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Author(s): James E. Klaunig, Peter J. Goldblatt, David E. Hinton, Michael M. Lipsky, Jacob Chacko, Benjamin F. Trump

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MOUSE LIVER CELL CULTURE

I. Hepatocyte Isolation

JAMES E. KLAUNIG,¹ PETER J. GOLDBLATT, DAVID E. HINTON,
MICHAEL M. LIPSKY, JACOB CHACKO, AND BENJAMIN F. TRUMP

*Department of Pathology, Medical College of Ohio, Toledo, Ohio 43699 (J. E. K., P. J. G.);
Department of Pathology, University of Maryland School of Medicine, Baltimore, Maryland 21201
(M. M. L., J. C., B. F. T.); and Department of Anatomy, West Virginia University School
of Medicine, Morgantown, West Virginia 26506 (D. E. H.)*

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SUMMARY

A method for isolation of mouse liver cells by a two-step perfusion with calcium and magnesium-free Hanks' salt solution followed by a medium containing collagenase is described. Several variations of the commonly used procedure for rat liver cell isolation were quantitatively compared with respect to cell yield and viability. The optimal isolation technique involved perfusion through the hepatic portal vein and routinely produced an average of 2.3×10^6 viable liver cells/g body weight. Optimal perfusate collagenase concentration was found to be 100 U of enzyme activity per milliliter of perfusate.

Light and electron microscopic evaluation of liver morphology after several steps of the isolation showed distinct morphologic changes in hepatocytes and other liver cells during perfusion. After perfusion with Hanks' calcium- and magnesium-free solution, many hepatocytes exhibited early reversible cell injury. These changes included vesiculation and slight swelling of the endoplasmic reticulum as well as mitochondrial matrix condensation. Subsequent to perfusion with collagenase, the majority of hepatocytes appeared connected to one another only by tight junctional complexes at the bile canaliculi. Multiple evaginations were seen on the outer membrane resembling microvilli and probably represented the remains of cell-to-cell interdigitations between hepatocytes and sinusoidal lining cells from the space of Disse. The cytoplasmic injury seen after Hanks' perfusion was reversed after collagenase perfusion. After mechanical dispersion, isolated mouse hepatocytes were spherical in shape and existed as individual cells; many (80 to 85%) were binucleated under phase contrast light microscopy. By electron microscopy, cells appeared morphologically similar in cytoplasmic constitution to that seen in intact nonaltered liver cells.

Key words: mouse hepatocyte; isolation; perfusion; morphology.

INTRODUCTION

During the past decade methods for the isolation of high yields of viable adult rat liver cells have been developed (1) and improved (2-4). These methods have in common a two-step perfusion through the hepatic portal vein that involves initially a calcium-removing solution followed by collagenase-containing medium.

Hepatocytes isolated by this method have been cultured successfully in both suspension and conventional monolayer. Quantitation of perfusion conditions necessary for optimal isolation of viable rat liver cells has been investigated extensively (3-6). Various factors, including concentration of collagenase in the perfusate, method of hepatocyte dispersal after perfusion, and force of isolated cell pelleting have been shown to influence greatly the yield of viable rat hepatocytes.

Liver cells also have been isolated from the mouse. Early attempts to dissociate mouse liver with mechanical methods (7,8) and chelating

¹To whom request for reprints should be addressed at Department of Pathology, Medical College of Ohio, C.S. 10008, Toledo, Ohio 43699.

agents (9-11) were unsuccessful. More recently, Renton et al. (12) have isolated adult mouse hepatocytes by retrograde perfusion through the inferior vena cava and have cultured these cells for *in vitro* studies involving P-450 induction by interferon. Primary mouse liver cell cultures also have been used in the study of unscheduled DNA synthesis after carcinogen exposure (13). To date, however, no quantitative investigation of perfusion isolation techniques for mouse liver cells has been reported.

This report describes the development and quantitative comparison of several procedures for isolation of mouse hepatocytes by collagenase perfusion. The influence of perfusion variables such as collagenase concentration of perfusate, method of perfusion, and force of isolated cell centrifugation were explored. In addition, morphological analysis at several important steps of the isolation process was performed.

