, we would like to lay stress on the fixation procedure: very short fixation times of 1 min, as recommended in perfusion fixations (6), were used both for cell suspension

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FIGURE 11  Zonal profile of protein, DNA, RNA, glycogen, glucose-6-phosphatase, and density at 20°C along the 15-40% linear Ficoll gradient, buffered with a calcium-free Hanks' solution containing 2 mM EDTA and 2% serum albumin, after isopycnic centrifugation of isolated hepatocytes in a SW 27 rotor at 25,000 rpm for 3 h: 17 fractions of 2.5 ml each were collected. Four subdivisions of the gradient were performed. Overlay and tubes 1-3: nonsedimenting material; tubes 4-9: low density cell fraction (mean density: 1.10) composed of light hepatocytes; tubes 10-15: high density cell fraction (mean density: 1.14) where heavy hepatocytes are isopycnically banded; finally, a bottom fraction. Biochemical data corresponding to these profiles are listed in Table III.

| Gradient subfractions | Protein | DNA | RNA | Glucose-6-phosphatase | Glycogen |
|-----------------------|---------|-----|-----|-----------------------|----------|
|                       | %       | %   | %   | %                     | %        |
| Nonsedimented material| 5.9 ± 0.5| 8.5 ± 0.6| 5.0 ± 0.7| 6.9 ± 0.8| 2.2 ± 1.5 |
| Low density fraction  | 12.3 ± 1.8| 15.0 ± 2.6| 7.5 ± 2.0| 13.2 ± 0.5| 6.6 ± 2.9 |
| High density fraction | 21.7 ± 5.4| 23.3 ± 5.2| 14.5 ± 2.8| 22.5 ± 5.5| 15.3 ± 7.2 |
| Bottom                | 21.3 ± 3.3| 30.7 ± 4.4| 20.9 ± 6.1| 12.5 ± 3.0| 14.9 ± 8.4 |
| Recovery              | 61.1 ± 5.9| 76.7 ± 7.9| 47.8 ± 3.7| 55.0 ± 4.8| 38.2 ± 16.1 |

Mean values and standard deviations calculated for five experiments.
FIGURE 12 Light hepatocyte. Constant morphological characteristics are detected in these cells: an extended smooth ER, composed of numerous profiles of smooth tubules (ser), a few α-glycogen particles dispersed within the network of the reticulum, and numerous small mitochondria. × 8,500.

and for resuspended cell pellets. The usual fixation times of 1 or 2 h, recommended for tissues, are unsatisfactory in many instances. Although Howard et al. (13) found that calcium ions were very important for structural integrity, we notice that even the use of EDTA did not interfere with cell preservation provided fixation was carried out for 1 min only.
The criteria of cell preservation which were used in the course of this work consist of observing fixed and embedded cells in semithin sections for light microscopy and ultrathin sections for electron microscopy. The low magnifications in electron microscopy are particularly indicated for a critical evaluation of the degree of preservation. The correlation between these ultrastructural criteria and the trypan blue test used in many laboratories remains to be specified.
FIGURE 14 Size measurements of hepatocyte profiles. First column: size distribution histograms of 1,500 diameters measured on sections of the original cell population (OP), the low density subfraction (LD), and the high density subfraction (HD). Dotted lines correspond to size distributions corrected for the small diameters. Second column: size distribution histograms of the corresponding cell diameters (equatorial diameters) calculated according to the method of Wicksell (25). Third column: probability plot of the cumulated size frequencies on a logarithmic abscissa. Mean diameters, surfaces, and volumes of cells are recorded in Table IV.

The final efficiency of the technique was evaluated at about 65% isolated cells recovered from the whole liver, which corresponds to about 50–75 \( \times 10^6 \) cells per g of liver tissue. These figures are lower than those reported by Seglen (22). It might be that we retain more cells on the last 28-\( \mu \)m filter than Seglen who uses a 62-\( \mu \)m nylon mesh. Since we need perfectly isolated cells to layer on top of density gradients, it was imperative to obtain single cell suspensions.

