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The Isolation of Functionally Heterogeneous Hepatocytes of the Proximal and Distal Half of the Liver Acinus in the Rat

JORGE J. GUMUCIO, MARYANN MAY, CAROLINE DVORAK, JOSE CHIANALE AND VERONICA MASSEY
Department of Internal Medicine, Section of Gastroenterology, Veterans Administration Medical Center and the University of Michigan School of Medicine, Ann Arbor, Michigan 48105

The objective of this study was to isolate hepatocytes of the proximal half (Zones 1 and 2) or distal half (Zones 2 and 3) of the liver acinus. The zonal origin of the isolated hepatocytes was recognized by: (a) the presence in hepatocytes of a fluorescent marker, acridine orange, selectively delivered to either the proximal or the distal half of the acinus by *in situ* perfusion prior to cell isolation and (b) the measurement of the induction of cytochrome P-450 by phenobarbital, an induction known to occur predominantly in the distal half of the acinus.

Following the selective labeling of the acinus with acridine orange, livers were perfused with collagenase in either the portal to hepatic vein direction (anterograde) or in the retrograde direction. Hepatocytes isolated by either an anterograde or a retrograde perfusion were separated by centrifugation in a Percoll density gradient. This procedure isolated populations of proximal or distal hepatocytes, respectively, which were intact and 90% fluorescent. In an effort of assessing the heterogeneity of the separated proximal and distal hepatocytes, each population was further fractionated by centrifugal elutriation. This resulted in the arbitrary separation of proximal or distal hepatocytes into five fractions. Total cytochrome P-450 was determined spectrophotometrically in each of the fractions isolated from controls and after 3 days of the *in vivo* administration of phenobarbital. On the basis of the pattern of fluorescence in isolated hepatocytes and on the cytochrome P-450 inductive response to phenobarbital administration, it is proposed that: (a) the anterograde or retrograde perfusion of the liver with collagenase separated hepatocytes predominantly of the proximal or distal half of the liver acinus, respectively and (b) that hepatocytes of the distal half of the liver acinus responded to phenobarbital administration with the highest level of cytochrome P-450 induction, indicating that the isolated hepatocytes conserved the functional heterogeneity observed *in vivo*.

Hepatocytes of the microvascular unit of hepatic parenchyma, the liver acinus, are heterogeneous (1, 2). This heterogeneity becomes apparent when morphological (3–5), biochemical (6, 7) or functional (8–11) characteristics of hepatocytes located around the terminal portal venule are compared with those of hepatocytes located around the hepatic venule. These two areas, which Rappaport (12) called Zones 1 and 3 of the hepatic acinus, are linked by numerous hepatocytes, identified as Zone 2, which present morphological and biochemical characteristics intermediate and thus transitional between Zones 1 and 3. It should be noted, however, that there are no anatomical boundaries between these acinar zones, and to that extent, the division of the acinus in three zones remains arbitrary.

Various attempts have been made to isolate hepatocytes originating in one or another acinar zone. The most common experimental approach has been to separate hepatocytes according to a physical characteristic which is expressed predominantly in one zone, i.e., cell size (13–15), cell density (16–18) and binding to lectins (19). Recently, a method of isolation of zonal hepatocytes involving the simultaneous perfusion of the liver via the portal and hepatic vein has been described (20). By perfusing the liver with buffer in a direction opposite to that of collagenase, it has been proposed that the diffusion of this enzyme within the acinus can be limited, resulting in the zonal separation of hepatocytes. All of these studies have shown that hepatocytes of the acinus can be fractionated into subpopulations. However, whether the separated subpopulations represent hepatocytes originating in different zones of the hepatic acinus or simply hepatocytes grouped according to a common physical characteristic regardless of their zonal origin has not been convincingly established. A major methodological obstacle to the assessment of the zonal origin of the separated subpopulations has been the lack of specific markers of hepatocytes of each zone. The criterion most frequently used to assess the zonal origin of isolated hepatocytes has been the measurement of the relative distribution of enzymes among the subpopulations isolated. However, since the differences in enzymatic content among hepatocytes of the acinus are gradual and in a dynamic state, i.e., the zonal content of one or another enzyme changes with variations in metabolic state (7), the relative distribution of enzymes has serious limitations when used as the sole criterion for recognition of the zonal origin of isolated hepatocytes. For these

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Address reprint requests to: Jorge J. Gumucio, M.D., Department of Internal Medicine, Section of Gastroenterology, Veterans Administration Medical Center, 2215 Fuller Road, Ann Arbor, Michigan 48105.

reasons, in this study we have first labeled hepatocytes located either in the proximal half (Zones 1 and 2) or in the distal half (Zones 2 and 3) of the acinus by selectively delivering an exogenous label into these hepatocytes while still in tissue. The distribution of this exogenous label in the isolated hepatocytes was subsequently followed. In addition, the response of the isolated hepatocytes to the *in vivo* administration of phenobarbital (PB) with induction of cytochrome P-450 was also measured. Cytochrome P-450 has been one of the enzymes frequently used to establish the zonal identity of the isolated hepatocytes, since it is induced by PB (21–25). Moreover, this induction occurs predominantly in hepatocytes located in the distal half of the liver acinus, providing a compelling example of the functional heterogeneity of the hepatic parenchyma (26, 27). This heterogeneous response of acinar hepatocytes allows the use of the cytochrome P-450 induction by PB as a suitable marker for the recognition of distal hepatocytes once they are isolated.

In this study, we describe a new method for the isolation of hepatocytes originating predominantly in either the proximal half (Zones 1 and 2) or distal half (Zones 2 and 3) of the hepatic acinus. The origin of the isolated hepatocytes was established by two criteria: (a) by the presence in hepatocytes of acridine orange, a fluorescent marker delivered selectively to proximal or distal hepatocytes by liver perfusion prior to cell isolation and (b) by the induction of cytochrome P-450 by PB.

The method of hepatocyte fractionation described here provides a tool for the study of the molecular mechanisms responsible for the heterogeneous expression of cytochrome P-450 within the liver acinus.

MATERIALS AND METHODS

Isolation and Fractionation of Proximal and Distal Hepatocytes

The entire method of cell isolation used in these experiments is illustrated as a flow chart in Figure 1.

The Selective Labeling of proximal or Distal Hepatocytes by In Situ Liver Perfusion with Acridine Orange: Male Charles River rats weighing between 190 and 260 gm were used in all of these experiments. Rats were kept under the same environmental conditions for at least 1 week prior to any experiment.

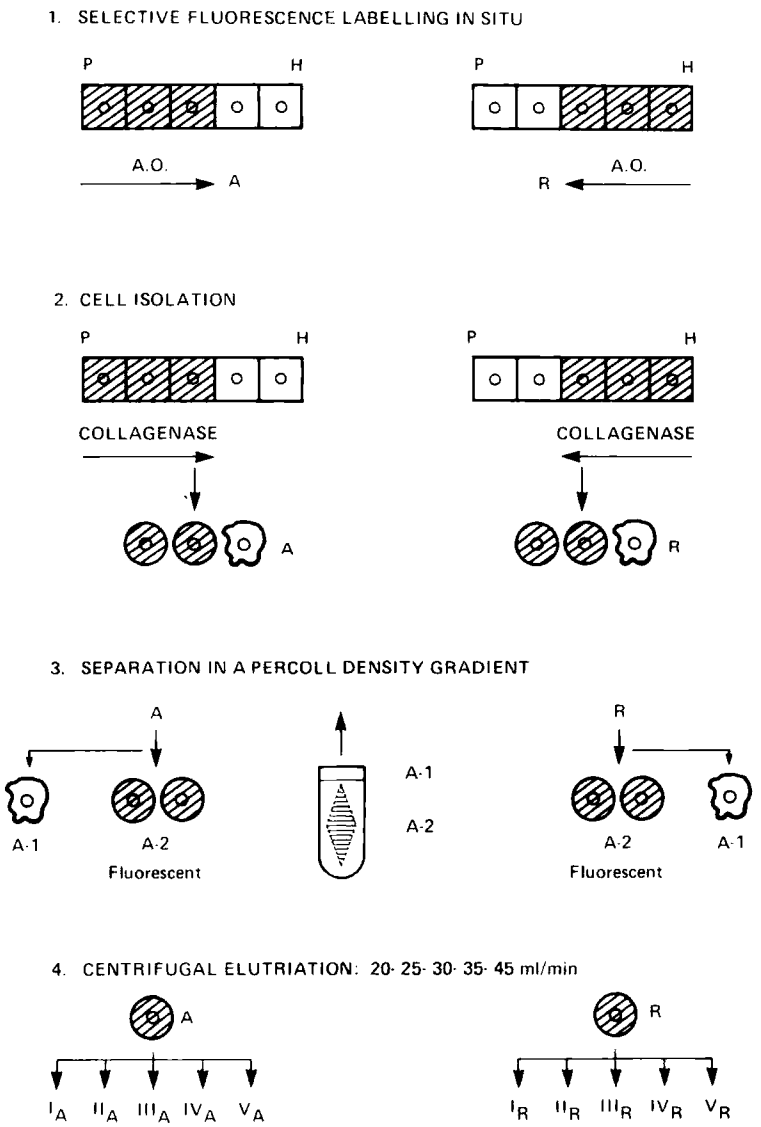

FIG. 1. *Isolation and fractionation of proximal and distal hepatocytes.* A-1 = hepatocyte fraction of light density obtained after centrifugation in a Percoll gradient. These hepatocytes represented partially damaged cells which had lost their fluorescence. A-2 = hepatocyte fraction of heavier density. These hepatocytes were well-preserved and fluorescent. This cell fraction was used for further fractionation of proximal and distal hepatocytes by centrifugal elutriation. IA to VA = hepatocyte fractions obtained by elutriation of hepatocytes separated by anterograde perfusion with collagenase. IR to VR = hepatocyte fractions obtained after elutriation of hepatocytes separated by retrograde perfusion with collagenase. *Shaded areas* illustrate the presence of fluorescence. P = terminal portal venule; H = terminal hepatic venule; A.O. = acridine orange; A = anterograde perfusion; R = retrograde perfusion.

