Separation of Periportal and Perivenous Rat Hepatocytes by Fluorescence-activated Cell Sorting: Confirmation with Colloidal Gold as an Exogenous Marker

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Periportal and perivenous hepatocytes are known to display various functional differences. In this study we present a new method to separate periportal and perivenous cells: after selectively loading zone 1 or zone 3 with the fluorescent label acridine orange in an antegrade or retrograde perfusion, respectively, we separated the isolated hepatocytes on a fluorescence-activated cell sorter. The common way to check on proper separation is to estimate activities of enzymes known to exhibit a heterogeneous acinar distribution. Using enzyme histochemistry, however, we found that already on short collagenase perfusion, some enzymes displayed a more shallow gradient than *in vivo*, making enzyme activities less suitable as zonal markers. We therefore used colloidal gold granules (17 nm) injected intravenously (2.5 mg) into the rat 2 to 3 hr before cell isolation. The gold is taken up predominantly by perivenous hepatocytes, probably because of the efficient removal of gold granules in zone 1 by competing Kupffer cells. We compared acridine orange fluorescence, presence of gold particles and activities of six marker enzymes, three biochemically and three histochemically determined. Acridine orange and gold both pointed to a high enrichment of the fractions, whereas most enzyme activities were more randomly distributed among the cells as a result of the isolation procedure. Our separation procedure yielded fractions highly enriched in either viable periportal or perivenous cells, both from one liver. The use of colloidal gold as a marker to monitor separation is a valuable alternative to the more risky estimation of enzyme activities. (HEPATOLOGY 1991;13:73-82.)

Hepatic parenchymal cells are known to exhibit several differences in relation to their localization in the acinus (1). Best documented are the various differences in metabolic functions. Several biochemical studies in the intact organ (2-4) are complemented with numerous enzyme histochemical studies that show an acinar heterogeneity in the activities of many enzymes (2, 5, 6).

These enzyme activities in the different zones are used to predict metabolic fluxes in the liver, but they are also used as markers for cells prepared from the different zones of the hepatic acinus. In the past 10 yr several methods have been designed to separate periportal (pp) (zone 1) from perivenous (pv) (zone 3) cells, mostly with limited success. At first, conventional cell separation techniques were used, such as centrifugal elutriation (7, 8) or gradient centrifugation methods (9, 10), but unfortunately cells from the acinar zones differ only marginally in volume and density. More recent reports concern a collagenase gradient perfusion (11, 12) and a more or less conventional hepatocyte isolation after selectively damaging upstream cells with a short digitonin pulse (13, 14). The latter method is effective and currently the most generally used technique, although cells from only one zone are obtained. In addition, the cells contain some digitonin immediately after isolation, which has disappeared after 24 hr of cultivation (R. Gebhardt, Personal communication, May 1990). Quistorff, Grunnet and Cornell (15) found at a concentration of digitonin of 6 mg/ml (higher than the 4 or 5 mg/ml used for the separation procedure) an extraction fraction of 85% during the first 50 sec, which then rapidly dropped to 10%. This implies that digitonin reaches the distal hepatocytes at a concentration of at least 15% of the inlet concentration.

A striking observation in comparing most separation studies is that marker enzymes do not seem to provide absolute evidence for the success of the attempted cell separation. Some enzymes indicate a high enrichment of the fractions in pp or pv cells (13, 14, 16, 17) as compared with microdissection data or enzyme histochemical data from whole liver slices (18-22), but in almost every study one or more enzyme activities deviate from the supposed pattern, implying a lower or no enrichment at all (13, 14, 16, 17, 23-25).

In this paper we present a new method to obtain both viable pp and viable pv cells from one liver using a

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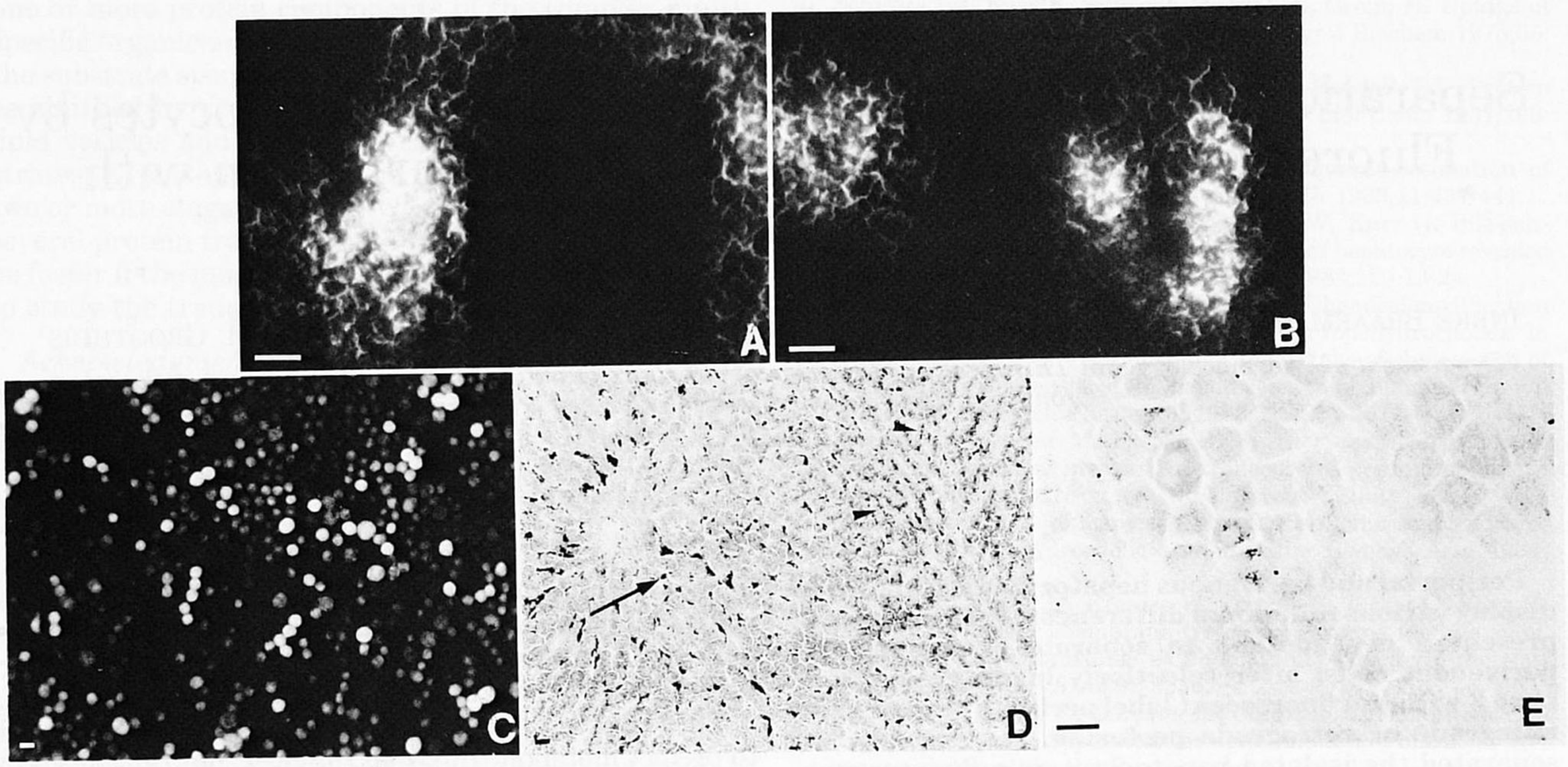

