Separation of Hepatocytes of Different Acinar Zones by Flow Cytometry[1]

T. Thalhammer, A. Gessl, I. Braakman, and J. Graf

Department of General and Experimental Pathology, University of Vienna, A-7090 Vienna, Austria (T.T., A.G., J.G.); Department of Pharmacology and Therapeutics, University of Groningen, Groningen, The Netherlands (I.B.)

Hepatocytes in the proximal (zone 1) and distal (zone 3) regions of the liver acinus are selectively stained by perfusion of the isolated rat liver with 0.2–20 μM acridine orange (AO). After 10–60 min of anterograde perfusion, AO fluorescence is visible in zone 1 cells, whereas retrograde perfusion stains cells of zone 3. In this paper, we describe a technique to isolate a mixed population of fluorescent and nonfluorescent hepatocytes (cells from all acinar zones, which do not loose the zone specific AO labeling) and to separate these cells according to their zonal origin by fluorescence activated cell sorting. The zonal populations obtained were either fluorescent or non-fluorescent (purity >95%). Separated cell fractions differed in their enzyme content (5' nucleotidase, succinate-dehydrogenase, β-glucuronidase). An unidentified AO metabolite, which is not found in bile after retrograde perfusion (not formed in zone 3 cells), is also absent after retrograde perfusion in sorted fluorescent cells (zone 3 cells), indicating zonal purity of sorted cells.

Key terms: Heterogeneity of hepatocytes, fluorescence staining, rat liver, acridine orange

The microcirculatory unit of the liver, the liver acinus (20) consists of hepatocytes, which are functionally and biochemically heterogeneous (number and size of cell organelles, enzyme content, biliary transport, mitotic rate, toxin sensitivity; for reviews, see 12,15). This heterogeneity results from the fact that cells within the acinus are not equally exposed to nutrients, oxygen, and other compounds: Blood supply originates from the portal triads containing the hepatic artery and portal venous branches (center of zone 1) and blood drainage is through the central vein (center of zone 3). The intermediate cells of zone 2 seem to adapt according to nutrient and oxygen supply either to zone 1 or to zone 3 cells (Fig. 1).

Some previous methods to isolate hepatocytes originating from different acinar zones relied on the zone-specific damage of liver cells by toxins (10,19) or modifications in zonal specific cell density by application of phenobarbital prior to cell isolation (27,29). Other methods use various centrifugation methods (1,5,7,23–25,28) and affinity chromatography with lectins (21) but have revealed preparations of variable purity. Identification of obtained cell fractions relying on zonal enzyme distribution remained unsatisfactory in that no distinct enzymatic heterogeneity was observed.

In a previous paper, Gumucio et al. (13) reported that several fluorescent compounds accumulate in zone 1 or zone 3 after anterograde or retrograde perfusion, respectively, and that cells can be isolated without redistribution of fluorescence. In this study we use acridine orange [3,6-bis(dimethylacridine)] (AO) for selective zonal staining, collagenase perfusion for cell isolation, and fluorescence-activated cell sorting on a Becton Dickinson FACS-III for separation of fluorescent and nonfluorescent cells. The method described is different from previous techniques in that it avoids zone specific toxic cell damage and provides simultaneously zone 1 and zone 3 cell populations of high purity, which allows study of intrinsic functional heterogeneity of liver cells of acinar zones 1 and 3, the yield of the cell preparation being limited by the speed of fluorescence-activated cell sorting.