Rats were fasted for 16 hr and anesthesized with sodium pentobarbital (4 mg per 100 gm body weight, i.p.). An *in situ* liver perfusion was established as previously described (28), with some modifications. All experiments started with the perfusion of the liver via the cannula in the portal vein. The perfusate consisted of 0.01 *M* Hanks buffer (29) (pH 7.4) with an osmolality of 300 to 320 mOsm per liter, which was constantly equilibrated with 95% O_2 and 5% CO_2. The temperature of the perfusate measured at the tip of the cannula was 37°C. The rate of perfusion was maintained at 30 ml per min and the perfusion pressure between 15 and 25 cm of water. The perfusate was allowed to exit the liver via a cannula placed in the inferior vena cava. This cannula was maintained open to attain a nonrecirculating condition. Once the liver had been cleared from blood, 1×10^{-5} *M* acridine orange was added to the perfusate. From this point on, direct illumination of the liver was avoided. Previous experiments (28) had shown that perfusion of the liver via the cannula in the portal vein (anterograde perfusion) with up to 8×10^{-5} *M* acridine orange for 5 min labeled the proximal one-half of the hepatic acinus (corresponding to Zones 1 and 2).

In those experiments in which the distal half of the acinus was to be labeled, the liver was perfused under the same conditions described above, but 1×10^{-5} *M* acridine orange was perfused via the cannula in the inferior vena cava (retrograde perfusion). Under these conditions, the cannula in the portal vein served as the outlet of the perfusate.

Isolation of Hepatocytes: After 5 min of perfusion with acridine orange, hepatocytes were isolated according to Ingebretsen et al. (30), with some modifications. Cell isolation was performed by collagenase perfusion via either an anterograde (portal to hepatic vein direction) or a retrograde perfusion (hepatic to portal vein direction). Since the initial zonal labeling with acridine orange was also performed via either an anterograde or a retrograde perfusion, two initial experimental groups resulted: Group A—acridine orange-anterograde, collagenase-anterograde and Group B—acridine orange-retrograde, collagenase-retrograde. The perfusate used in all cell isolations consisted of 0.01 *M* Hanks buffer without calcium (pH 7.4), with an osmolality between 300 to 320 mOsm per liter. Otherwise, conditions were identical to the ones described above. After 10 min of perfusion with this calcium-free buffer, the perfusate was changed to a 0.01 *M* Hanks buffer, containing 2.5 m*M* $CaCl_2$ and 50 units of collagenase per ml of perfusate (usually between 0.02 and 0.04% collagenase), until the liver became soft and channels were observed on its surface. Other than the direction of perfusion, the experimental conditions were maintained constant during anterograde or retrograde perfusions. The liver was rapidly removed and placed in a Petri dish containing ice-cold 10 m*M* Hepes buffer (pH 7.4) with an osmolality of 300 to 320 mOsm per liter. The liver capsule was opened and the cell suspension gently stirred on ice in a clinical rotator. After 3 min, the cell suspension was centrifuged at 40 $\times$ *g* for 3 min. The cell pellet was washed 3 times with 10 m*M* Hepes buffer. The final pellet was resuspended in Hepes, and aliquots were used to determine cell counts and cell size (Coulter counter ZBI coupled to a H-4 channelizer and recorder), fluorescence (Leitz fluorescence microscope) and viability, as described below.

Separation of Isolated Heptocytes in Percoll Density Gradients: Since it has been shown (31) that centrifugation of freshly isolated hepatocytes in a Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient enables the separation of intact hepatocytes from partially damaged cells, the remainder of the cell suspension was placed in a Percoll gradient. This gradient was prepared by mixing 9 parts of Percoll with 1 part of 0.05 *M* Tris buffer (pH 7.4) containing 2.5 *M* sucrose and 1% bovine albumin. This stock solution was diluted to 63% (v:v) with 5 m*M* Tris buffer (pH 7.4) containing 0.25 *M* sucrose and 0.1% bovine albumin. The cell suspension was diluted 1:3 (v:v) with 10 m*M* Hepes buffer (pH 7.4), and 5 to 7 ml of this suspension were mixed with 30 ml of the 63% Percoll solution. Preliminary experiments showed that after centrifugation at 40,000 $\times$ *g* per min for 20 min at 4°C, a continuous density gradient between densities of 1.06 and 1.16 was formed. After centrifugation of the cell suspension under the same conditions, the layer of cells floating on top of the gradient referred as A-1 was removed. The remainder of hepatocytes distributed inside the gradient, most of them equilibrating between densities of 1.12 and 1.14. These hepatocytes were collected as a single fraction subsequently called A-2. This A-2 fraction was diluted 1:5 (v:v) with 10 m*M* Hepes buffer (pH 7.4) and centrifuged once at 120 $\times$ *g* for 3 min. The cell pellet obtained was washed twice with the same buffer and centrifuged at 40 $\times$ *g* for 3 min. The final cell pellet (A-2) was resuspended in 10 m*M* Hepes buffer (pH 7.4), and aliquots were used to determine cell counts, cell size, proteins and cytochrome P-450, as described below.

Centrifugal Elutriation: Analysis of the initial data showed that the anterograde or retrograde perfusion of the liver with collagenase, followed by separation of intact cells in Percoll gradients, separated populations of hepatocytes originating in the proximal or distal half of the acinus, respectively. However, it was also apparent that proximal or distal hepatocytes contained intermediate (Zone 2) hepatocytes as "contaminants." In an attempt to assess the presence of functionally heterogeneous subpopulations, within proximal or distal hepatocytes, a third optional step was introduced to the procedure. Hepatocytes separated in A-2, proximal or distal, were further fractionated by centrifugal elutriation, according to Bernaert et al. (13), with some modifications. This method fractionates hepatocytes mainly, although not exclusively according to size (13). Using a Beckman J 21-C centrifuge and a JE-6 rotor, A-2 hepatocytes separated after either anterograde or retrograde perfusions with collagenase were loaded into the separation chamber at a concentration of 5×10^6 cells per ml in Hepes buffer at a rate of 15 ml per min. The centrifuge was maintained at 14°C and 840 rpm. By changing counterflow rates to 20, 25, 30, 35 and 45 ml per min, A-2 was separated into five cell fractions. Cell fraction I corresponded to hepatocytes obtained at a counterflow rate of 20 ml per min, and Fraction V was collected at 45 ml per min. Each fraction was centrifuged at 40 $\times$ *g* per min for 3 min and the cell pellet resuspended in 10 m*M* Hepes buffer (pH 7.4). This cell suspension was used for determinations of cell counts, size, viability and fluorescence.

The Assessment of Cell Number and Size, Cell Viability and Cell Fluorescence in the Isolated Hepatocytes

For the determinations of cell number and size, hepatocytes isolated in each fraction were analyzed in a ZBI Coulter counter coupled to a H-4 channelizer. The H-4 channelizer was previously calibrated with microspheres of known size. About 20 to 40 μl of a 1:3 dilution of the cell pellet were completed to 20 ml with Isotone (Coulter Electronics, Inc., Hialeah, Fla.). Using a probe with an aperture of 100 μm, cell counts were obtained from the ZBI. Cell size and a cell size histogram were obtained from data provided by the H-4 channelizer.

$$\text{Cell size } (u^3) = [(\text{channel number} \times \text{ww}/100) + \text{BCT}] \times \text{TF}$$

where

ww = window width
BCT = base channel threshold, and
TF = threshold factor.

In addition, and since some aggregated forms were detected by light microscopy, hepatocytes were counted in each fraction using a hemocytometer. Hepatocytes counted by light micros-

copy have been used when the data have been expressed per 10^6 cells.

Cell viability was initially assessed by Trypan blue exclusion criterion (32). However, light microscopic examination revealed that some hepatocytes, even though excluding Trypan blue, presented an irregular contour with several blebs as well as a granular cytoplasm. These hepatocytes have been called "granular" in the text. In contrast, other hepatocytes presented a sharp and regular contour as well as a transparent, smooth cytoplasm. These hepatocytes have been called "intact" in the text. Finally, some hepatocytes incorporated Trypan blue and have been called "nonviable" in the text.

Acridine orange fluorescence was determined in the isolated hepatocytes by fluorescence microscopy. Under appropriate filters, a drop of the cell suspension which had been maintained in the dark was placed on a slide, covered and hepatocytes were counted. Several fields were chosen at random, and the total number of hepatocytes as well as the number of fluorescent hepatocytes were determined. Three patterns of fluorescence were encountered. Some hepatocytes did not show any fluorescence at all. In some, only the nucleus showed fluorescence. For purposes of calculations, these two cell patterns were considered "not fluorescent." Finally, some hepatocytes were intensely fluorescent. In these hepatocytes, both the cytoplasm as well as the nucleus exhibited brilliant fluorescence. In the calculations, these cells were considered "fluorescent" hepatocytes.