FIG. 1. AO localization and gold localization during the isolation procedure. Bar = 25 μm. (A) Periportal AO fluorescence after 8 min antegrade collagenase perfusion. (B) Perivenous AO fluorescence after 8 min retrograde collagenase perfusion. (C) AO fluorescence of total cell suspension to be sorted. (D) Gold localization 2 hr after intravenous injection of 2.5 mg gold granules. Gold is present in Kupffer cells in the pp area *(arrow)* and in hepatocytes in the pv area *(arrowhead)*. (E) Gold localization in total cell suspension.

fluorescence-activated cell sorter (FACS). Preliminary experiments have been published by Thalhammer et al. (25). Gumucio et al. (26) first reported the possibility of labeling a subset of hepatocytes with a fluorescent substance. We labeled upstream hepatocytes with acridine orange (AO) and separated the cells accordingly. We also show that enzyme activities may not be the best parameters to measure the actual enrichment of the separated fractions. We found an alternative in the form of an external marker. In 1985 Hardonk, Harms and Koudstaal (27) reported a zonal heterogeneity in the *in vivo* uptake of colloidal gold granules with a diameter of 17 nm. The granules were selectively taken up into zone 3 hepatocytes on intravenous injection, probably a result of a higher uptake of gold by Kupffer cells in zone 1 (27). This marker is expected to stay inside the cells during the isolation and separation procedures because the gold is directed to the lysosomes through the endocytic pathway. We show that colloidal gold is an excellent marker for zone 3 hepatocytes and hence can be used to determine the quality of pp and pv cell separation.

MATERIALS AND METHODS

Materials. Male Wistar rats (230 to 260 gm), fed *ad libitum*, were used as liver donors. Animal protocols were approved by the Bioethical Committee of Groningen University. The following compounds were obtained from the indicated sources: demineralized BSA from Organon Teknika (Oss, The Netherlands), AO from Eastman Kodak Company (Rochester NY), polyvinylpyrrolidone (PVP) of 40 kD and collagenase were from Sigma Chemical Company (St. Louis MO), Percoll was from Pharmacia Fine Chemicals (Woerden, The Netherlands), DePeX mounting medium "Gurr" from BDH Limited (Poole, England) and the silver enhancement kit (Intense II) was from Janssen Chimica (Beers, Belgium). All other chemicals were of analytical grade.

Preparation of Colloidal Gold Granules. Gold granules with a diameter of about 17 nm were prepared according to Frens (28). Three mg PVP per 100 ml gold sol was added to stabilize the granules.

Hepatocyte Isolation. Colloidal gold granules (2.5 mg/animal) were injected into the penile vein while the animals were under light ether anesthesia. Two to three hours after injection, the isolation procedure was started. Hepatocytes were isolated in an antegrade (from portal to hepatic vein) or retrograde (vice versa) fashion, depending on where the fluorescence label was to be introduced.

The portal vein was cannulated and a single-pass preperfusion was started with HBSS (112 mmol/L NaCl, 5.4 mmol/L KCl, 0.88 mmol/L KH_2PO_4, 0.66 mmol/L Na_2HPO_4, 25 mmol/L $NaHCO_3$, 10 mmol/L glucose, saturated with O_2/CO_2 [95:5], pH 7.45) without Ca^{2+} and Mg^{2+} and with 0.5 mmol/L EGTA for 5 min at 37° C. In a retrograde isolation the direction of flow was changed immediately after cannulation of the vena cava. During preperfusion the liver was carefully excised and placed in a holder on top of a funnel. AO was added to a concentration of 4 μmol/L and perfusion was continued for 5 min. This procedure led to the selective labeling of about one third of the acinus with AO, being zone 1 in an antegrade and zone 3 in a retrograde perfusion. EGTA was then washed out of the liver by a 2 min perfusion with HBSS with 2 mmol/L Ca^{2+}; collagenase was added to a concentration of 0.03% and the effluent of the liver was directed to the medium reservoir to obtain recirculation. The collagenase perfusion was carried out at 37° C until the liver

tissue was very soft and leaky (about 10 min). During the whole procedure, flow rate was about 30 ml/min and liver entry pressure did not exceed 9 cm H_2O.

The liver was transferred to a Petri dish on ice and the cells were freed from the Glisson's capsule. To prevent the AO from redistributing among the cells, all subsequent steps were carried out on ice. The suspension was filtered through nylon gauzes with 100 and 50 μm mesh, respectively, and washed three times with 100 ml Krebs-Henseleit bicarbonate buffer (KHB; 118 mmol/L NaCl, 5.0 mmol/L KCl, 1.1 mmol/L $MgSO_4$, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 10 mmol/L glucose, saturated with O_2/CO_2 [95:5], pH 7.45) supplemented with 10 mmol/L HEPES and 1% BSA. Cells were collected by centrifugation at 40 *g* for 2 min. The final cell pellet was resuspended in 60% Percoll (in 0.25 mol/L sucrose/5 mmol/L HEPES/0.1% BSA, pH 7.40) to prevent the hepatocytes from rapid sedimentation during separation with the FACS. Cell viability was assessed by trypan blue exclusion and was always greater than 90%.

Hepatocyte Separation. Cells were separated into a fluorescent and a nonfluorescent population based on their green AO fluorescence using a FACS 440 (Becton Dickinson, Sunnyvale CA). After gating out debris and clumped cells using forward and perpendicular light scatter (FSC and SSC, respectively), the populations to be sorted were chosen as the 30% of the cells with the highest fluorescence and the 30% of the cells with the lowest fluorescence, because this was the percentage of the acinus labeled with AO. Sorted cells were collected in KHB with 10 mmol/L HEPES and 4% BSA and kept at 4° C.