MATERIALS AND METHODS

Male unfasted 150–200 g rats (strain Louvain; obtained from Institut für Versuchstierzucht, Himberg,
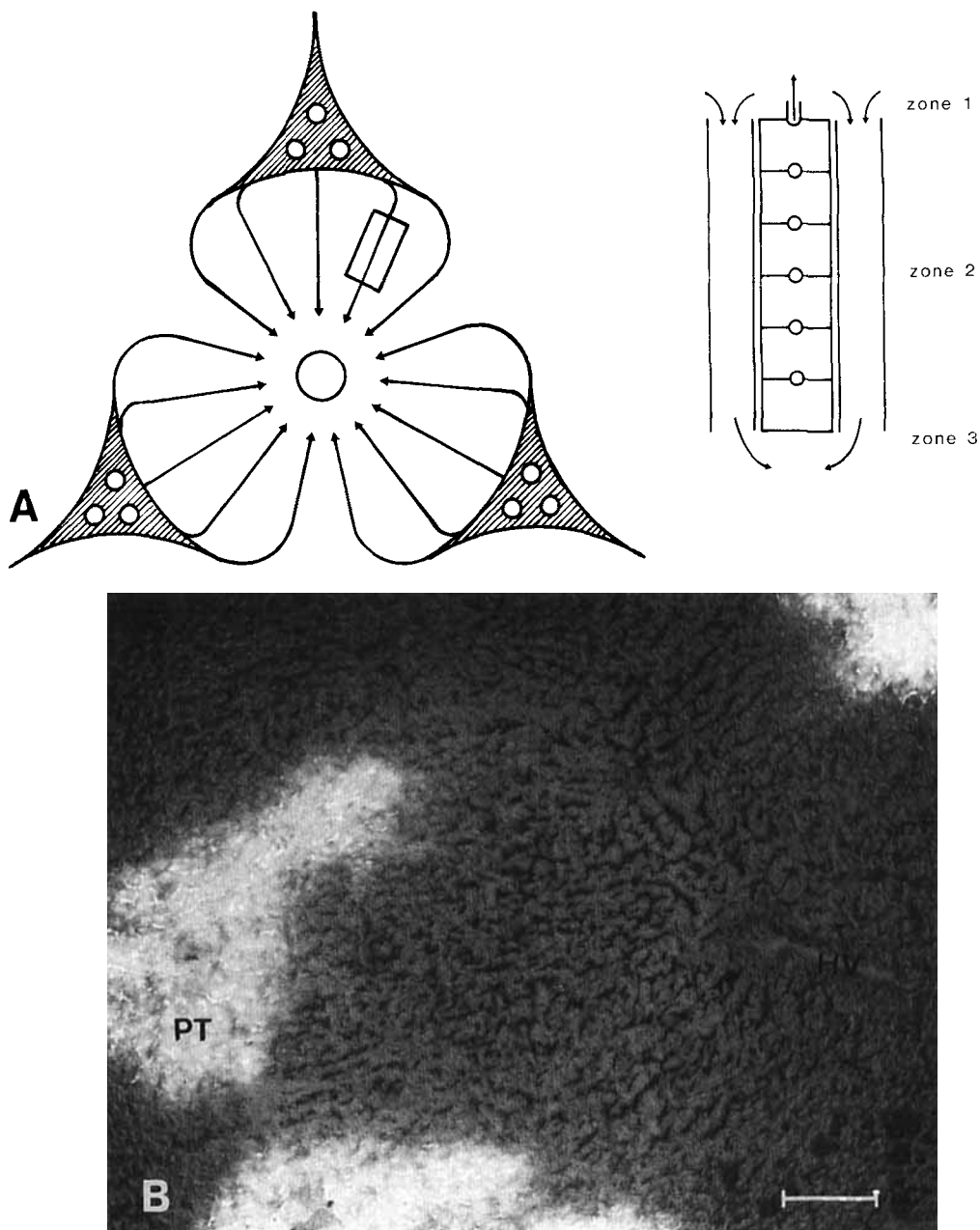
FIG. 1. **A:** Schematic drawing of liver architecture. This idealized scheme shows three portal fields (triangular, hatched areas) and one central vein. The portal fields contain terminal branches of the hepatic artery, the portal vein, and the bile duct (portal triads). The liver parenchyma (area between portal fields and central vein) receives mixed blood supply from the hepatic artery and the portal vein. Blood flows through the liver sinusoids as indicated by the arrows and is drained by the central vein. Thus the central vein, as the center of a liver "lobule," receives blood from three portal fields. Conversely, each portal field, as the center of a liver "acinus," provides blood supply to three central veins; only the one common to three acini is shown. **Inset:** Liver parenchymal cells form flat plates, having contact to sinusoidal blood on both sides. According to the direction of blood flow from the center of a liver acinus to the center of a lobule, parenchymal cells are allocated to zones 1–3 of the liver acinus. Bile flows around liver cells in the reverse direction to blood flow, and bile is drained into the bile ducts of the portal fields. **B:** Fluorescence micrograph of a rat liver lobulus section. After anterograde perfusion with 2 μM AO, cells in zone 1 around the portal triad (PT) are fluorescent, whereas cells of zone 3 around the hepatic venule (HV) remain unstained. Bar = 100 μm.

Austria) were anesthetized with 20 mg thiopental per 100 g body weight, and the liver was perfused at 37°C in a nonrecirculating perfusion system at a rate of 3 ml · min^{-1} · liver^{-1} with Krebs-Henseleit buffer as described elsewhere (9), either through the portal vein (anterograde) or through the vena cava inferior (retrograde). AO fluorescence in the effluent perfusate and bile was measured on an Aminco (American Instru-

ment Comp., Silver Springs, MA) fluorescence spectrophotometer (excitation 493 nm, emission 575 nm).