It should be noted that, as described (30), contamination of these preparations with nonparenchymatous cells was negligible.

The Assessment of Fluorescence in Tissue

In initial experiments, the distribution of acridine orange in hepatocytes of the liver acinus of controls and PB-treated rats was determined. Five minutes after starting the perfusion with 1×10^{-5} *M* acridine orange, samples of each liver lobe were removed and immediately frozen in a mixture of absolute ethanol and dry ice. Cryostat sections 8 μm thick were prepared and examined by fluorescence microscopy (Leitz, Dialux). In addition to this qualitative assessment, the zonal distribution of fluorescence was assessed semiquantitatively by laser-activated microphotometry, as previously described in this laboratory (28). To determine whether the zonal distribution of fluorescence changed during collagenase perfusion, similar sections of liver were prepared 7.5 min after initiation of collagenase. Subsequent to this time interval, recognition of tissue details became difficult.

Control Experiments

The Zonal Labeling with Acridine Orange and the Separation of Labeled Hepatocytes: In order to assess further the correlation of the patterns of fluorescence labeling in tissue with those of the isolated hepatocytes, several control experiments were performed. These experiments resulted in two additional groups: Group C—acridine orange-anterograde, collagenase-retrograde and Group D—acridine orange-retrograde, collagenase-anterograde. The purpose of these two groups was to determine, by comparison with Groups A and B, whether hepatocytes located in the acinar half distal to the direction of collagenase perfusion were also separated.

Preliminary experiments showed that perfusion of acridine orange labeled the entire hepatic acinus when perfused by anterograde perfusion for 5 min, followed by an additional 5 min of perfusion with this fluorescent substance in the retrograde direction. This model was used to assess the loss of fluorescence during collagenase perfusion (Group E). After labeling the entire acinus with acridine orange, collagenase was perfused as usual in the anterograde direction.

In a series of additional experiments, the simultaneous double perfusion of the liver described above (20) was used in an attempt to compare the patterns of fluorescence obtained by that approach with the patterns observed with our method. The simultaneous double perfusion of the liver (20) also involves the treatment of the isolated hepatocytes with a mixture of trypsin and DNAase as a means of digesting the partially damaged hepatocytes. Therefore, we compared fluorescence and viability of isolated hepatocytes when incubated with these enzymes with that obtained after cell separation in Percoll gradients. These experiments are described in Groups F and G:

Group F. Acridine orange-anterograde, followed by collagenase-anterograde, concomitantly with the simultaneous perfusion with Hanks buffer via retrograde perfusion.

Group G. Acridine orange-retrograde, followed by collagenase-retrograde and simultaneous perfusion with Hanks buffer via an anterograde perfusion.

Both of these perfusions were performed exactly as described (20).

Finally, in an attempt to determine the percentage of *total hepatocytes* of the acinus which were actually labeled with acridine orange, the simultaneous double perfusion described above was modified. Acridine orange was perfused in either anterograde or retrograde direction while collagenase was added to both reservoirs and thus perfused simultaneously in both directions (in an attempt to separate all hepatocytes). This resulted in two additional groups:

Group H. Acridine orange-anterograde, collagenase perfused simultaneously in both directions.

Group I. Acridine orange-retrograde, collagenase perfused in both directions.

The Persistence of the Original Pattern of Zonal Labeling with Acridine Orange during Cell Separation: As already described, livers were first labeled with acridine orange, and tissue sections were performed 7.5 min after the initiation of collagenase perfusion.

In separate experiments, the effluent obtained during collagenase perfusion of a liver previously labeled with acridine orange was saved in the dark and immediately used to perfuse another liver. The purpose of these experiments was to determine whether any fluorescence leaking out of hepatocytes into the sinusoidal space during collagenase perfusion was capable of labeling hepatocytes of the "second" liver. This was used as an approach to assess whether acridine orange leaking out of the hepatocytes originally labeled may have resulted in intense fluorescence of downstream hepatocytes.

The Separation in Percoll Density Gradients of Fluorescent and Nonfluorescent Hepatocytes: The purpose of these control experiments was to assess whether the separation of hepatocytes in A-2 depended on the presence of acridine orange in hepatocytes. For this purpose, hepatocytes were isolated from a liver not labeled with acridine orange. An aliquot of the hepatocytes obtained in A-2 was incubated *in vitro* with 1×10^{-5} *M* acridine orange for 10 min. Mixtures of the hepatocytes labeled *in vitro* with unlabeled hepatocytes were performed in 1:2, 1:4 and 1:8 ratios. These mixtures were centrifuged again in a Percoll gradient of the same characteristics. The percentage of labeled hepatocytes recovered in A-2 after this second gradient centrifugation was compared with the percentage of fluorescent hepatocytes originally loaded into this second centrifugation. The results have been expressed as a ratio of fluorescent hepatocytes before and after the second Percoll gradient centrifugation.

The Induction of Cytochrome P-450

Sodium PB (80 mg per kg) was administered i.p. between 8 a.m. and 9 a.m. for three consecutive days. Control rats received an equal volume of the solvent (benzyl alcohol:propylene glycol:water, 1:30:19, v:v, respectively). Total cytochrome P-450 was determined spectrophotometrically according to Omura and Sato (33). These determinations were performed in the A-2 fractions as well as in the five fractions isolated after centrifugal elutriation of proximal or distal hepatocytes. Each cell fraction was homogenized in 1.15% KCl (1:3, v:v). Using sodium dithionite as a reducing agent, the absorbance spectrum was determined simultaneously in the carbon monoxide-treated sample and in the control between 400 and 500 μm. To assess the effect of turbidity on cytochrome P-450 measurements, all samples in the initial experiments were measured in both a Cary and in an Aminco double beam spectrophotometer. Differences in absorbance between the control cuvette and the sample cuvette were measured at 490 and 450 μm. An extinction coefficient of 91 m$M \times \text{sec}^{-1}$ was used in the calculations, as described (34). Cytochrome P-450 content has been expressed both as nmoles per milligram cellular protein as well as nmoles per 10^6 hepatocytes. Protein concentration in hepatocytes was determined as described by Lowry et al. (35). In preliminary experiments, cytochrome P-450 was determined immediately after isolation of the fractions. However, due to the length of the experiments, all measurements reported here were performed after the cell pellets had been stored at −20°C overnight. This storage resulted in less than 15% of cytochrome P-450 loss.

Once the origin of hepatocytes separated by anterograde or retrograde perfusion with collagenase was established for each of the groups used in this study (controls and PB-treated), the labeling with acridine orange was omitted from the procedure. It should be noted that the initial experiments showed that the presence of acridine orange inside hepatocytes did not interfere with the spectrophotometric detection of cytochrome P-450 in controls nor with the determination of its induction by PB. The decision to omit acridine orange in subsequent experiments was therefore one of convenience. The experiments could be performed under light and more importantly, it was not known whether other determinations, to be performed in future experiments, may be altered by the intracellular presence of acridine orange. Cytochrome P-450 measurements presented here were performed in hepatocytes not exposed to acridine orange.

Statistics

Differences between means have been calculated by a Student's t test. Values of p larger than 0.05 have been considered not significant.

RESULTS

Fluorescence Distribution in Tissue and in Isolated Hepatocytes

Figure 2 (A and B) shows the patterns of fluorescence visualized in tissue after anterograde (portal to hepatic vein direction) and retrograde (hepatic to portal vein direction) perfusion of the liver with acridine orange. The proximal half of the liver acinus became fluorescent following forward perfusion, while the retrograde perfusion of the liver with acridine orange labeled predominantly the distal half of the acinus. This pattern of labeling persisted half-way through collagenase perfusion, as shown in Figure 2B. Moreover, in some experiments, the effluent obtained during collagenase perfusion was immediately used to perfuse another liver. Hepatocytes of the "second" livers did not become fluorescent in any of these experiments.

The qualitative assessment of fluorescence distribution within the acinus was corroborated by semiquantitative measurements of fluorescence performed by laser microphotometry, as shown in Figure 3. The anterograde or retrograde perfusions of the liver with acridine orange labeled the proximal or distal half to two-thirds of the liver acinus, respectively. This was observed in controls and in PB-treated rats.

The fluorescence pattern obtained in hepatocytes isolated immediately after collagenase perfusion is shown in Figure 4. Two patterns were predominantly observed: (a) hepatocytes exhibiting intense fluorescence in both the nucleus and in the cytoplasm, which have been called "fluorescent hepatocytes" and (b) hepatocytes containing acridine orange only in the nucleus and subsequently called "nonfluorescent hepatocytes."

Percentage of Fluorescent Hepatocytes in the Populations Isolated

Table 1 shows that about two-thirds of hepatocytes were fluorescent at zero time (immediately after collagenase perfusion and prior to separation in Percoll gradients) whether the perfusion was in the anterograde or in the retrograde direction (Groups A and B). In contrast, when acridine orange was placed in proximal hepatocytes by an anterograde perfusion and collagenase was perfused in the retrograde direction or vice versa (Groups C and D), only about 30 to 35% of the isolated hepatocytes were fluorescent. When the entire liver acinus was labeled by perfusing acridine orange first via an anterograde and subsequently via a retrograde perfusion (Group E), about 80% of hepatocytes were fluorescent at zero time. This indicated that some hepatocytes are damaged during isolation, resulting in the loss of fluorescence.