The FACS was equipped with an argon laser emitting 0.3 W at 488 nm. Green fluorescence was collected through a 530/30 BP filter (Becton Dickinson, Sunnyvale CA). To avoid cell damage we used a nozzle tip with an 80 μm diameter; the flow rate was kept at 1,500 events/sec. A typical sort lasted for about 3 hr. The yield was usually 10^6 cells/hr/fraction.

Fluorescence Microscopy Studies. Toward the end of the isolation procedure (after 8 min collagenase perfusion) pieces of liver were immediately frozen in liquid Freon (−96° C). Sections (8 μm) were cut in a cryostat at −20° C. Isolated hepatocytes (the starting suspension and the separated fractions) were centrifuged (5 min at 40 *g*) onto a slide in a cytocentrifuge and air-dried.

Slides with dried liver sections and hepatocytes both were used for staining reactions and fluorescence microscopy. Fluorescence was studied in unmounted sections with a Leitz Wetzlar ortholux microscope (Leitz; Wetzlar, FRG) with the appropriate fluorescence filters (excitation 450 to 490 nm; emission > 515 nm). Photographs were recorded on Kodak Ektachrome II film at 400 ASA.

Staining Reactions. Metallic gold was visualized with a silver enhancement kit. After enhancement for 10 min twice at room temperature, the sections and cells were counterstained with eosin or directly embedded in DePeX.

For histochemical staining the cells and sections were fixed in acetone at −20° C for 30 min and air-dried. After incubation for 45 min at 37° C with the proper medium for each enzyme, sections and cells were rinsed for 2 min with distilled water. Postfixation with 4% formaldehyde, 5.4% macrodex and 1% $CaCl_2$ in 0.81% NaCl was followed by rinsing again for 2 min with distilled water and subsequent mounting in glycerine-gelatine (29).

To visualize NADPH-tetrazolium reductase activity (NADPH-red), incubation was performed in 50 mmol/L phosphate buffer (pH 7.6), 0.61 mmol/L nitroblue tetrazolium and 1.2 mmol/L NADPH.

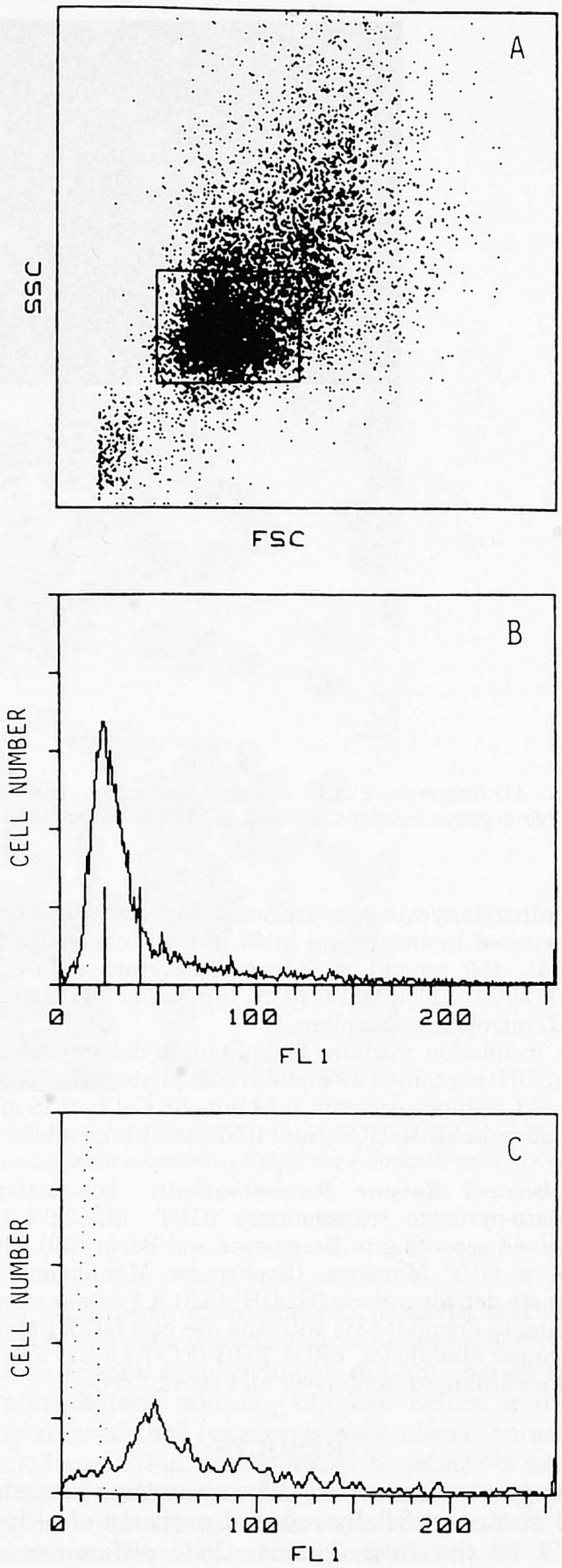

FIG. 2. Analysis of scatter features and AO fluorescence by FACS. (A) Scatter features of the total cell suspension. Rectangular area indicates gates that were set for FSC and SSC to eliminate debris (low FSC and SSC) and cell clumps (high FSC) from the sort. Only cells within the rectangle were sorted. (B,C) Analyses of AO fluorescence *(FL1)* of total suspension during sorting (B) and separated fractions reanalyzed after sorting (C). Vertical bars in B denote gates for FL1. (C) · · · · · · = AO-negative fraction (FL1 between 0 and left gate); ——— = AO-positive fraction (FL1 between right gate and 250). All parameters are in arbitrary units.