Fluorescence labeling of different acinar zones was achieved by perfusion for 20 min with Krebs-Henseleit buffer containing 2 μM AO. Perfusion was either made anterograde to label cells of zone 1 or retrograde to label cells of zone 3. To localize AO within the acinus, after different times of perfusion, the tissue was frozen in liquid nitrogen, and 8–12 μm cryostat sections were cut and examined under the fluorescence microscope. Similarly, small pieces of liver were examined after 5–10 min of collagenase perfusion.

Cells were isolated according to Ingebretsen et al. (14), with several modifications: After 20 min perfusion with AO, the perfusion medium was switched to a Ca^{2+}-free Hanks I buffer. Perfusion was continued in the same direction for 10 min in the open perfusion system, then perfusion was switched to a recirculating system containing Hanks II buffer supplemented with 2.5 mM $CaCl_2$ and 0.05% collagenase Type I (Sigma, St. Louis, MO) (80 ml). Perfusion was finished after 10–15 min, when the liver became soft, and fluid appeared on the surface. Then, the liver was immersed into precooled, supplemented Hanks II medium and all the following procedures were performed at 4°C to avoid redistribution of AO. The liver capsula was opened. Cells were carefully separated with a spatula and transferred into a modified Krebs/bicarbonate/Hepes medium (modified Krebs buffer; see below). After shaking of the suspension (approximately 200 ml) for 3 min at 4°C, aggregates were removed by filtrating twice through a 60 μm nylon mesh (PA-60/33; ICI, Mellingtree, England). Cells were centrifuged (40g, 3 min) and washed twice with the modified Krebs buffer. Finally, cells were suspended in phosphate-buffered Leibowitz 15 cell culture medium (L 15) to a final concentration of 2–3 × 10^6 cells/ml.

Modified Krebs buffer contained (mM) 107.7 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.19 KH_2PO_4, 0.13 $CaCl_2$, 25 $NaHCO_3$, 10 Hepes, 10 glucose, and 0.5–1% bovine serum albumin, fraction V (BSA; Sigma). Before addition of BSA, the buffer was preequilibrated with 95% O_2 and 5% CO_2, and the pH was titrated to 7.4 at 4°C.

To separate intact hepatocytes from partially damaged cells (cells showing a granular cytoplasm or protrusions of the plasma membrane, although excluding trypan blue) and nonviable cells (cells that incorporate trypan blue), hepatocytes were loaded on a self-forming Percoll gradient according to Gumucio et al. (11). This method was modified as follows. The gradient solution was prepared from a stock solution of 2.5 M sucrose, 50 mM Hepes, and 1% BSA. One part of this stock solution was diluted 1:10 with distilled water (solution A), and for solution B one part of the stock solution was mixed with nine parts of Percoll (v/v). Solution A (~100 ml) was mixed with solution B (~120 ml) in order to obtain an osmolarity of about 300, $n_D^{4°C}$ = 1.3550 and titrated to pH 7.4 with 0.1 N HCl.

Centrifugation was carried out in a Beckman Ti 50

fixed-angle rotor at 15,500 rpm for 20 min. Intact hepatocytes were isolated at a density of 1.12–1.14, whereas damaged cells were floating on the top of the gradient. Intact hepatocytes were collected, washed once with modified Krebs buffer, resuspended in L 15 medium, counted, and assessed for viability, which was greater than 90% by trypan blue exclusion.

In this suspension, the percentage of fluorescent cells was analyzed with the fluorescence microscope (Leitz, Wetzlar, BRD) equipped with an I 2/3 filter block (band-pass filter 450–490; reflexion short-pass filter 520; long-pass filter 515) (Leitz Nr. 513604). In accordance with Gumucio et al. (13), cells showing bright green cytoplasmic fluorescence as well as green fluorescence of the nucleus were considered as "fluorescent," whereas cells showing no or weak cytoplasmic fluorescence were considered as "nonfluorescent."