Further separation of hepatocytes isolated at zero time by centrifugation in Percoll density gradients resulted in the isolation of a group of hepatocytes, 90% of which were intensely fluorescent (A-2). Percoll centrifugation separated A-2 hepatocytes from a band of light hepatocytes (A-1), 25 to 30% of which were fluorescent.

The fluorescence pattern of hepatocytes isolated by the single perfusion of the liver (Groups A and B) compared with the pattern obtained by the simultaneous double perfusion of the liver (Groups F and G) indicated that the percentage of fluorescent hepatocytes released by both approaches was similar. This similarity was established at zero time as well as after Percoll separation into A-1 and A-2 fractions. Furthermore, density gradient centrifugation provided as good a separation of viable and fluorescent hepatocytes from nonfluorescent, granular hepatocytes, as the trypsin-DNAase treatment used in the original description of the double perfusion method (20). Finally, and since these data suggested that the single perfusion of the liver with collagenase separated some but not all acinar hepatocytes, the simultaneous double perfusion was modified in an attempt to isolate all acinar hepatocytes. In these experiments (Groups H and I), either the proximal or the distal half of the acinus was labeled by anterograde or retrograde

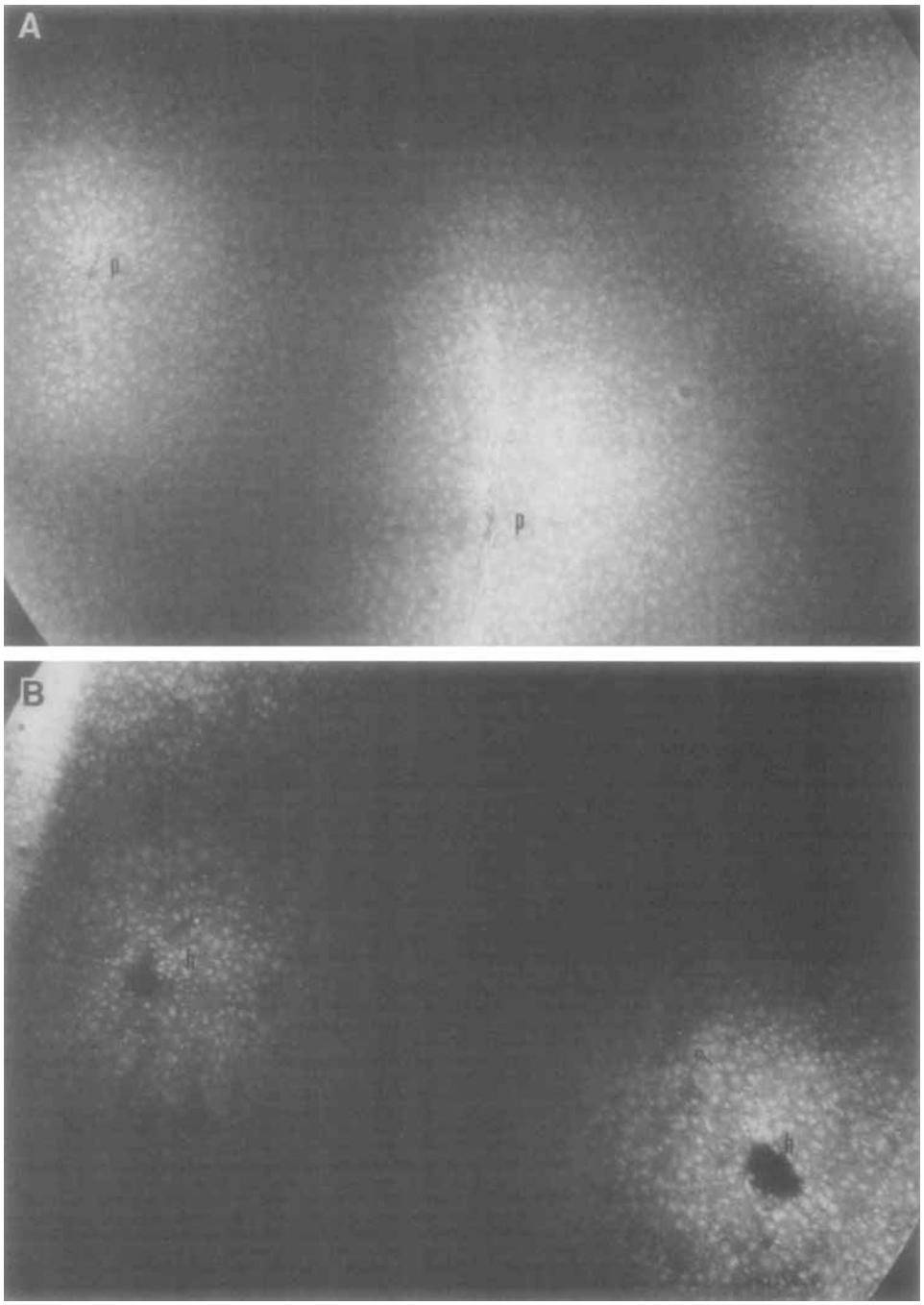

FIG. 2. *Patterns of fluorescence obtained after forward or retrograde perfusion of the liver with acridine orange.* (A) *Anterograde perfusion.* Hepatocytes surrounding two terminal portal venules (p) appeared intensely fluorescent. Both cytoplasm and nucleus were fluorescent. These hepatocytes have been called in the text hepatocytes of the proximal half of the acinus or anterograde hepatocytes. During the anterograde perfusion of the liver with acridine orange, hepatocytes of the distal half of the acinus or retrograde hepatocytes contained acridine orange mainly in the nucleus. (B) *Retrograde perfusion.* In this experiment, acridine orange was perfused via a retrograde perfusion. The procedure of cell isolation with collagenase was initiated, and this liver section was obtained 7.5 min into the retrograde perfusion with collagenase. Hepatocytes surrounding terminal hepatic venules (h) appeared intensely fluorescent. Both nucleus and cytoplasm were fluorescent. Channels between cells indicating partial separation of hepatocytes were visible (*arrows*).

perfusion with acridine orange, respectively. This was followed by the perfusion of the liver with collagenase, simultaneously, in both directions. As expected, since nonlabeled hepatocytes should also be released during this double perfusion with collagenase, only 30 to 35% of the isolated hepatocytes were fluorescent. Table 2 shows that the distribution of fluorescence in hepatocytes separated by either an anterograde or a retrograde perfusion with collagenase after 3 days of PB administration was similar to that of controls. About 85% of hepatocytes isolated in A-2 after PB were intensely fluorescent.

To assess whether the separation of proximal or distal hepatocytes in A-2 depended on the intracellular presence of acridine orange, hepatocytes were separated into the A-2 fraction from a liver not labeled with acridine orange. Half of these hepatocytes were incubated with 1×10^{-5} *M* acridine orange *in vitro*. Mixtures 1:2, 1:4 and 1:8 of *in vitro* labeled and unlabeled hepatocytes were prepared, placed again in Percoll and centrifuged. The percentage of fluorescent hepatocytes expressed as the ratio of values obtained before and after the second centrifugation in Percoll was 1.03 ± 0.15 (S.E.) in controls and 1.06 ± 0.2 (S.E.) after PB ($n = 18$ in each

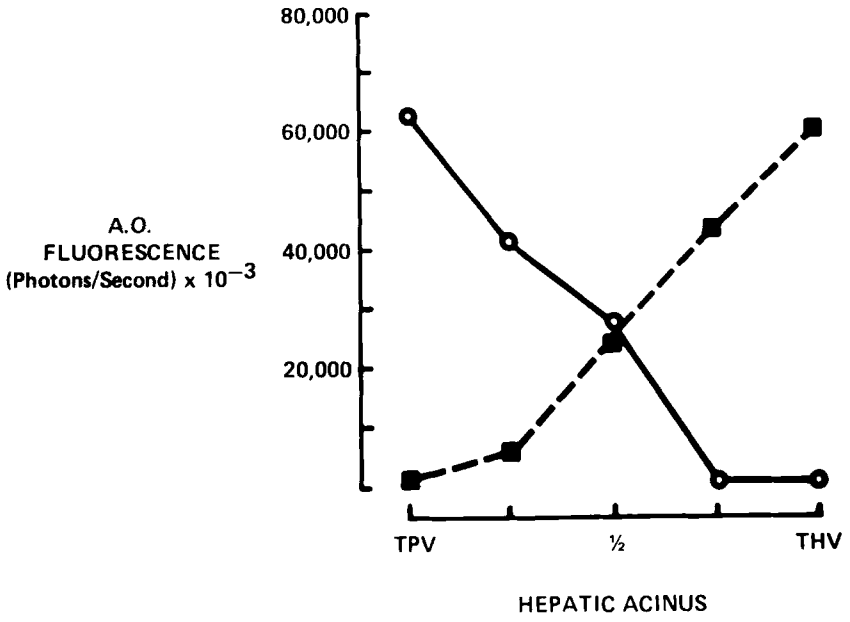

FIG. 3. *Semiquantitative assessment of the relative distribution of acridine orange (A.O.) within the hepatic acinus by laser-activated microphotometry.* Mean ± S.E., n = 5 experiments per group; ○ = anterograde perfusion; ■ = retrograde perfusion; TPV = terminal portal venule; THV = Terminal hepatic venule. Details of this method of assessing fluorescence in individual hepatocytes have been described (28).