MATERIALS AND METHODS

Animals. Adult male BALB/c mice (Charles River Laboratories, North Wilmington, MA) weighing 30 to 35 g each were used. Animals were housed two to three per cage in a 12 h light/12 h dark cycle animal room and given water and Purina Laboratory Chow *ad libitum*. To minimize diurnal variations, all liver cell isolations were initiated between 9:00 and 10:00 AM.

Cell isolation. The primary goal of this work was to develop a perfusion isolation procedure that would produce a maximum yield of individual viable hepatocytes. Two different methods of liver perfusion were compared with respect to isolated cell yield and viability.

Mouse hepatocytes were isolated initially using a modification of the hepatic portal perfusion method for rat liver cell isolation reported by Seglen (2,3) as modified by Williams and co-workers (4,5). Instead of recirculating the perfusate however, both the Hanks' and the collagenase solutions were allowed to run as waste through a cut made in the subhepatic inferior vena cava. It was deemed unnecessary to recirculate the collagenase perfusate for the sake of collagenase conservation. Perfusion with calcium and magnesium-free Hanks' balanced salt solution containing 0.5 mM ethylene glycol-bis-(β -amino ethyl) *N,N'*-tetracetic acid (EGTA) and 0.05 M *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES) (pH 7.3) maintained at 37° C was begun immediately. As the liver began to blanch, the ligature around the lower abdominal vena

cava was tightened and the perfusate was allowed to run to waste. Perfusion with Hanks' solution was continued for 4 min. The enzyme solution containing collagenase (Sigma Chemical Co., St. Louis, MO; Type I; 100 U/ml solution) dissolved in Leibovitz modified L15 medium (14) (pH 7.4) maintained at 37° C was perfused for 12 min. A perfusion rate of 10 ml/min was maintained for both perfusates for the entire procedure. After the perfusion was terminated, the liver was excised rapidly from the body cavity and transferred to a sterile petri dish containing 15 ml of the collagenase perfusate. The gall bladder was removed and the hepatocytes were stripped from the connective tissue stroma with a stainless steel dog comb (2,3,5). After combing, the residual white fibrous tissue plug was discarded. Cells were further separated by repeated pipetting (10 times) with a wide-mouth pipette and passed through a single thickness of Swiss nylon monofilament mesh fabric (TETKO, Elmsford, NY; Nitek HC3-253) into a 50 ml sterile centrifuge tube. The cells were pelleted at 50 \times g for 5 min at 10° C in a refrigerated centrifuge. The cell pellet was resuspended in modified L15 medium containing 10% heat inactivated fetal bovine serum (GIBCO, Grand Island, NY) and gentamicin (50 μ g/ml). A 0.1 ml portion of the cell suspension was combined with 0.5 ml of trypan blue dye solution (0.4% in isotonic saline) and allowed to incubate for 5 min at 25° C. The viable and nonviable cells were counted in a hemocytometer using trypan blue dye exclusion as the criterion of viability. The total number and percentage of viable cells isolated was calculated. The entire perfusion protocol from anesthesia to cell counting was completed within 1 h.

The yield and percentage of viable cells isolated using this method were considerably lower (per gram body weight) than reported for rat liver (4,6). In an effort to increase the yield of viable cells, modification of the portal perfusion technique involving the intermittent occlusion with forceps of the lower abdominal vena cava (during perfusion with the collagenase solution) was performed. The occlusion of the vena cava followed a 15 s closed/15 s open cycle that when closed allowed the liver to swell to twice the normal volume. When the forceps were released and the vena cava opened, the swollen liver drained rapidly.

A second method, a modification of that reported by Renton et al. (12), using cannulation of the thoracic inferior vena cava via the right

atrium and perfusion of the liver in a retrograde manner also was performed and the results were compared to the hepatic portal perfusion method described above. Perfusion times and subsequent manipulation of the liver following perfusion was exactly as described above for the portal perfusion.