For cell sorting, a FACS-III cell sorter (Becton Dickinson; Sunnyvale, CA) was used. The suspension obtained from the Percoll gradient was diluted in L 15 medium (1:1; v/v) and filtered once through the 60 μm nylon mesh. The presence of Percoll reduced sedimentation of cells. To avoid dye redistribution during sorting, the bulk of the cell suspension (~15 ml) was kept on ice, and 1 ml aliquots were added to the sample reservoir. Phosphate-buffered saline was used as the diluting transport vehicle (sheath fluid). Cells were sorted according to green fluorescence intensity (excitation 488 nm and emission >525 nm, linear gain). The fluorescence light was collected through a LP 515 glass and LP >520 multilayer optical filter. Technical data of separation were: 70 μm nozzle tip, 14 psi sheath fluid pressure, 17 psi sample pressure, excitation with a 5 W argon laser at a 300 mW laser output (Spectra Physics Series 2000; Mountain View, CA); 1,000–2,000 cells per sec were sorted. The data were processed in a Nuclear Data ND 100 multichannel analyzer. Data were stored in an Industrial Micro System 5,000 SX Computer (IMS International, Carson City, NV). Customer-written software acquired histogram data from the cytometer and was used to store and print. Sorted cells were collected in Eppendorf vials containing cold modified Krebs/Hepes buffer, pooled, washed once in cold modified Krebs-Hepes buffer, and used for further determinations.

For cell enzyme determinations, sorted cells were homogenized by sonication (Sonifier cell disruptor, Branson Sonic Power Co., Heusenstamm, FRG) in 250 mM sucrose, 10 mM Hepes/KOH, pH 7.4. 5'-Nucleotidase was determined according to Sigma Technical Bulletin No. 675; glucose-6-phosphatase according to de Duve et al. (4), with liberated inorganic phosphate being measured according to Fiske and Subbarow (8); succinate-dehydrogenase according to Earl and Korner (6); and β-glucuronidase according to Sigma Technical Bulletin, No. 325, with phenolphthalein-glucuronic acid as substrate. Protein was determined according to Bradford (3), with BSA as standard.

Thin-layer chromatography (TLC) of AO and its con-

jugates in single bile drops collected from the perfused liver and in cell homogenates (10 μl) was carried out on G 1500/LS 254 thin-layer plates (Schleicher & Schüll, Dassel, FRG). Plates were developed at room temperature in the solvent system n-propanol/HCOOH (70/30 v/v). AO and its various biotransformation products were visualized by ultraviolet (UV) illumination, and photographs were taken on Polaroid (type 107) films. The presence of glucuronide and sulfate conjugation products in bile was studied by incubation with β-glucuronidase and arylsulfatase for 2–24 hr at pH 6.8 and 7.4 at 37°C, respectively (2).

AO was obtained from Kodak-Eastman (Rochester, NY). BSA fraction V enzyme substrates, and collagenase (Sigma Type I) were purchased from Sigma. Percoll was obtained from Pharmacia (Uppsala, Sweden) and thiopental from Sanabo (Vienna, Austria). All other chemicals were purchased from Merck (Darmstadt, FRG) and were of analytical grade. Leibowitz L 15 cell culture medium was obtained from Gibco Bio-Cult Ltd. (Paisley, England).

RESULTS

Localization of AO in the Liver Acinus, Its Biliary Metabolism, and Labeling of Hepatocytes

In preliminary experiments, the isolated rat liver was perfused with solutions containing 0.2–20 μM AO for 10–60 min. This dye is effectively cleared from the perfusate, accumulates in the liver, and is concentrated in bile. With 2 μM AO concentration in the perfusion liquid, uptake of AO into the liver is complete, and, after 60 min, uptake is still above 98%. Enrichment in bile depends on AO concentration in the perfusate. At an AO concentration of 2 μM in the perfusion liquid, fluorescence in bile exceeds AO concentration in the perfusate 200-fold, whereas at 20 μM AO biliary fluorescence is 40-fold enriched. Bile flow was not affected during 60 min of perfusion. AO is rapidly biotransformed in the liver, and biotransformation products are seen in bile already after 5 min of anterograde or retrograde perfusion (shown in Fig. 2). A glucuronidation product in bile is seen after antero- and retrograde perfusion. Sulfated products were not identified. During retrograde perfusion a chemically unidentified metabolite is absent from bile and does not appear even after 40 min. This metabolite is apparently produced only by cells from zone 1, and during retrograde perfusion these cells are not reached by the AO due to complete uptake in zone 3.