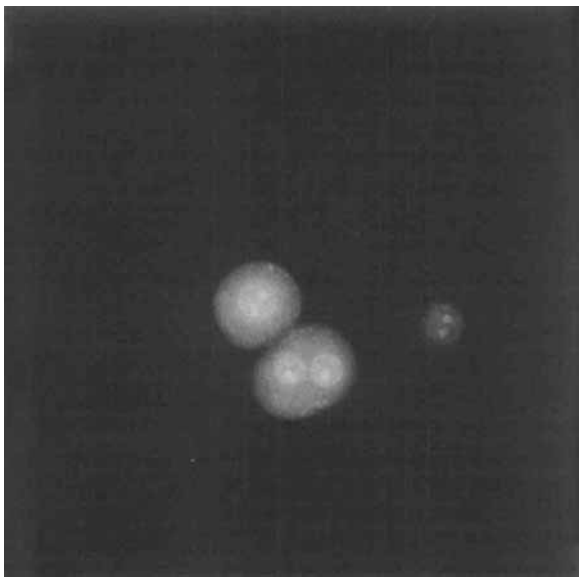

FIG. 4. *Fluorescence in isolated hepatocytes.* Hepatocytes immediately after isolation with collagenase. Hepatocytes were considered "fluorescent" if both the nucleus and the cytoplasm contained brilliant fluorescence. Two fluorescent, nondissociated hepatocytes containing acridine orange in the nuclei and cytoplasm are visible. Hepatocytes containing fluorescence only in the nucleus were considered "nonfluorescent." A nonfluorescent hepatocyte containing acridine orange in the nucleus and nucleoli is also visible. The cytoplasm of this hepatocyte is barely visible in the background.

group). Since no differences were found among the various ratios of fluorescent/nonfluorescent hepatocytes, all experiments have been combined. Also, no differences were found in these ratios between hepatocytes separated by anterograde or retrograde perfusions. These results indicated that acridine orange did not change the sedimentation behavior of control or PB-treated hepatocytes in Percoll gradients and could therefore be omitted.

Cell Yield and Cell Viability

Table 3 shows the yield of hepatocytes at zero time and the percentage distribution of hepatocytes recovered after Percoll density centrifugation. Cell yield and the percentage of hepatocytes recovered in cell fractions A-1 and A-2 were similar after an anterograde or a retrograde perfusion of the liver with collagenase. The recovery of the total number of hepatocytes loaded into the density gradient was about 85%, regardless of the direction of collagenase perfusion.

Table 4 illustrates the percentage of hepatocytes which were intact, granular and nonviable at zero time and after separation of hepatocytes in a Percoll gradient. While most hepatocytes separated in either A-1 or A-2 fractions excluded Trypan blue, A-1, the fraction containing hepatocytes of density lower than 1.06, was comprised of a high percentage of granular cells. Moreover, by comparison with the distribution of acridine orange shown in Table 1, it can be appreciated that A-1 also contained a high percentage of hepatocytes which were not fluorescent. Therefore, it is conceivable that A-1 may have represented hepatocytes which were partially damaged during isolation and separation. In contrast, hepatocytes separated in A-2 exhibited high levels of fluorescence, and only about 20% of them showed granularity of the cytoplasm. Consequently, centrifugation of hepatocytes obtained at zero time in Percoll gradients mainly accomplished the separation of well-preserved and fluorescent hepatocytes from partially damaged, nonfluorescent cells.

Cytochrome P-450 Content of Hepatocytes Separated by Density Gradient Centrifugation

Total cytochrome P-450 content (nmoles per milligram cellular protein) was determined in hepatocytes separated in A-2 after either anterograde or retrograde perfusions with collagenase. The term "total cytochrome P-450" is used here since there are at least four forms of

TABLE 1. Percentage distribution of fluorescence in isolated hepatocytes[a]

Experimental group	Direction of perfusion		Zero time	Percoll		Trypsin DNAase
	AO	Collagenase		A-1	A-2	
	→	→				
A (n = 7)	A	A	64.0 ± 2.2	26.4 ± 6.6	89.8 ± 2.5	89.0 ± 4.1
	←	←				
B (n = 7)	R	R	66.3 ± 1.6	30.4 ± 3.8	90.0 ± 0.5	91.0 ± 3.8
	→	←				
C (n = 3)	A	R	33.5 ± 0.8			
	←	→				
D (n = 3)	R	A	32.4 ± 1.2			
	→←	→				
E (n = 3)	A R	A	80.1 ± 2.5			
	Double Perfusion					
	→	→				
F (n = 4)	A	A	68.5 ± 2.9	19.5 ± 2.8	84.6 ± 3.1	91.0 ± 0.3
	Double Perfusion					
	←	←				
G (n = 4)	R	R	65.5 ± 2.1	20.8 ± 2.4	86/7 ± 3.1	90.0 ± 0.4
	Double Perfusion					
	→	→←				
H (n = 3)	A	A R	31.5 ± 1.8			
	Double Perfusion					
	←	→←				
I (n = 3)	R	A R	33.4 ± 2.3			

[a] Each value represents the mean ± S.E. of the percentage of the total number of hepatocytes which were fluorescent after the various perfusions with acridine orange (AO) and collagenase. The arrows indicate the direction of perfusion with acridine orange and collagenase. A = anterograde perfusion. R = retrograde perfusion. Trypsin DNAase = group of hepatocytes released by collagenase perfusion and incubated with these enzymes in an attempt to digest partially damaged cells and thus segregate a population of viable hepatocytes (20). Zero time = hepatocytes obtained immediately after collagenase perfusion. Percoll = hepatocytes obtained after centriguation in a Percoll density gradient. A-1 and A-2 are as described in Figure 1. Criteria for the assessment of "fluorescent" and "nonfluorescent" hepatocytes are as described in Figure 4.

TABLE 2. Distribution of fluorescence in hepatocytes separated after 3 days of PB administration[a]

Experimental group	Direction of perfusion		Zero time	Percoll	
	AO	Collagenase		A-1	A-2
PB	→	→			
(n = 8)	A	A	61.3 ± 4.2	30.1 ± 5.2	86.7 ± 2.5
PB	←	←			
(n = 12)	R	R	65.6 ± 3.5	24.9 ± 6.2	85.2 ± 3.7

[a] The significance of the terms anterograde, retrograde, A-1, A-2 and AO is the same as described in Table 1. Values are mean ± S.E.

TABLE 3. Cell yield[a]

Direction of perfusion	Zero time (hepatocytes × 10^8)	% recovered cells	
		A-1	A-2
Anterograde	3.18 ± 0.4	47 ± 11.0	42 ± 3.6
Retrograde	3.24 ± 0.3	48 ± 7.8	42 ± 2.7

[a] Zero time = hepatocytes isolated immediately after collagenase perfusion and prior to Percoll centrifugation. A-1 = partially damaged hepatocytes of density lower than 1.06 which floated on top of the Percoll gradient. A-2 = intact hepatocytes of higher density which remained inside the Percoll gradient. Results for A-1 and A-2 were expressed as percentage of hepatocytes recovered after Percoll gradient centrifugation. Values are mean ± S.E., n = 12 experiments per group.

cytochrome P-450 in the PB-inducible family (24), and moreover, even other families of cytochrome P-450 may respond to this inducer, although at a lower level. The spectrophotometric method used here should have measured the response of all of these forms. In controls, there was no difference in the cytochrome P-450 content of hepatocytes separated by an anterograde or a retrograde perfusion with collagenase. The mean and S.E. were 0.119 ± 0.02 and 0.113 + 0.01, n = 9, respectively (p = not significant). After 3 days of PB administration, the mean and S.E. were 0.316 + 0.04 and 0.497 + 0.06, n = 12, for anterograde and retrograde perfusions, respectively ($p < 0.05$). While PB induced total cytochrome P-450 in both proximal and distal hepatocytes, the difference in cytochrome P-450 content between induced hepatocytes separated by anterograde or retrograde perfusions was slight. This difference was certainly of a lesser magnitude than that expected from the results of the experiments using immunofluorescence techniques (26, 27). The possibility was raised that intermediate, PB-inducible hepatocytes of Zone 2 may have "contaminated" each preparation and therefore masked the heterogeneous response of these populations to PB. In an attempt to isolate subpopulations within proximal or distal hepatocytes which may show more clear-cut functional differences, hepatocytes of A-2 obtained by either anterograde or retrograde perfusions were submitted to centrifugal elutriation.

TABLE 4. Cell viability[a]

Direction of perfusion		Zero time			Percoll					
					A-1			A-2		
AO	C	Intact	Granular	Nonviable	Intact	Granular	Nonviable	Intact	Granular	Nonviable
→	→									
A	A	27.7 ± 3.5	64.3 ± 3.1	6.3 ± 0.5	27.6 ± 4.8	65.3 ± 6.1	7 ± 3.5	75.0 ± 1.1	24.2 ± 1.2	0.6 ± 0.10
←	←									
R	R	36.1 ± 3.0	54.1 ± 2.8	8.7 ± 1.2	29.7 ± 5.4	58.3 ± 4.3	12 ± 7.0	75.0 ± 3.5	24.5 ± 3.4	0.3 ± 0.08

[a] AO = acridine orange; C = collagenase; A = anterograde perfusion; R = retrograde perfusion. Zero, A-1 and A-2 are as defined in Figure 1. Intact = hepatocytes which excluded trypan blue and on light microscopy presented a distinct, regular contour and a smooth cytoplasm. Granular = hepatocytes which although excluding trypan blue presented an irregular surface and a granular cytoplasm. Nonviable = hepatocytes incorporating trypan blue. Values are mean ± S.E., n = 12 experiments per group.