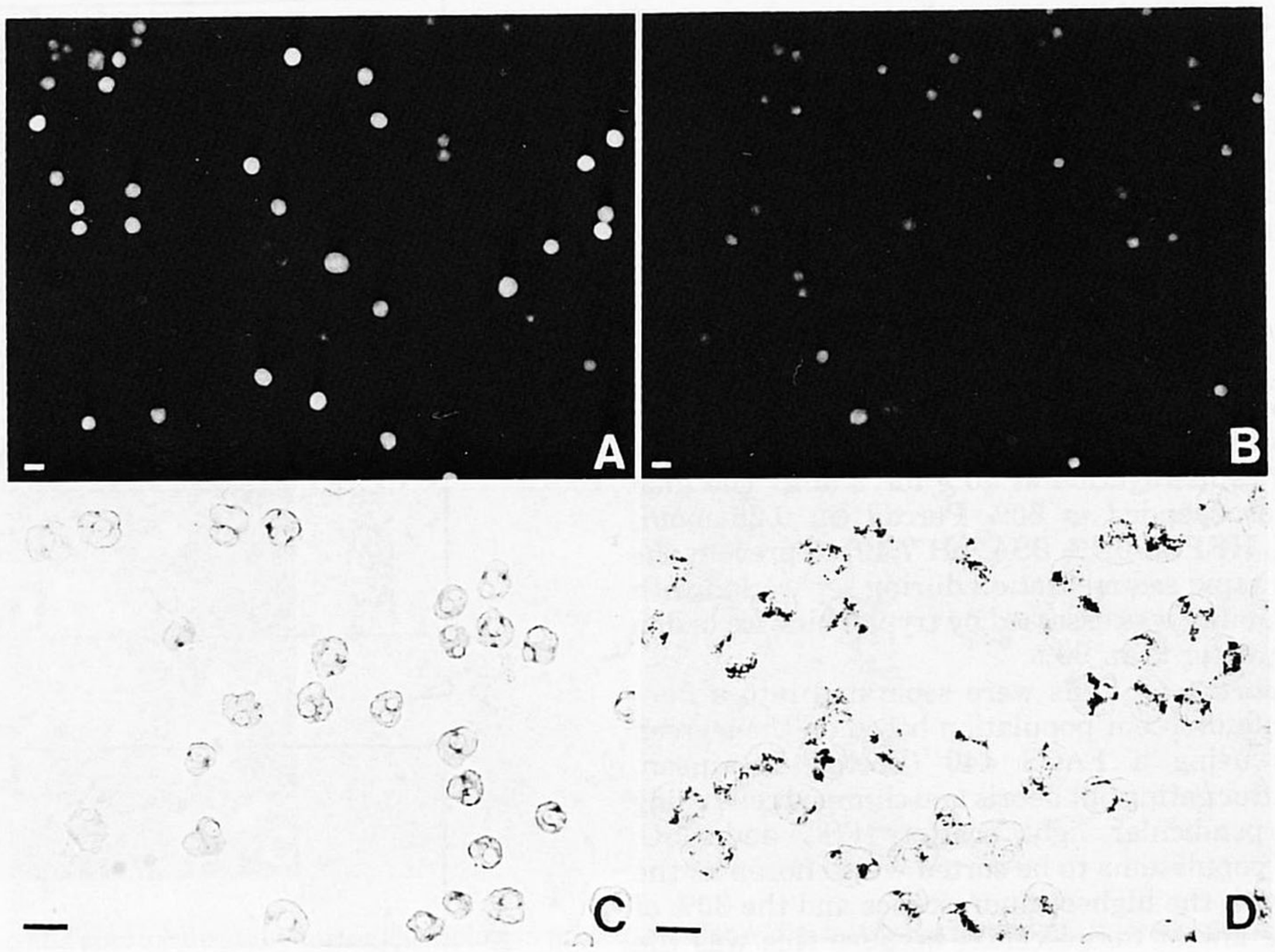

FIG. 3. AO fluorescence (A,B) and gold localization (C,D) in the separated fractions after antegrade isolation. AO-positive fractions in A and C, AO-negative fractions in B and D. Gold is only present in the AO-negative fraction. Bar = 25 μm.

β-Hydroxybutyrate dehydrogenase activity (βHBDH) was demonstrated by incubation in 67 mmol/L phosphate buffer (pH 7.4), 450 mmol/L DL-3-hydroxybutyrate sodium, 0.75 mmol/L NAD^+, 11 mmol/L KCN, 5 mmol/L $MgCl_2$ and 0.61 mmol/L nitroblue tetrazolium.

The incubation medium for succinate dehydrogenase activity (SDH) contained 47 mmol/L phosphate buffer (pH 7.6), 47 mmol/L sodium succinate, 0.14 mmol/L $CaCl_2$, 0.19 mmol/L $AlCl_3$, 28 mmol/L $NaHCO_3$ and 0.57 mmol/L nitroblue tetrazolium.

Biochemical Enzyme Determinations. The activity of glutamate-pyruvate transaminase (GPT; EC 2.6.1.2) was determined according to Bergmeyer and Bernt (30) with the use of a GPT Monotest (Boehringer Mannheim, FRG). Glutamate dehydrogenase (GLDH; EC 1.4.1.3) was measured according to Schmidt (31) with the use of a GLDH Monotest (Boehringer Mannheim, FRG). LDH (EC 1.1.1.27) was determined according to Bergmeyer and Bernt (32).

RESULTS

Hepatocyte Separation. The separation procedure is based on an artificially induced property of either the zone 1 or the zone 3 cells: their difference in AO fluorescence. It therefore is essential to check the localization of the fluorescent label up to the separation. Immediately after AO labeling, before the collagenase perfusion, fluorescence was only seen in a small pp area on antegrade perfusion, whereas in retrograde perfusion pv cells were labeled (not shown). The gradient was steep; downstream cells were completely devoid of AO. The collagenase perfusion caused the gradient to shallow a little, but AO was still absent in the downstream cells. This final zone 1 labeling is shown in Figure 1A and the zone 3 labeling in Figure 1B. The washed hepatocyte suspension consisted of cells with a large variety in labeling intensity: from highly fluorescent to nonfluorescent, as shown in Figure 1C. This total suspension was used for the separation procedure. Unless otherwise stated, all photographs presented are from a typical antegrade perfusion, resulting in a pp AO labeling (one third of the acinus).

Analysis of the fluorescence intensity of the fractions indicated a clear separation of the cells into an AO-negative and an AO-positive population (Fig. 2C). This picture agreed with Figure 3A and B. For all parameters measured and observed except the AO gradient, no difference existed between the antegrade and the retrograde isolation procedure. These parameters being gross appearance of the liver, medium flow, portal pressure, fluorescence distribution and colloidal gold distribution, trypan blue exclusion, liver and hepatocyte volume, enzyme activities and distribution after collagenase perfusion, FACS scatter and FACS fluorescence parameters. Comparison of the fluorescence of the sorted fractions (Fig. 2C) with fluorescence of the original sample (Fig. 2B) indicates that the single tailing peak of the total suspension had a fluorescence level between that of the separated fractions.