Complete uptake of AO in early segments of the sinusoids is also documented by fluorescence microscopy (Fig. 1). After 30 min perfusion with 2 μM AO, a fluorescent zone extends to approximately one-third of the sinusoid length, and centroacinar or centrolobular (zone 1 or 3) staining is seen after antero- and retrograde perfusion, respectively. With increasing concentrations, AO fluorescence extends further downstream of the sinusoids, a phenomenon also seen with other compounds that are effectively cleared from the blood stream (16). Therefore, 30 min of perfusion and 2 μM of AO were used in standard protocol. Continuing the perfusion with Ca^{2+}-free Hanks buffer for 10 min results in an insignificant release into the effluent perfusate (3% of fluorescence accounted for) of the dye. Also, after subsequent perfusion with collagenase for 10 min, the zonal staining with AO remains unchanged. Therefore, reversing the perfusion direction after dye loading appeared unnecessary. In accordance with the extent of cell labeling seen from frozen sections, 20–30% of isolated cells exhibited intensive fluorescence.

After isolation and Percoll gradient centrifugation, the final cell viability is >90%. To assess whether dye release from labeled cells and uptake into unlabeled cells occurs during cell preparation, unlabeled cells were mixed with fluorescent cells labeled in vitro with 2 μM AO for 5 min at 25°C. After gradient centrifugation, the same percentage of fluorescent cells was recovered. No difference in the equilibrium density (1.12–1.14) of cells isolated by anterograde or retrograde perfusion was observed.

Cell Separation by Flow Cytometry

Analysis of isolated cells by FACS III cytometry showed bright fluorescence of 20–30% of total cells as already observed by fluorescence microscopy. As seen on the respective dot plot (fluorescence intensity vs. forward-angle light scatter), some cells exhibit an intermediate fluorescence between extensive labeling and background (autofluorescence). These cells with low AO most likely originate from intermediate regions of the acinus (zone 2) downstream of the intensely labeled cells (Fig. 3). To exclude cells with low AO fluorescence and a high background fluorescence, appropriate windows were set to obtain subpopulations with >95% either fluorescent or nonfluorescent cells as calculated by fluorescence microscopy. Obviously, higher purities were obtained at lower sorting rate (<2,000 cells/sec). Cell viability after sorting decreased to 60–80%, perhaps due to a relatively high sensitivity of these epithelial cells to mechanical or irradiation damage, whereas recovery was >80%.

To identify sorted cells, enzyme activities, whose different acinar localisation had been previously determined, mainly by histochemical techniques (15,18,22), were measured. Hepatocytes from zone 1 exhibit higher activities of succinate-dehydrogenase and β-glucuronidase, whereas 5′-nucleotidase is slightly enriched in cells from zone 3. No difference in glucose-6-phosphatase was seen (Table 1).