Centrifugal Elutriation of Hepatocytes Separated in A-2 by Either Anterograde or Retrograde Perfusions with Collagenase

By changing the rate of counterflow, five fractions were arbitrarily separated from A-2 hepatocytes derived from either proximal or distal hepatocytes. Cell size histograms of each fraction are illustrated in Figure 5 (A and B). Each fraction contained hepatocytes of several sizes, although the overall trend was to separate larger hepatocytes with faster counterflow rates. The possibility that some of the large hepatocytes could represent associated forms was explored (since the pore of the ZBI probe used was 100 μm, and the average diameter of hepatocytes is about 20 to 22 μm). Results are presented in Table 5. While most hepatocytes of Fractions I and II consisted of single forms, Fractions III, IV and V contained significant percentages of associated forms. The percentage distribution of hepatocytes in Fractions I to V, corrected by the mean percentage of doublets and triplets (Table 5), was 41, 18, 16, 14 and 11%, respectively. This distribution was similar regardless of the direction of perfusion or of PB administration. Due to the presence of associated forms in each fraction, the real number of hepatocytes per fraction was assessed independently by light microscopy. Only these values have been used in the expression of results. Figure 6 shows the total cytochrome P-450 content of the various fractions separated by centrifugal elutriation. No differences were found in the total cytochrome P-450 content of "anterograde" or "retrograde" fractions isolated from control livers either when expressed as nmoles per milligram of microsomal protein or as nmoles per 10^6 hepatocytes. Total cytochrome P-450 content measured in these fractions after 3 days of PB administration was expressed in relationship to the cytochrome P-450 content of corresponding control fractions. The pattern of response to PB is shown in Figure 6. When the data were expressed per milligram of cellular protein (Figure 6A), it was apparent that the highest levels of cytochrome P-450 induction were attained in some fractions isolated from hepatocytes separated by retrograde perfusion. When results were expressed as nmoles per 10^6 hepatocytes (Figure 6B), the inductive response revealed that there were: hepatocytes responding poorly to PB (Fraction I anterograde); hepatocytes responding to PB in an intermediate manner (Fractions II, III and IV anterograde and Fraction I retrograde), and finally, hepatocytes responding with the highest levels of cytochrome P-450 induction represented by Fraction V anterograde and Fractions II, III, IV and V retrograde. Centrifugal elutriation therefore, enabled a better definition of the heterogeneous patterns of functional response of acinar hepatocytes to the PB-mediated induction of cytochrome P-450.

DISCUSSION

It is proposed that hepatocytes separated in A-2 by either anterograde or retrograde perfusion with collagenase originated predominantly in either the proximal or the distal half of the liver acinus, respectively. The validity of this claim rests on two findings: (a) that the selective labeling of proximal or distal hepatocytes with acridine orange was followed by the recovery in A-2, of hepatocytes, 85 to 90% of which were intact and intensely fluorescent, regardless of the direction of collagenase perfusion and (b) that isolated hepatocytes showed a heterogeneous pattern of response to the PB-mediated induction of total cytochrome P-450. Furthermore, that the highest levels of induction were observed in some fractions isolated from distal hepatocytes, in agreement with the data generated *in vivo* by studies using immunofluorescence (26, 27) and microspectrophotometry (25).

Three assumptions had to be tested before accepting the use of acridine orange as a suitable marker for the recognition of the zonal origin of the isolated hepatocytes: (a) proximal or distal hepatocytes had to be selectively labeled while still in tissue; (b) the fluorescent label had to remain in the same hepatocytes originally labeled throughout the method of cell isolation, and (c) the fluorescent hepatocytes had to be selectively harvested.

Selective labeling of either proximal or distal hepatocytes was demonstrated in controls and after PB administration, both by visual inspection as well as by laser-activated microphotometry. The pattern of fluorescence obtained in tissue, i.e., intensely fluorescent hepatocytes showing fluorescence in nuclei and cytoplasm, and hepatocytes containing some fluorescence only in the nucleus was entirely similar to the two patterns of fluores-

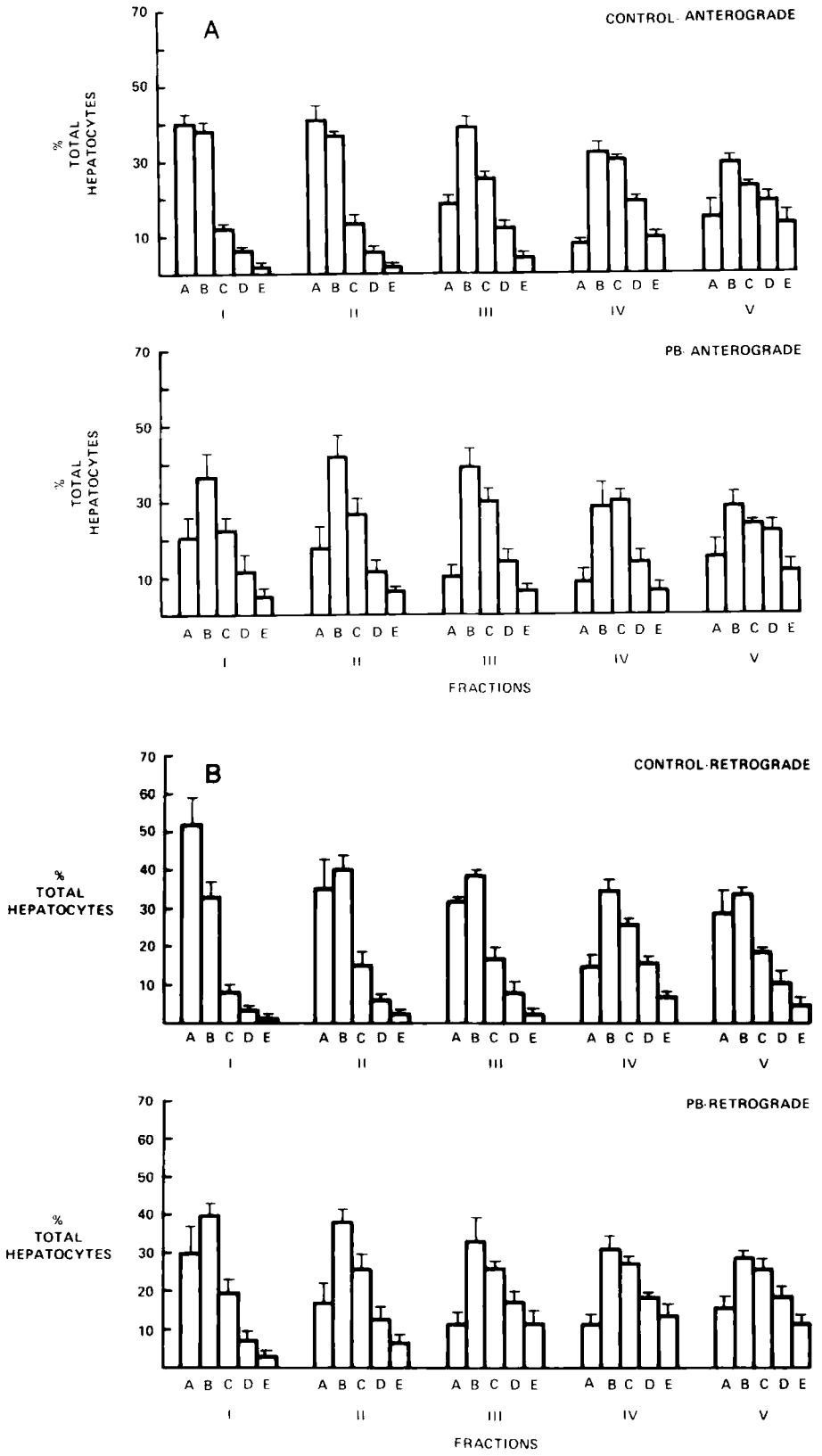

FIG. 5. *Hepatocyte size distribution in cell fractions obtained by centrifugal elutriation.* Histograms of cell size distribution in each of the five fractions obtained by centrifugal elutriation. Cell size was determined in a Coulter counter ZBI linked to a H-4 channelizer. This system was calibrated with microspheres of known size. In the *ordinate*, results were expressed as percentage (of hepatocytes of a determined size) of the total number of hepatocytes in each fraction. (A) Anterograde and retrograde control groups. (B) Hepatocytes obtained by anterograde or retrograde perfusions with collagenase of livers treated with PB for 3 days. In the *abscissa*, I to V represent the five hepatocyte fractions obtained by changing the rate of counterflow used during centrifugal elutriation. Counterflow rates were 20, 25, 30, 35 and 45 ml per min for the separation of Fractions I to V, respectively. A to E represent size of hepatocytes encountered in each of the five fractions elutriated: A = hepatocytes up to 16.43 μm in diameter; B = hepatocytes from 16.44 to 20.70 μm in diameter; C = hepatocytes from 20.71 to 23.70 μm in diameter; D = hepatocytes from 23.71 to 26.09 μm in diameter; E = hepatocytes from 26.10 to 28.01 μm in diameter. Mean ± S.E., n = 6 experiments per group.

cence observed in isolated hepatocytes. The presence of acridine orange in nuclei of hepatocytes located downstream from the entry of the perfusate may be explained by the high affinity of acridine orange for nucleic acids (36). Small amounts of acridine orange which were either not taken up by proximal hepatocytes, or, which diffused back into the sinusoidal space, may have become available for uptake and binding to high-affinity binding sites in nuclei of distal hepatocytes. It is possible, however, that during collagenase perfusion, acridine orange may have moved into hepatocytes other than those originally labeled. Several experiments indicated that this possibil-

ity was unlikely. When the pattern of fluorescence in tissue was examined 7.5 min after the initiation of collagenase perfusion, the original zonal pattern of labeling was still present. Moreover, when acridine orange was placed in proximal hepatocytes and collagenase perfused in the retrograde direction or vice versa, the percentage of fluorescent hepatocytes isolated at zero time decreased to about 32%. This indicated that the percentage of fluorescent hepatocytes recovered at zero time depended on the zonal location of acridine orange and on the direction of perfusion with collagenase.