Quality of the Cells. Viability of the cells before and during separation was checked by trypan blue exclusion and by measuring the FSC and SSC of the cells. These parameters represent size, shape, viability and several other cellular characteristics (33). Separation of the

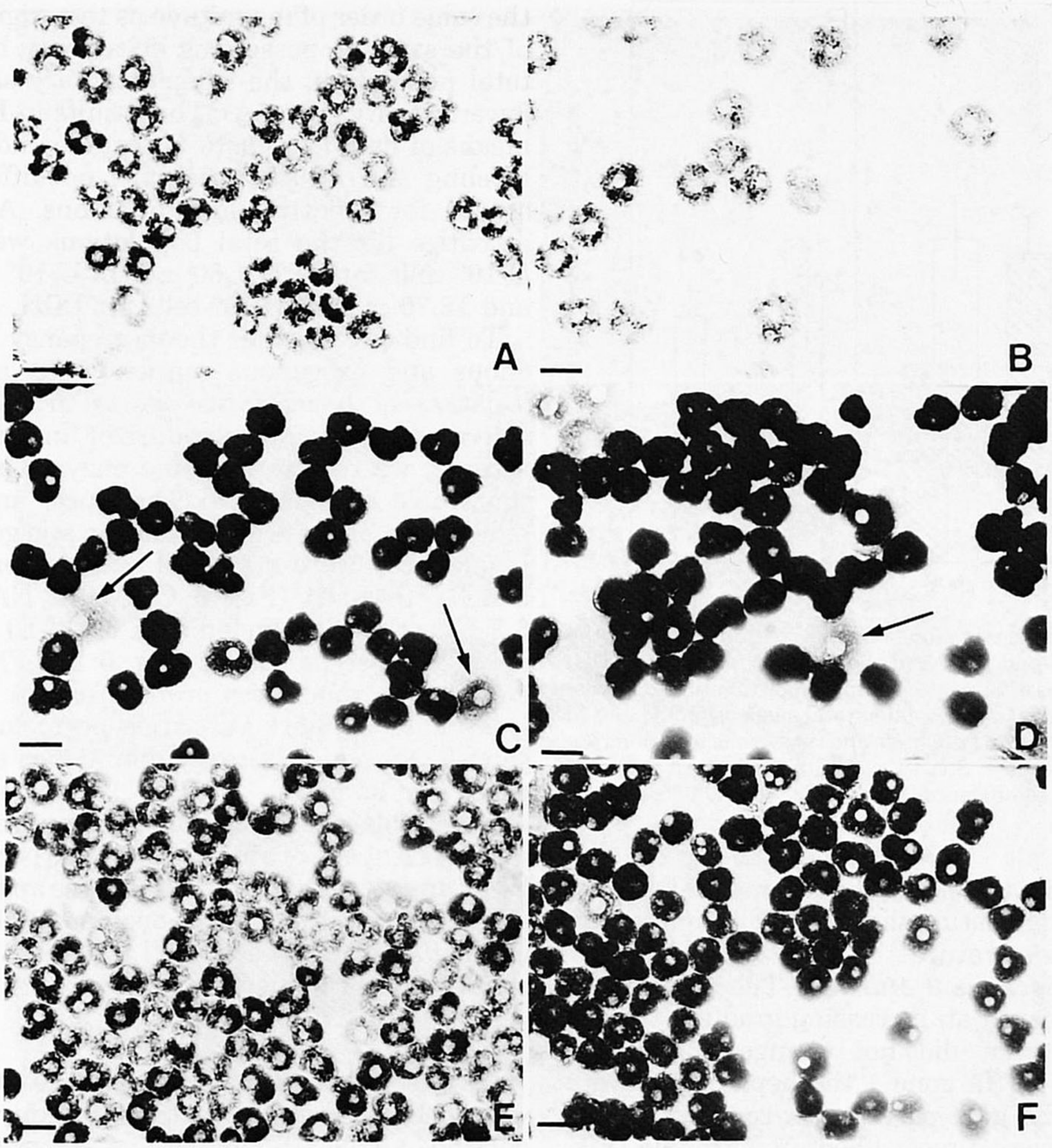

FIG. 4. Enzyme histochemical determinations of zonal marker enzymes. The "zone 1 marker" SDH (A,B) and the "zone 3 markers" βHBDH (C,D) and NADPH-red (E,F) are shown. AO-positive fractions from an antegrade isolation procedure (A,C,E) are compared with AO-negative fractions (B,D,F). Arrows point to "smashed" cells (see text). Bar = 25 μm.

hepatocytes did not damage the cells. Viability before sorting was always >90%; on the average, separation decreased cell viability by less than 10%. Viability determined with trypan blue was always >80% and not significantly different for the fluorescence-positive and fluorescence-negative population. This was confirmed by the equal pattern of FSC and SSC for both groups (not shown). Nonviable and injured cells are different from viable cells in their FSC and SSC patterns. By gating out the cells with deviant characteristics, as well as cell clumps (with high FSC) and debris (low SSC and FSC) (Fig. 2A), they are omitted from the sort. The largest part of the populations stayed inside the gates after separation; hence the cells did not change significantly with respect to these parameters. Although sorting was started immediately after isolation, the portions of the isolated hepatocyte suspension that were sorted last were stored on ice for a maximum of 3 hr. After 3 hr on ice and after separation, the AO features and scatter features of the cells were the same as immediately after isolation. Trypan blue exclusion did not change either; acid phosphatase staining of cells before and after sorting showed that lysosomes were always intact (not shown). In addition, unseparated hepatocytes are routinely tested in the laboratory for transport capacities. Freshly isolated cells and cells stored for 4 to 5 hr on ice performed comparably when incubated at 37° C in a shaking water bath for transport studies.

Under the conditions that we used for the experiments, a redistribution of AO among the cells was not seen. When a cell suspension loaded with AO was added to a suspension of unstained cells, two separate fluorescence peaks were seen on the histogram immediately after mixing. When the suspension was left at a temperature above 10° C, the peaks approached each other and finally fused until one single peak remained.