DISCUSSION

The method described in this study takes advantage of the capability of hepatocytes to remove the fluorescent dye AO effectively from the blood stream. Therefore, labeling of cells of the acinar zones 1 and 3 is obtained by anterograde and retrograde perfusion, respectively. Labeled cells retain the dye during cell
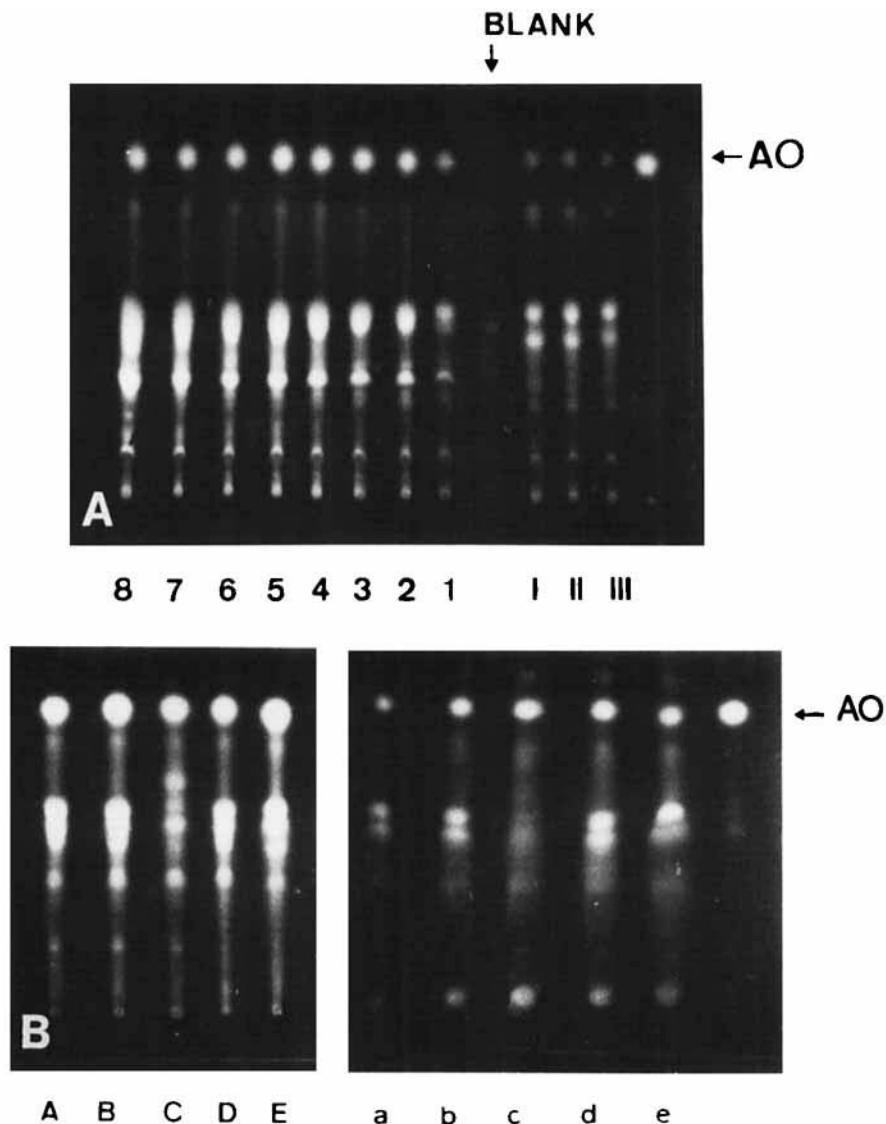
FIG. 2. **A:** Thin-layer chromatography of bile samples after antero-grade and retrograde perfusion with 2 μM AO. Bile samples 1–8 were collected every 5 min during anterograde and samples I, II, and III after 10, 25, and 40 min of retrograde perfusion. **B:** Identification of the AO glucuronide in bile. **Left:** After anterograde perfusion. **Right:** After retrograde perfusion. A,a, bile untreated; B,b, bile incubated at pH 6.8; C,c, bile incubated at pH 6.8 with β-glucuronidase; D,d, bile inbuated at pH 7.4; E,e, bile incubated at pH 7.4 with arylsulfatase.

isolation with collagenase, and fluorescent and non-fluorescent cells can be separated by fluorescence-activated cell sorting. Similar studies have been carried out using fluorescence diacetate as a marker (17). As far as dye staining and dye metabolism are concerned, cell populations obtained by the technique described appear highly enriched in cells either originating from zone 1 or zone 3.

In the liver, xenobiotic compounds are metabolized in the endoplasmatic reticulum by two sequential processes. Phase 1 metabolism involves reactions by oxidoreductases and hydrolases; phase 2 reactions result in formation of better water-soluble compounds by glucuronidation and sulfation. Our studies showed that AO is metabolized and glucuronidated in cells of both acinar zones. However, one metabolite not excreted into bile after retrograde perfusion is not found in zone 3 cells and confirms the enzymatic and fluorescence cell identification. This metabolite, which is more hydrophilic than AO and its glucuronide and so far determined to be no sulfate conjugate, is thus exclusively produced in cells of acinar zone 1. This is interesting in that heterogeneity of metabolizing enzymes with some preference for zone 3 cells has been reported (26) and requires further investigations.

Measurements of enzyme markers in the populations are in good agreement with previous reports on the zonal distribution (15,22) and AO fluorescence distri-
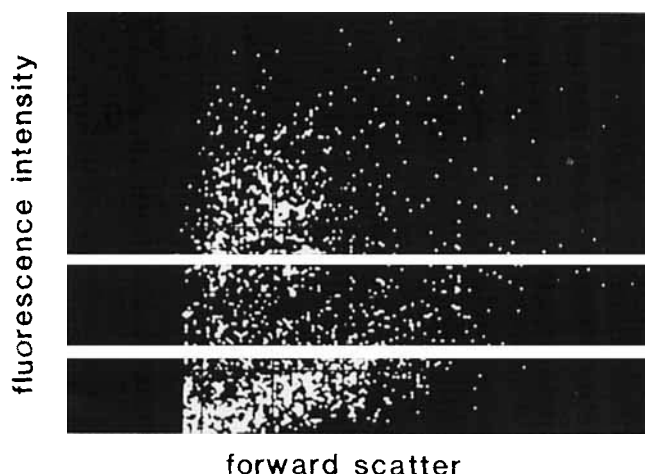
forward scatter

FIG. 3. Separation of AO-stained rat hepatocytes on a FACS-III flow cytometer. Dot plot was obtained after 30 min of anterograde perfusion with 2 μM AO. Sorting windows are indicated by the two lines.