TABLE 5. Per cent distribution of single and aggregated forms of hepatocytes after centrifugal elutriation[a]

Fraction	Hepatocytes (%)			
	Single	Doublets	Triplets	Multiple aggregates
I	83 ± 3.8	13 ± 2.9	2.9 ± 1.1	0.7 ± 0.1
II	81 ± 3.8	13 ± 3.6	4.8 ± 1.0	0.8 ± 0.1
III	68 ± 11.4	27 ± 9.2	4.3 ± 1.5	0.4 ± 0.1
IV	51 ± 8.1	32 ± 4.7	13.4 ± 3.4	3.8 ± 1.2
V	46 ± 11.5	31 ± 1.9	14.1 ± 3.3	8.7 ± 2.2

[a] The percentage of single or aggregated forms of hepatocytes was determined by light microscopic counting of aliquots extracted from each fraction. Results were similar for controls and after P, regardless of the direction of collagenase perfusion. All values have been combined. Values are mean ± S.E., n = 8 experiments per group.

Finally, when the outflow collected during collagenase perfusion of a liver labeled with acridine orange was kept in the dark and immediately used to perfused a second liver, hepatocytes of this second liver did not become fluorescent. This was not surprising since any back diffusion of acridine orange into the sinusoidal space should have resulted in dilution of the label with the nonlabeled incoming perfusate. The end result should have been fewer rather than more hepatocytes presenting a pattern of intense fluorescence. These results suggested that even though some acridine orange may have back diffused into the sinusoidal space, this movement did not result in the labeling of downstream hepatocytes. Therefore, it is concluded that the intensely fluorescent-isolated hepatocytes originated from the zones originally labeled with acridine orange.

Why was it possible to selectively separate fluorescent hepatocytes after Percoll centrifugation regardless of the direction of collagenase perfusion? Partially damaged or nonviable hepatocytes attain lighter densities than do intact hepatocytes. This has allowed the use of Percoll

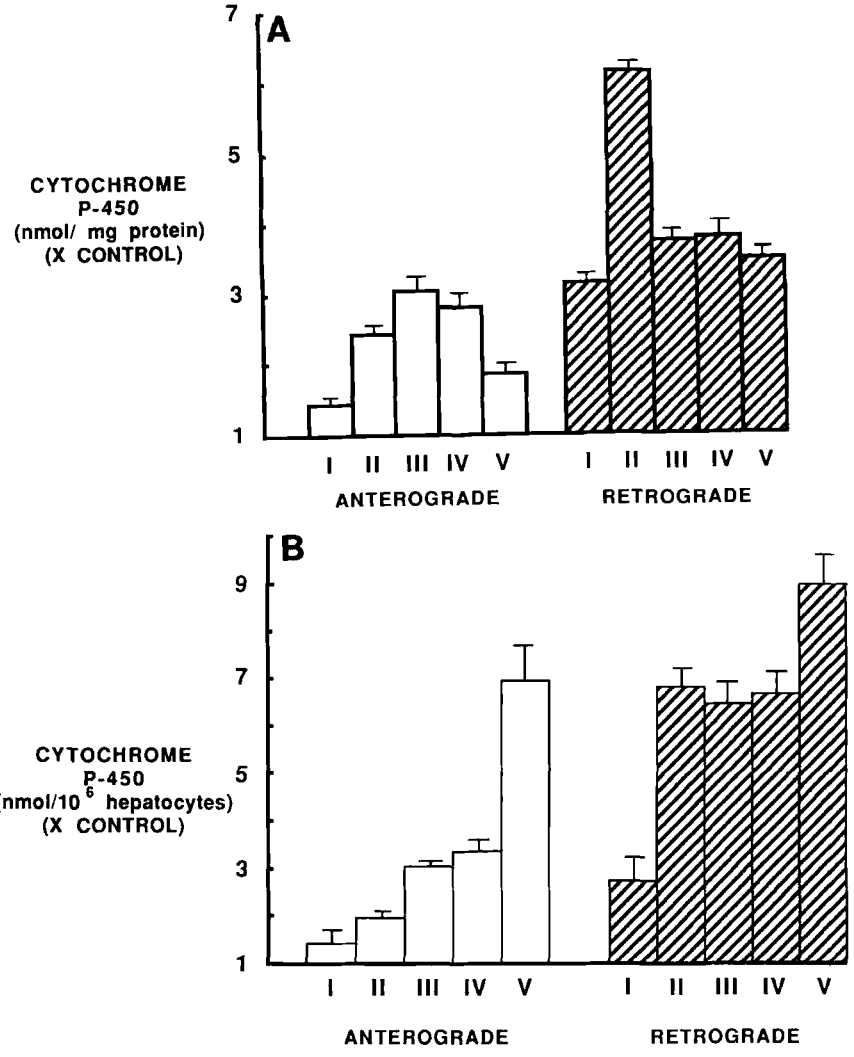

FIG. 6. *Cytochrome P-450 content in hepatocytes fractionated from anterograde or retrograde preparations by centrifugal elutriation.* (A) In the *ordinate*, values of total cytochrome P-450 (as determined spectrophotometrically) are presented relative to corresponding control values (× control) and expressed as nmoles per milligram cellular protein. In the *abscissa*, Fractions I to V correspond to each of the five fractions obtained by centrifugal elutriation. Anterograde perfusions = *clear bars*; retrograde perfusions = *shaded bars*. Mean ± S.E., n = 5 experiments per group. (B) In the *ordinate*, cytochrome P-450 is expressed as nmoles per 10^6 hepatocytes and in relationship to the corresponding control values. Hepatocyte number per fraction was determined in a hemocytometer. The *abscissa* represents the five fractions obtained after centrifugal elutriation of hepatocytes isolated by anterograde or retrograde perfusions with collagenase. Mean ± S.E., n = 5 experiments per group.

to separate well-preserved from partially damaged cells (31). In these experiments, about two-thirds of the hepatocytes isolated at zero time were intensely fluorescent and morphologically intact both in controls and after PB. The remaining one-third of hepatocytes were not fluorescent. These nonfluorescent hepatocytes may have been partially damaged during isolation, losing the fluorescent label, they may have represented hepatocytes separated from the unlabeled zone distal to the direction of collagenase perfusion or both situations may have occurred. Centrifugation of hepatocytes obtained at zero time in a Percoll gradient resulted in an increment in the percentage of fluorescent hepatocytes from 66 to about 90%. This increment had to be secondary to the separation of partially damaged hepatocytes, which had lost their fluorescence, from the intact, fluorescent cells. Indeed, when the light fraction of hepatocytes, A-1, was examined, most cells were partially damaged and nonfluorescent. Therefore, perfusion with collagenase in either direction did not result in the release of cells representative of all hepatocytes, as it is commonly assumed, but rather in the predominant release of those hepatocytes first perfused with this enzyme (which in this design were also the hepatocytes labeled with acridine orange). The nonfluorescent hepatocytes released at zero time represented mainly damaged cells rather than nonlabeled "contaminants." The percentage of nonlabeled "contaminant" hepatocytes was better appreciated in the A-2 fraction. In this fraction, all hepatocytes were well-preserved, but even so there were about 10% of them not fluorescent. These hepatocytes had to originate from the nonlabeled zones distal to the entry of the collagenase perfusate. Therefore, centrifugation in Percoll density gradients separated the intact, intensely fluorescent hepatocytes (A-2) from the partially damaged cells (A-1) which had lost their fluorescence. Of interest, the pattern of fluorescence assessed in isolated hepatocytes was similar in experiments using the conventional single perfusion or the double perfusion approach. Moreover, separation of hepatocytes in Percoll obtained as high a percentage of viable and fluorescent hepatocytes as the trypsin-DNAase treatment used in the double perfusion. This may represent a significant advantage in experiments in which the presence of these enzymes is not desirable.

The fact that these results did not depend on the intracellular presence of acridine orange was shown by the similar sedimentation behavior in Percoll of hepatocytes labeled *in vitro* with acridine orange and of the unlabeled hepatocytes separated from the same liver. For this reason, once the origin of the hepatocytes isolated by anterograde or retrograde perfusions with collagenase had been established in all experimental groups used in this study, acridine orange labeling was omitted. However, periodic "quality control" experiments with acridine orange were performed to ensure reproducibility of the original data.