The recovery of the different compounds and, in particular, of glycogen is another expression of the degree of cell preservation. The total loss of about 40% of the glycogen during the entire process of cell preparation partly occurs during the perfusion with the Hanks' solution which does not contain glucose. Berry and Friend (3) reported similar observations. In unpublished experiments, we ob-
TABLE IV

Morphometric Data Obtained from Cellular Profiles in Sections

| Fractions                  | Standard deviation of diameter | Mean area | Mean volume |
|----------------------------|-------------------------------|-----------|-------------|
|                            | μm μm² μm³ μm³ |            |             |
| Original population        | 20.0 3.1 1,296 4,547          |           |             |
| Hepatocytes of low density | 20.5 3.3 1,349 4,835          |           |             |
| (d: 1.10)                  |                               |           |             |
| Hepatocytes of high density| 19.0 3.0 1,160 3,839          |           |             |
| (d: 1.14)                  |                               |           |             |

TABLE V

DNA, RNA, Glycogen Content, and Glucose-6-Phosphatase Activity of Isolated Hepatocytes, Expressed Per Milligram of Protein

| Low density fraction (d: 1.10) | High density fraction (d: 1.14) |
|--------------------------------|---------------------------------|
| DNA 0.031 ± 0.005              | 0.025 ± 0.001                   |
| RNA 0.029 ± 0.005              | 0.027 ± 0.006                   |
| Glycogen 0.075 ± 0.009         | 0.106 ± 0.007                   |
| Glucose-6-phosphatase 0.045 ± 0.007 | 0.042 ± 0.003 |   |

The standard deviations were calculated for four experiments.

to distinguish two cell populations along the gradient: light hepatocytes with a mean density of 1.10, and heavy hepatocytes with a mean density of 1.14. Both cell fractions are equally rich in DNA and in RNA, and present about the same glucose-6-phosphatase activity, but the glycogen content differs markedly (Table V). The dissimilarity between light and heavy cells is more striking morphologically because the distribution and localization of some cell constituents differ; this is the case for the smooth ER which is relatively expanded in the light hepatocytes and less developed in the heavy cells; it is also true for the glycogen particles which are dispersed within the fine network of the smooth ER in the light cells but are packed in large aggregates in the heavy cells. These descriptions correspond to those which can be made in the liver lobule, considering the centro- and perilobular hepatocytes. Loud (15), basing his demonstration on quantitative stereological techniques, has shown that centrolobular hepatocytes, representing 20% of the total liver parenchymal cells, are characterized by the presence of isolated rosettes of glycogen associated with membranes of smooth-surfaced ER. The morphometric studies of that author have also quantified the relative volume occupied by the mitochondria. In the centrolobular cells, the numerous mitochondria are small and occupy only 12.9% of the total cell volume, whereas in the perilobular cells the chondriome constitutes 20% of the cell volume. For the reasons given above, we suggest that the light hepatocytes correspond, with a high probability, to centrolobular cells and that the heavy hepatocytes arise mainly from the perilobular region. Our conclusion in this respect appear to be in contradiction with those formed by Castagna and Chauveau (4), since these authors ascribe the light hepatocytes to those cells located in the perilobular region of the lobule. In attempting to explain this discrepancy, a main difference in the experimental conditions is to be noticed: the rats of the Wistar strain used by Castagna and Chauveau were fasted for 18 h, which results in a glycogen depletion of the hepatocytes. Starvation may also induce in some strains a proliferation of the smooth ER which may interfere with the density of the cells to be dispersed in the gradient. Some contradictions remain unclarified, however. It may be of interest to mention here that the use of a new gradient medium, such as metrizamide (19), influencing only slightly the osmotic pressure, modifying slightly the viscosity, and remaining inactive against the

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usual biochemical reagents, has actually been proposed and may solve technical problems.

The glucose-6-phosphatase activity was also nicely preserved in the isolated hepatocytes, after short glutaraldehyde fixation. Lead phosphate deposits were evenly distributed in the cisternae of the endoplasmic reticulum. These observations suggest that enzymatic activity remained intact during the isolation procedure. Biochemical assays of glucose-6-phosphatase activity confirmed these findings and revealed that 70% of the enzymatic activity present in a liver homogenate was recovered in the isolated liver cells.

The diameters of isolated liver cells maintained in suspension were measured by means of a Coulter counter. The cell profiles in thick sections were also measured and the data were transformed so that the influence of fixation and embedding could be determined. Unfixed and fixed hepatocytes in suspension presented, respectively, a mean diameter of 22.8 µm and 21.3 µm and a mean volume of 6,186 µm³ and 5,114 µm³. This reduction in size is due to the fixative. On the other hand, the mean diameter and volume of hepatocytes, calculated from cellular profiles in sections, fell to 20.0 µm and 4,547 µm³, respectively. Dehydration and embedding would account for this additional size reduction. These latter morphometric values, obtained for isolated spherical cells, were similar to those obtained by Loud (15) for rat hepatocytes measured in situ: 21.0 µm ± 0.3 µm for cell diameter and 5,100 µm³ for cell volume. Although cells appeared transformed into spheres during the isolation procedure, their diameters and volumes were thus not modified.