Table 1
Distribution of Marker Enzymes in Zone 1 and Zone 3 Hepatocytes[a]

	zone 1	zone 3
5'-Nucleotidase	3.33 ± 0.48	6.51 ± 0.58**
Succinate dehydrogenase	3.38 ± 0.82	2.21 ± 0.37*
β-Glucuronidase	1.05 ± 0.26	0.57 ± 0.17**
Glucose-6-phosphatase	2.62 ± 1.20	2.55 ± 0.70

[a]Activities are given in μmoles $\cdot$ hr^{-1} $\cdot$ mg protein^{-1}. Values are means $\pm$ SD. Cells of zones 1 and 3 were obtained by anterograde and retrograde perfusion, respectively.

Metabolites of AO in the fluorescent cell population were also analyzed (Fig. 4). In accordance with thin-layer chromatograms of bile, the glucuronide was identified in both cell populations, whereas an unidentified metabolite was absent in cells of zone 3 obtained by retrograde perfusion with AO.

*$P < 0.05$ (Student's t test).
**$P < 0.001$ (Student's t test).

bution. However, because functional heterogeneity is dynamic depending on adaption of hepatocytes to different metabolic requirements rather than being due to stable (11) differences in enzyme content in subpopulations, the differences between populations, although significant, are not as high as might be expected. Furthermore, it should be noted that there is a continuous gradient for these enzymes rather than a unique localization. This might also be the reason why for glucose-6-phosphatase no clear zonal distribution was found in our study.

In conclusion, this study shows that liver perfusion with AO and isolation of labeled cells by fluorescence-activated cell sorting allows one to obtain cell populations from zone 1 and zone 3 of the liver acinus at very high purity. The duration of cell sorting imposes a limit on the cell yield, though, so this separation method is recommended for applications when high cell purity rather than high cell count is required.
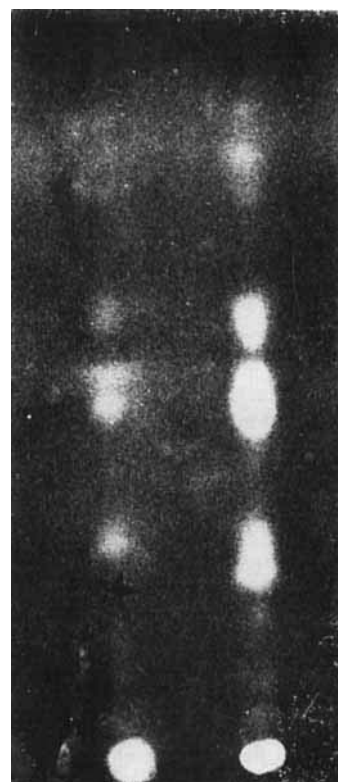

FIG. 4. Thin-layer chromatography of AO metabolites in FACS-III-sorted hepatocytes. Cells were labeled with 2 μM AO either by 30 min of retrograde (left) or anterograde (right) perfusion and sorted. The fluorescent subpopulation was homogenized, and 10 μl was used for chromatography. Note absence of a metabolite (star) in cells after retrograde perfusion.

LITERATURE CITED

1. Bengtsson BG, Kiessling KH, Smith-Kielland A, Morland J: Partial separation and biochemical characteristics of periportal and perivenous hepatocytes from rat liver. Eur J Biochem 118:591–967, 1981.
2. Braakman I, Groothuis GMM, Meijer DKF: Acinar redistribution and heterogeneity in transport of the organic cation rhodamine B in rat liver. Hepatology 7:849–855, 1978.
3. Bradford M: A rapid and sensitive method for the quantition of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254, 1976.
4. de Duve C, Wattiaux R, Baudhuin P: Distribution of enzymes between subcellular fractions in animal tissues. Adv Enzymol 24:291–358, 1962.
5. Drochmans P, Wanson JC, Mosselmans R: Isolation and subfractionation on ficoll gradients of adult rat hepatocytes. J Cell Biol 66:1–22, 1975.
6. Earl DCN, Korner A: The isolation and properties of cardiac ribosomes. Biochem J 94:721–734, 1965.
7. Epstein CJ: Cell size, nuclear content, and the development of polyploidy in the mammalian liver. Proc Natl Acad Sci USA 57: 327–334, 1967.
8. Fiske CH, Subbarow Y: The colorimetric determination of phosphorus. J Biol Chem 66:375–400, 1925.