Hence, the labeling of the acinus with acridine orange enabled the assessment of the zonal origin of the hepatocytes released by either anterograde or retrograde conventional perfusions with collagenase. Centrifugation in Percoll density gradients resulted in the harvesting of hepatocytes located predominantly in the half of the acinus that first made contact with collagenase both in controls and after PB.

The functional heterogeneity of the isolated hepatocytes was assessed by measuring the induction of cytochrome P-450 in response to the *in vivo* administration of PB. Differences in total cytochrome P-450 content between hepatocytes separated in A-2 after an anterograde or a retrograde perfusion with collagenase were significant, but not as striking as predicted from immunofluorescence data (25–27). This result was interpreted as suggestive of the presence in anterograde or retrograde A-2 fractions of transitional, PB-inducible hepatocytes (Zone 2). It was reasoned that if proximal or distal hepatocytes could be further separated into fractions, functional differences among hepatocytes comprising each population may become apparent. After centrifugal elutriation of A-2, more clear-cut patterns of heterogenous response were observed. Within populations separated by anterograde or retrograde perfusions, there were hepatocytes responding poorly to PB induction, others responding in an intermediate manner and finally, others responding with high levels of cytochrome P-450 induction. It also became apparent that since there was a group of hepatocytes separated by centrifugal elutriation in Fraction I, which represented 40% of all hepatocytes, the response of proximal or distal hepatocytes previously separated in A-2 was mainly determined by this fraction. The highest inductive responses to PB occurred in hepatocyte fractions which represented between 15 and 25% of all hepatocytes. These inductive responses would have been overlooked had centrifugal elutriation of proximal and distal hepatocytes not been performed. The zonal origin of each of the fractions obtained after centrifugal elutriation, however, will have to be determined by a more direct approach than the one offered in this study. It is also possible that a better definition of the functional differences among hepatocytes may have resulted from the measurement of the inductive response of specific PB-inducible cytochrome P-450 forms in microsomal fractions. However, the limited number of hepatocytes obtained in each fraction precluded these additional determinations.

In conclusion, it is proposed that the directional perfusion of the liver with collagenase, followed by the separation of the hepatocytes in a Percoll density gradient, isolated hepatocytes originating predominantly in either the proximal or distal half of the liver acinus. Centrifugal elutriation of proximal and distal hepatocytes uncovered various patterns of inductive response of cytochrome P-450 to PB, opening the possibility for the study of the molecular mechanisms responsible for this heterogeneous induction.

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REFERENCES

1. Gumucio JJ, Miller DL. Functional implications of liver cell heterogeneity. Gastroenterology 1981; 80:393–403.
2. Jungermann K, Katz N. Functional hepatocellular heterogeneity. Hepatology 1982; 2:385–395.
3. Loud AV. Quantitative stereological description of the ultrastructure of normal rat liver parenchymal cells. J Cell Biol 1968; 37:27–46.
4. Jones AL, Schmucker DL, Mooney JS, et al. Morphometric analysis of rat hepatocytes after total biliary obstruction. Gastroenterology 1976; 71:1051–1061.
5. Grisham JW. A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver. Autoradiography with ^{3}H thymidine. Cancer Res 1962; 22:842–849.
6. Novikoff AB. Cell heterogeneity within the hepatic lobule of the rat (staining reactions). J Histochem Cytochem 1959; 7:240–244.
7. Katz N, Jungermann K. Autoregulatory shift from fructolysis to lactate gluconeogenesis in rat hepatocyte suspensions. Hoppe-Seyler's Z Physiol Chem 1976; 357:359–375.
8. Gumucio JJ, Balabaud CP, Miller DL, et al. Bile secretion and liver cell heterogeneity in the rat. J Lab Clin Med 1978; 91:350–362.
9. Jones AL, Hradek GT, Renston RH, et al. Autoradiographic evidence for hepatic lobular concentration gradient of bile acid derivative. Am J Physiol 1980; 238:G233–G237.
10. Groothius GMM, Hardonk MJ, Katja P, et al. Autoradiographic and kinetic demonstration of acinar heterogeneity of taurocholate transport. Am J Physiol 1982; 243:G455–G462.
11. Thurman RG, Kaufmann FC. Sublobular compartmentation of pharmacological events (SCOPE): metabolic fluxes in periportal and pericentral regions of the liver lobule. Hepatology 1985; 1:144–157.
12. Rappaport AM. The microcirculatory hepatic unit. Microvasc Res 1973; 6:212–228.
13. Bernaert D, Wanson JC, Mosselmans R, et al. Separation of adult rat hepatocytes into distinct subpopulations by centrifugal elutriation. Morphological, morphometrical and biochemical characterization of cell fractions. Biol Cellulaire 1979; 34:159–174.
14. Sweeney GD, Garfield RE, Jones KG, et al. Studies using sedimentation velocity on heterogeneity of size and function of hepatocytes from mature male rats. J Lab Clin Med 1978; 91:423–430.
15. Epstein CJ. Cell size, nuclear content, and the development of polyploidy in the mammalian liver. Proc Natl Acad Sci 1967; 57:327–334.
16. Wanson JC, Drochmans P, May C, et al. Isolation of centrilobular and perilobular hepatocytes after phenobarbital treatment. J Cell Biol 1975; 66:23–41.
17. Sumner IG, Freedman RB, Lodola A. Characterisation of hepatocyte sub-populations generated by centrifugal elutriation. Eur J Biochem 1983; 134:539–545.
18. Gumucio JJ, DeMason L, Miller DL, et al. Induction of cytochrome P-450 in a selective sub-population of hepatocytes. Am J Physiol 1978; 234:C102–109.
19. Rojkind M, Portales ML, Cid ME. Isolation of rat liver cells containing concanavalin-A receptor sites. FEBS Lett 1974; 4711–4714.
20. Vaananen H, Lindros KO, Salaspuro M. Selective isolation of intact periportal or perivenous hepatocytes by antero or retrograde collagenase gradient perfusion. Liver 1983; 3:131–139.
21. Yuan PM, Ryan DE, Levin W, et al. Identification and localization of aminoacid substitutions between two phenobarbital-inducible rat hepatic microsomal cytochromes P-450 by microsequence analysis. Proc Natl Acad Sci 1983; 80:1169–1173.
22. Adesnik M, Bar-Nun S, Maschio F, et al. Mechanism of induction of cytochrome P-450 by phenobarbital. J Biol Chem 1981; 256:10340–10345.
23. Hardwick JP, Gonzalez FJ, Kasper CB. Transcriptional regulation of rat liver epoxide hydratase, NADPH-cytochrome P-450 oxidoreductase, and cytochrome P-450 genes by phenobarbital. J Biol Chem 1983; 258:8081–8085.
24. Walz FG, Vlasuk GP, Omieenski CJ, et al. Multiple, immunoidentical forms of phenobarbital-induced rat liver cytochromes P-450 are encoded by different mRNAs. J Biol Chem 1982; 257:4023–4026.
25. Gooding PE, Chayen J, Sawyer B, et al. Cytochrome P-450 distribution in rat liver and the effect of sodium phenobarbitone administration. Chem Biol Interactions 1978; 20:299–310.
26. Baron J, Rednick JA, Guengerich P. An immuno-histochemical study on the localizations and distributions of phenobarbital and 3-methyl-cholanthrene-inducible cytochromes P-450 within the livers of untreated rats. J Biol Chem 1981; 256:5931–5937.
27. Baron J, Rednick JA, Guengerich P. Effects of 3-methyl-cholanthrene, β-naphtoflavone, and phenobarbital on the 3-methyl-cholanthrene-inducible isozyme of cytochrome P-450 within centrilobular, midzonal, and periportal hepatocytes. J Biol Chem 1982; 257:953–957.
28. Gumucio JJ, Miller DL, Krauss MD, et al. Transport of fluorescent compounds into hepatocytes and the resultant zonal labelling of the hepatic acinus in the rat. Gastroenterology 1981; 80:639–646.
29. Hanks JH, Wallace RE. Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc Soc Exp Biol Med 1949; 71:196–201.
30. Ingebretsen WR, Wagle SR. A rapid method for the isolation of large quantities of rat liver parenchymal cells with high anabolic rates. Biochem Biophys Res Commun 1972; 42:408–410.
31. Singh B, Borrebaeh B, Osmundsen H. Separation of different cell populations of rat liver by density gradient centrifugation in a vertical rotor with self-generated Percoll gradients. Acta Physiol Scand 1983; 117:497–505.
32. Baur H, Kasperek S, Pfaff E. Criteria of viability of isolated liver cells. Hoppe-Seyler's Z Physiol Chem 1975; 356:827–838.
33. Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. J Biol Chem 1964; 239:2379–2385.
34. Estabrook RW, Peterson J, Baron J, et al. Principles of spectrophotometric measurements of turbid suspensions. Meth Pharmacol 1972; 2:303–350.
35. Lowry HO, Rosebrough NJ, Farr AL, et al. Protein measurement with Folin phenol reagent. J Biol Chem 1951; 193:265–275.
36. Adams LR, Kamentsky LA. Machine characterization of human leukocytes by acridine orange fluorescence. Acta Cytol 1971; 15:289–292.