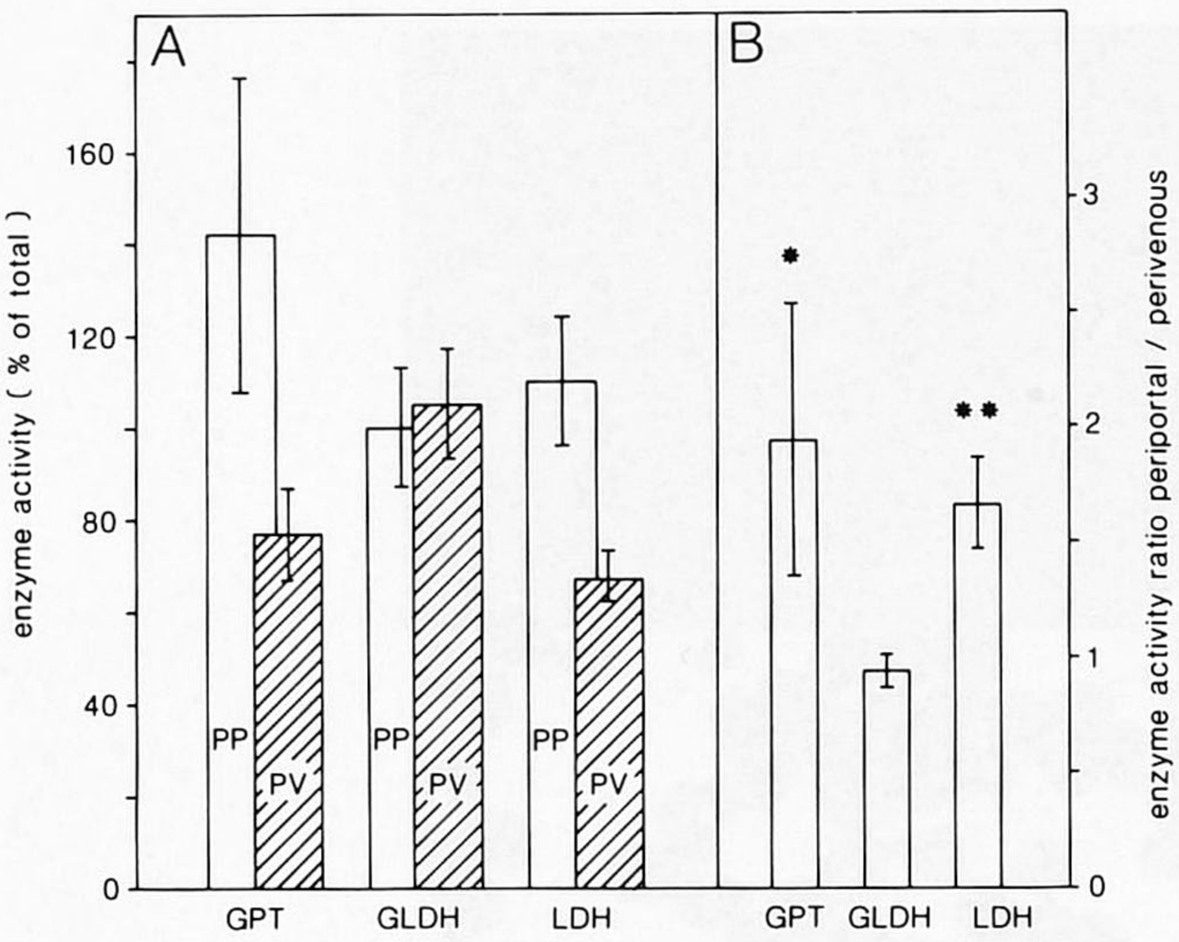

FIG. 5. Biochemical determinations of zonal marker enzymes: the "zone 1 markers" GPT and LDH and the "zone 3 marker" GLDH. Specific enzyme activities of the separated fractions (in units/10^6 cells) are related to the activities of the isolated total suspension (A) and to each other (B). Data from both antegrade and retrograde isolations are combined. Values are means ± S.D. (n = 6). Student's *t* test was used to determine statistical significance: *$p < 0.05$, **$p < 0.005$.

At 4° C, however, the two peaks did not merge but kept the same distance, indicating that redistribution hardly occurred at that temperature.

Colloidal Gold as Zone 3 Marker. The gold grains *in vivo* indeed displayed an increasing gradient toward zone 3 (Fig. 1D) that did not change during the collagenase perfusion. In zone 1 the hepatocytes were almost devoid of the gold particles in contrast to the Kupffer cells that were almost filled up with gold (Fig. 1D, *arrow*). After isolation, the gold granules were still seen in only a part of the hepatocytes (Fig. 1E). The AO-positive cells (Fig. 3A), isolated from zone 1 in this example, were almost devoid of colloidal gold (Fig. 3C), whereas all the AO-negative cells (Fig. 3B) contained the gold grains (Fig. 3D). After a retrograde isolation, gold and AO were seen in the same cells.

Marker Enzyme Activities. Although the external zonal markers, AO and gold, pointed to a high zonal enrichment of the cell fractions, the enzyme activities suggested otherwise. A moderate difference existed in the histochemically determined activities of SDH (Fig. 4 A,B) and NADPH-red (Fig. 4 E,F), but hardly any difference was seen in the activity of βHBDH (Fig. 4 C,D). This is in contrast with the activity gradient observed *in vivo* (cf. Fig. 6). In addition, assumed pp and pv fractions still gave a heterogeneous staining pattern. Cells "smashed" onto the slide surface by the cytocentrifugation procedure could be clearly distinguished from intact cells that prevented them from biasing the results (Fig. 4, *arrows*). The biochemical enzyme determinations showed clear zonal differences for GPT and LDH, whereas GLDH activity was equal in the two fractions (Fig. 5). The enzyme activities were always in the same order of magnitude as the original sample: one of the samples possessing an activity higher than the total population, the complementary sample having a lower activity (Fig. 5A). The results in Figure 5 are the means of data from both antegrade and retrograde AO labeling and isolation because no difference was observed for the two flow directions. Average enzyme activities for the total populations were 0.46 ± 0.02 U/10^6 cells for GPT, 1.60 ± 0.14 U/10^6 cells for GLDH and 12.70 ± 2.56 U/10^3 cells for LDH.

To find out whether the discrepancy between endogenous and exogenous markers was a result of the isolation of hepatocytes *per se* or the fluorescence-activated separation procedure or any kind of analytical artifact, we monitored some enzyme activities during the whole experimental procedure, analogous to the monitoring of the AO distribution and gold distribution. Liver slices were stained for activities of SDH (Fig. 6 A,B), βHBDH (Fig. 6 C,D) and NADPH-red (Fig. 6 E,F) without perfusion (Fig. 6 A,C,E) and after 8 min of collagenase perfusion (Fig. 6 B,C,F). All three gradients were much less pronounced on the collagenase treatment. A short Ca^{2+}-free perfusion, before collagenase was added, already diminished the zonal differences (not shown).

A possible cause for changing enzyme activity gradients is leakage of enzymes into the perfusion medium. We therefore collected all perfusates and measured total release of activities of three enzymes: 0.06% of liver GPT content and 0.02% each of GLDH and LDH activity was released into the medium.

DISCUSSION

Hepatocyte Separation. From the fluorescence data we concluded that our sorting procedure yielded highly enriched viable cell populations, separated according to their zonal origin. The cells from both zones were obtained from one liver, which is a significant advantage over the presently much used digitonin method (13, 14).

AO is a highly lipophilic compound that probably enters the cell mainly by diffusion across the plasma membrane (34). Hence uptake is extremely fast and a steep acinar gradient appears. This gradient strongly depends on the dose of AO: at a higher medium concentration more downstream cells were labeled, at a lower dose only few of the most upstream cells contained AO (not shown).