9. Graf J, Peterlik M: Ouabain mediated sodium uptake and bile formation in the isolated perfused rat liver. Am J Physiol 230: 876–885, 1976.

10. Gumucio JJ, Katz ME, Miller DI, Balabaud CP, Greenfield JM, Wagner RM: Bile salt transport after selective damage to acinar zone 3 hepatocytes by bromobenzene in the rat. Toxicol Appl Pharmacol 50:77–856, 1979.

11. Gumucio JJ, May M, Dvorak C, Chianale J, Massey V: The isolation of functionally heterogeneous hepatocytes of the proximal and distal half acinus in the rat. Hepatology 6:932–944, 1986.

12. Gumucio JJ, Miller DJ: Functional implications of liver cell heterogeneity. Gastroenterology 80:393–403, 1981.

13. Gumucio JJ, Miller DL, Krauss MD, Zanolli CC: Transport of fluorescent compounds into hepatocytes and the resultant zonal labelling of the hepatic acinus in the rat. Gastroenterology 80: 639–646, 1981.

14. Ingebretsen WR Jr., Wagle SR: A rapid method for the isolation of large quantities of rat liver parenchymal cells with high anabolic rates. Biochem Biophys Res Commun 47:403–410, 1972.

15. Jungermann K: Functional heterogeneity of periportal and perivenous hepatocytes. Enzymes 35:161–180, 1986.

16. Nakatsugawa T, Bradford WL, Usui K: Hepatic disposition of parathion: uptake by isolated hepatocytes and chromatographic translobular migration. Pesticide Biochem Physiol 14:13–25, 1980.

17. Nakatsugawa T, Timoszyk J: Fluorescence labelling and cell-sorter separation of periportal and centrilobular rat hepatocytes. Hepatology 5:197, 1985.

18. Novikoff A: Cell heterogeneity within the hepatic lobule of the rat (staining reactions). J Histochem Cytochem 7:240–244, 1959.

19. Quistorff B: Digitonin perfusion in the study of metabolic zonation of the rat liver: potassium as an intracellular concentration reference. Biochem Soc Transact 15:361–363, 1987.

20. Rappaport AM: Anatomic considerations. In: Diseases of the Liver. Vol 1, Schiff L (ed). JB Lippincott, Philadelphia, 1975, pp 1–50.

21. Rojkind M, Portales MI, Cid ME: Isolation of rat liver cells containing concanavalin-A receptor sites. FEBS Lett 47:11–24, 1974.

22. Shank RE, Morrison G, Chen CH, Karl I, Schwartz R: Cell heterogeneity within the hepatic lobule (quantitative histochemistry). In: The Biochemistry Cytological of Liver (10th Annual Meeting of the Histochemical Society, Atlantic City, NJ), 1985, pp 237–239.

23. Stacey NH, Klaasen CD: Uptake of galactose, ouabain and taurocholate into centrilobular and periportal enriched hepatocyte subpopulations. J Pharmacol Exp Ther 216:634–639, 1981.

24. Sumner IG, Freedman RB, Lodola A: Characterisation of hepatocyte sub-populations generated by centrifugal elutriation. Eur J Biochem 134:539–546, 1983.

25. Sweeney GD, Garfield RE, Jones KG, Latham AN: Studies using sedimentation velocity on heterogeneity of size and function of hepatocytes from mature male rats. J Lab Clin Med 91:423–433, 1978.

26. Thurman RG, Kauffman FC, Baron J: Biotransformation and zonal toxicity. In: Regulation of Hepatic Metabolism. Intra- and Intercellular Compartmentation. Thurman RG, Kauffman FC, Jungermann K (eds.) Plenum Press, New York, 1986, pp 321–382.

27. Wanson JC, Drochmans P, May C, Penasse W, Popowski A: Isolation of centrolobular and perilobular hepatocytes after phenobarbital treatment. J Cell Biol 66:23–41, 1975.

28. Weigand K, Otto I, Schopf R: Ficoll density separation of enzymatically isolated rat liver cells. Acta Hepatogastroenterol 21: 245–253, 1974.

29. Willson RA, Liem HH, Miyai K, Müller-Eberhard U: Initial rate of sodium taurocholate uptake in isolated elutriated hepatocytes from untreated and phenobarbital-treated rats. Biochem Pharmacol 34:4257–4262, 1985.