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REFERENCES

1. Anderson, N. G. 1953. The mass isolation of whole cells from rat liver. Science (Wash. D. C.). 117:627.
2. Baudhuin, P., and J. Berthet. 1967. Electron microscopic examination of subcellular fractions. Quantitative analysis of the mitochondrial population isolated from rat liver. J. Cell Biol. 35:631.
3. Berry, M. N., and D. S. Friend. 1969. High-yield preparation of isolated rat liver parenchymal cells. J. Cell Biol. 43:506.
4. Castagn, M., and J. Chauveau. 1969 Séparation des hépatocytes isolés de rat en fractions cellulaires métaboliquement distinctes. Exp. Cell Res. 57:211.
5. Curtis, A. 1962. Cell contact and adhesion. Biol. Rev. (Camb.). 37:82.
6. Drochmans, P., W. Penasse, and D. Menard. 1972. Adaptation of the fine structural demonstration of glucose-6-phosphatase to the study of the distribution and local deletions of the enzyme. Proceedings of the Fifth European Congress on Electron Microscopy, Manchester. The Institute of Physics, London. 276.
7. Emmelot, P., and E. L. Benedetti. 1967. Structure and function of isolated plasma membranes from liver. In Proteides de la Biological Fluids. Peeters, editor. Elsevier Scientific Publishing Company, Amsterdam. 15:315.
8. Fiske, C. H., and Y. Subbarow. 1925. Colorimetric determination of phosphorus. J. Biol. Chem. 66:375.
9. Hays, R. M., B. Singer, and S. Malamed. 1965. The effect of calcium withdrawal on the structure and function of the toad bladder. J. Cell Biol. 25:195.
10. Hems, R., B. D. Ross, M. N. Berry, and H. A. Krebs. 1966. Gluconeogenesis in the perfused rat liver. Biochem. J. 101:284.
11. Hers, H. G., and C. De Duve. 1950. Le système hexose-phosphatase. Répartition de l'activité glucose-6-phosphatase dans les tissus. Bull. Soc. Chim. Biol. 32:20.
12. Howard, R. B., A. K. Christensen, F. A. Gibbs, and L. A. Pesch. 1967. The enzymatic preparation of isolated intact parenchymal cells from rat liver. J. Cell Biol. 35:675.
13. Howard, R. B., J. C. Lee, and L. A. Pesch. 1973. The fine structure, potassium content, and respiratory activity of isolated rat liver parenchymal cells prepared by improved enzymatic techniques. J. Cell Biol. 57:642.
14. Krisman, C. A. 1962. A method for the colorimetric estimation of glycogen with iodine. Anal. Biochem. 4:17.
15. Loud, A. V. 1968. A quantitative stereological description of the ultrastructure of normal rat liver parenchymal cells. J. Cell Biol. 37:27.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265.
17. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409.

DROCHMANS, WANSON, AND MOSSELMANS Isolation of Adult Rat Hepatocytes 21
18. Mills, D. M., and D. Zucker-Franklin. 1969. Electron microscopic study of isolated Kupffer cells. *Am. J. Pathol.* 54:147.

19. Munthe-Kaas, A. C., and P. O. Seglen. 1974. The use of metrizamide as a gradient medium for isopycnic separation of rat liver cells. *Fed. Eur. Biochem. Soc.* Lett. 43:252–256.

20. Overton, J. 1968. The fate of desmosomes in trypsinized tissue. *J. Exp. Zool.* 168:203.

21. Schneider, W. C. 1945. Extraction and estimation of deoxypentose nucleic acid and of pentose nucleic acid. *J. Biol. Chem.* 161:293.

22. Seglen, P. O. 1973. Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. *Exp. Cell Res.* 82:391–398.

23. Wachstein, M., and E. Meisel. 1956. On the histochemical demonstration of glucose-6-phosphatase. *J. Histochem. Cytochem.* 4:592.

24. Wanson, J. C., P. Drochmans, C. May, W. Penasse, and A. Popowski. 1974. Isolation of centrolobular and perilobular hepatocytes after phenobarbital treatment. *J. Cell Biol.* 66:23–41.

25. Wicksell, S. D. 1925. The corpuscle problem. A mathematical study of a biometric problem. *Biometrika.* 17:84–91.

26. Wisse, E., J. v.d. Meulen, J. M. van 't Noordende, and J. J. Emeis. 1971. Electron microscopy at low magnification. A histological application. *Philips Tech. Bull.* February 1971.