The disadvantage of a fast diffusional uptake is the possibility of an equally fast cellular efflux. Intracellular binding of AO, however, limits the extent of redistribution to other cells. We observed that redistribution of fluorescence to unlabeled cells was absent at 4° C. We therefore assumed the level of fluorescence to be a reliable determinant of the zonal origin of the cells. In contrast to Gumucio et al. (12), we isolate cells from all zones of the acinus, the highly fluorescent as well as the nonfluorescent hepatocytes.

The AO gradient in Figure 1A was reflected in a single tailing peak in the fluorescence histogram in Figure 2B. A large part of the cells contained an intermediate

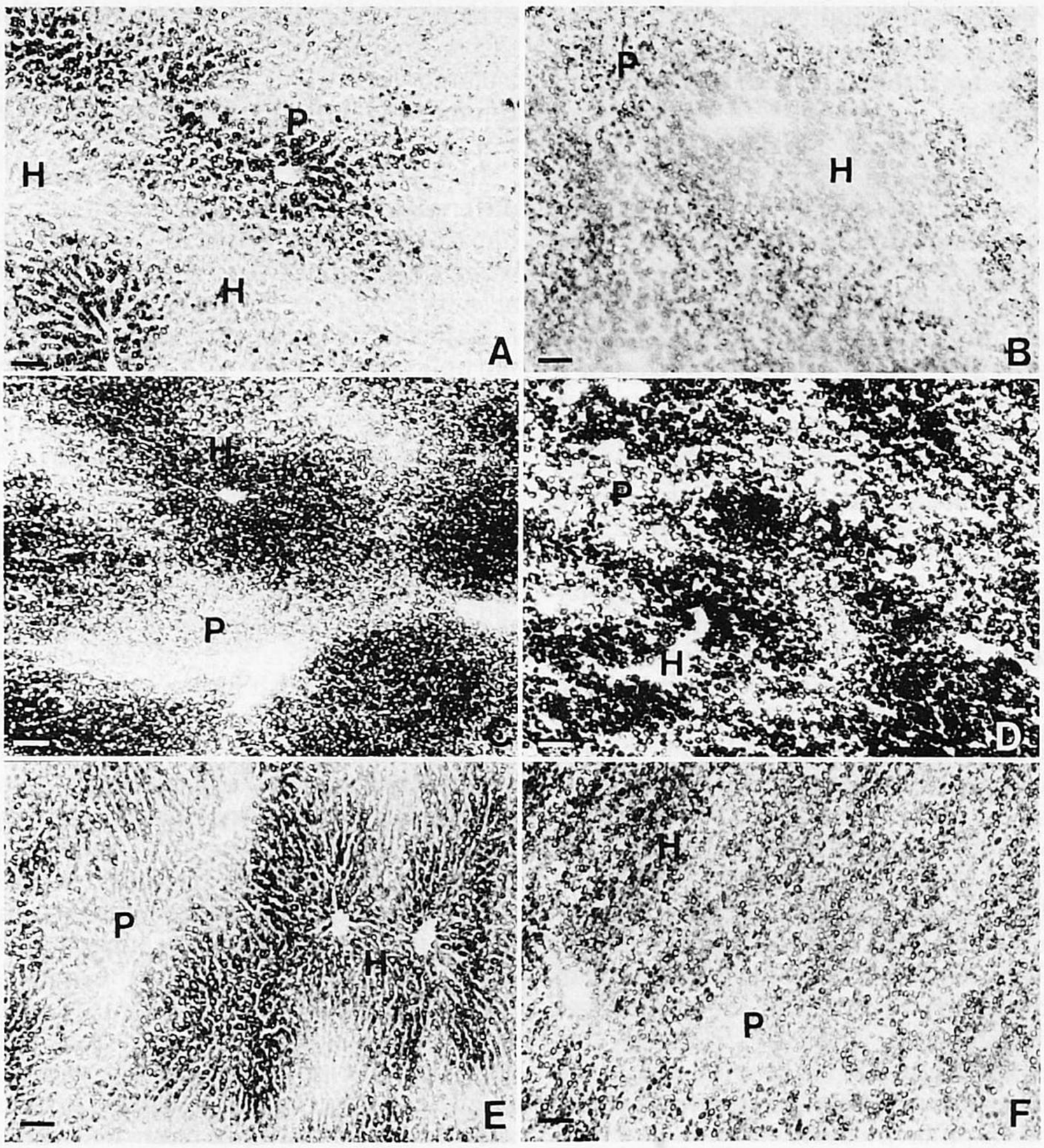

FIG. 6. Influence of collagenase on enzyme activity distributions. Enzyme histochemical determination of the zonal marker enzymes SDH (A,B), βHBDH (C,D) and NADPH-red (E,F). A comparison is made between enzyme gradients *in vivo* (A,C,E) and on 8 min collagenase perfusion (B,D,F). Bar = 25 μm. *P* = area around terminal portal venule; *H* = area around terminal hepatic venule.

amount of AO. The left slope of the peak is formed by the AO-negative cells that are quite homogeneous in their background fluorescence levels, and the right, tailing part of the histogram consists of the highly fluorescent population. The large intermediary group originated from the midzonal area, where fluorescence faded toward the downstream cells. In addition to the gradient in AO fluorescence, the heterogeneity in acinus length and sinusoid tortuosity may further smooth the histogram.

Quality of the Cells. Viability of the cells measured by scattering was not affected by the separation procedure nor were any of the other parameters that determine FSC and SSC. Trypan blue showed only a small decrease in viability. Moreover, cell structure was unaffected, as seen by enzyme histochemistry of cells before and after sorting. Obviously, the AO concentration was low enough to avoid photodamage. Figures 1, A to C, and 3, A and B, show that the AO concentration actually was too low to extensively stain the nucleus and lysosomes, a picture that is normally seen for this dye (34).

Viability of the AO-negative fraction in principle could be lower because leaky cells lose their AO. Contamination of the AO-negative suspension with damaged cells from the other zone would then result. The absence of this phenomenon, however, is a feature of the FACS. Damaged cells appear in the lower left-hand corner in the SSC against FSC dot plots and hence can be gated out during the sort. In addition, the SSC vs. FSC plots of the separated fractions were identical. We therefore concluded that cell viability was quite stable for some hours on ice during the sorting procedure. This probably was partly due to the composition of the sorting medium. The albumin-containing Percoll solution appeared to be a good preservation medium: overnight storage of hepatocytes suspended in a Percoll medium kept the amount of trypan-blue–excluding cells at 50% (tested after removing the Percoll), whereas in the same

medium without Percoll, cell viability decreased to less than 10% (unpublished observation). Cells in culture have also been shown not to be impaired in growth and function by Percoll (35).

Colloidal Gold as a Zone 3 Marker. Since AO fluorescence was used as the criterion for separation, an extra zone-specific marker was needed. Colloidal gold appeared to be a useful zone 3 marker. The grains were taken up preferentially by pv hepatocytes, possibly because Kupffer cells accumulate more gold in zone 1 because of their predominantly pp localization (27). This explanation is consistent with our observation that at a higher dose (5 mg), pp cells endocytosed gold granules as well although still less than the pv hepatocytes (not shown). Concomitantly, a lower dose only labeled Kupffer cells (not shown). Hepatocytes in the entire acinus thus have the capacity to internalize small gold grains. Another or additional explanation for the heterogeneous gold labeling may be the 30% higher porosity of the pv endothelial lining (27). Independent of the mechanism, uptake of the gold particles is very slow (27), which causes the blood concentration of gold to be virtually constant over the acinus. This implies that uptake by hepatocytes and Kupffer cells is determined only by local anatomical conditions and the capacities of the respective cells.

The entry pathway of this marker (endocytosis) leads to a persistent storage of the gold in the lysosomes, as confirmed with electron microscopy (27, and unpublished observations). As long as the lysosomes are intact, the distribution of this exogenous label will also remain intact, despite a slow biliary excretion of the grains over weeks. The results obtained with the colloidal gold granules confirmed the fluorescence results: the hepatocyte populations we obtained after sorting were viable and highly enriched in pp or pv cells.

Marker Enzyme Activities. The endogenous markers, however, presented a far lower enrichment (Figs. 4 and 5). Although the "zone 1 enzymes" GPT, LDH and SDH (Figs. 4 A,B and 5) did have a higher activity in the supposed pp cell fraction, and the "zone 3 enzymes" NADPH-red andβHBDH were more active in the supposed pv population (Fig. 4 C to F), the GLDH activity (Fig. 5) did not differ between the fractions. GLDH is often used as perivenous marker (13, 17, 23, 36). Lamers et al. (37), however, reported a different pattern, with a decrease in GLDH activity from zone 1 to zone 3. The pp/pv activity ratios of the presently used enzymes reported in the literature from *in vivo* studies vary largely: for GPT between 1.6 and 5.0 (18-20), for LDH between 1.3 and 1.8 (18, 22), both measured with a microbiochemical method after microdissection of the tissue; GLDH gave a ratio between 0.5 and 0.8 (18, 22). The studies on cell separation are equally inhomogeneous in their reported ratios. GPT ratios of 1.37 to 3.1 (12-14, 17, 23, 36, 38) were published, and LDH ratios of 1.3 (13, 17, 38) and about 1 (12). GLDH ratios found were 0.7 to 0.8 (13, 17, 23, 36). Our findings were within the range of the literature data but did not confirm the high enrichment anticipated from the AO and gold evidence. This is not due to unjustified use of quantitative enzyme histochemistry on tissue sections and isolated cells. This technique has proven to be very reliable and reproducible in measuring enzyme activities (39, 40).

In 1975 Sasse et al. (41) claimed that the zonal difference in enzyme activity was preserved in isolated hepatocytes. This often is the basic assumption made when enzyme activities are used as zonal markers. The activity gradients, however, already changed during the hepatocyte isolation (Fig. 6). The gradients observed in normal tissue (Figs. 6 A,C,E) considerably shallowed (Figs. 6 B, D,F). The reason for this effect is not known. It cannot be an artifact of tissue disruption resulting in downstream migration of hepatocytes because AO and gold were still in place at the end of the collagenase perfusion. The perfusion medium during isolation differs from the medium in a nondigesting perfusion only in two aspects: the absence of Ca^{2+} in the first medium and the presence of collagenase in the second.

The proteolytic activity of the collagenase preparation might interfere with membrane proteins as is shown for the bile canalicular membrane-bound enzyme leucyl-β-naphthylamidase (42). However, the enzymes from this study are intracellularly localized. Their activity could only be affected if the proteolytic component would enter the cells. Deprivation of Ca^{2+} during the precollagenase perfusion temporarily destabilizes the cell membrane (43, 44), possibly allowing proteins to enter the cell. This is very unlikely, though, because there was no leakage of LDH, GPT or GLDH out of the cells.

On the other hand, Ca^{2+} omission changes the intracellular Ca^{2+} concentration: mitochondrial potential drops (45) and (mitochondrial) enzyme activity might be affected. This latter explanation, although speculative, is the most likely because the changes were seen already during the Ca^{2+}-free perfusion. Since enzyme activities do not necessarily reflect enzyme contents, this explanation also agrees with the rise in activity seen in some cells (Figs. 6 D,F). The separation procedure did not further affect the enzymes because no difference in mean specific activity was seen between freshly isolated cells and the total of separated cells (Fig. 5). AO also had no effect on total enzyme activity, which makes the dye a good marker for the cells. The presence of AO in the separated cells does not exclude the use of these cells in transport experiments. The effect of AO on an experiment can be checked by comparing transport studies in total cell populations before and after labeling with AO.

From the combined data we conclude that the described separation procedure led to fractions highly enriched in pp and pv hepatocytes. Our study also shows that enzyme activities may underestimate the quality of a cell separation and may be replaced by small colloidal gold granules as a pv marker. This marker is reliable, reproducible, detected by presence and not by activity and it does not redistribute once inside the cell. Our FACS method has the disadvantage that the sortspeed and therefore the yield of the cells is quite low: about 10^6

cells/fraction may be sorted in 1 hr under our conditions. However, future generations of cell sorters with increased separation speed may overcome this handicap.

The present procedure also has many advantages. Viable cells are obtained from both zones out of one liver in contrast to the digitonin procedure (13, 14). The enrichment of the fractions is very high. By setting the sort gates more distal on the fluorescence histogram, purity may be further improved, though at the expense of yield. By using different labeling conditions, different parts of the acinus can be sorted, ranging from one cell layer to half the acinus per fraction. During sorting, the FACS may be used to study several cell functions simultaneously (24), such as enzyme activity (with a fluorescent substrate), uptake of a fluorescent compound, membrane potentials, intracellular pH or Ca^{2+} concentration (33). With labels that change fluorescence on biotransformation, the metabolic capacity of the cells can be monitored (46). In conclusion, this new separation method allows many applications and can be adapted to the needs of the researcher (e.g., a high purity of the sample or separation of the total population into more than two fractions). Colloidal gold may serve as an excellent zone 3 marker in the identification of the cells.

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