In an effort to develop a method for mouse liver cell isolation that would yield the maximal number of viable hepatocytes, several factors that have been shown in the rat to influence the cell isolation yield were investigated. One such variable, the concentration of collagenase used in the perfusate, has been reported (3-5) to influence the number of viable cells isolated directly. Therefore, three perfusate concentrations of collagenase (50 U/ml, 100 U/ml and 200 U/ml) were compared. Ten perfusions were performed for each concentration. Isolated cells were counted by trypan blue dye exclusion and the percentage of viable cells was calculated. Isolated cells from each variable were examined on an inverted phase contrast microscope and were also processed for high resolution light microscopy. The influence of the type of medium used as the collagenase carrying perfusate on cell yield also was examined. A comparison between the effectiveness of Williams' WE (4) and L15 media as the perfusate was made. The WE medium was supplemented with 0.05 HEPES to maintain the perfusate at a constant pH of 7.4. To analyze the effect of centrifugation force on yield and viability of the initial liver cell suspension, cell solutions from a single perfusion were divided into five equal portions and centrifuged in a 50 ml sterile centrifuge tube at either 1, 25, 50, 100, or 200 \times g for 5 min in a refrigerated centrifuge at 10° C. After centrifugation, the supernatant fluid was removed carefully and the resulting pellet was resuspended in L15 medium supplemented with 10% heat inactivated fetal bovine serum and gentamicin (50 μ g/ml) to make a total of 10 ml. Five portions of the resuspension were counted with a hemocytometer using trypan blue dye exclusion to assess cell viability. A total of 10 perfusions was used for the centrifugation experiments. Representative cells isolated by each centrifugation force were morphologically compared by phase contrast microscopy and high-resolution light microscopy (HRLM). For statistical analysis, an unpaired Student's *t*-test was used.

Cell morphology. To morphologically evaluate the perfusion, samples of liver tissue or cell were taken after several steps of the perfusion protocol

and processed for HRLM and electron microscopy. Several needle biopsies (1.0 to 1.5 mm³) of the liver were taken prior to perfusion (0 time) and after both the washout perfusion with Hanks' balanced salt solution and the perfusion with collagenase. Representative portions of liver cell suspensions also were taken after centrifugation and resuspension in medium. The residual plug that remained after the combing was likewise sampled. Isolated cells were processed in a micro-fuge tube for HRLM and electron microscopy.

Liver tissue and cells were fixed with 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) for 4 h, postfixed in 1% osmium tetroxide, dehydrated in increasing concentrations of ethanol followed by propylene oxide, and embedded in Epon 812. Representative sections, 0.5 to 1.0 μ m thick, were cut for HRLM and stained with toluidine blue (15). Isolated cells also were morphologically characterized by phase contrast light microscopy. Ultrathin sections for electron microscopy were cut on an ultramicrotome and mounted on 300 mesh copper grids. The sections were stained with uranyl acetate and lead citrate, observed, and photographed on a JOEL 100C electron microscope.

RESULTS

Table 1 summarizes the findings of the portal and retrograde perfusion techniques. The unmodified portal perfusion (Procedure 1A) resulted in an unsatisfactory low yield of viable hepatocytes. Modification of this basic procedure by intermittent occlusion of the vena cava (Procedure 1B) produced a significant increase ($P < 0.005$) in the yield of viable cells. The top 20% of the isolations using this method resulted in average yield of 2.9×10^6 viable cells/gram body weight (g body wt). This modification was used as the portal perfusion method of cell isolation in all subsequent experiments. Liver cell yields obtained by perfusion through the thoracic vena cava are summarized in Table 1 (Procedure 2A). Results of 30 experiments using this method gave an average yield of 2.13×10^6 viable cells/g body wt. The portal perfusion procedure (Procedure 1B), in our hands, yielded a significantly ($P < 0.005$) greater total number of isolated cells and total number of viable cells/g body wt. The top 20% of the portal perfusion viable cell yields also was significantly ($P < 0.005$) greater than the top 20% of the retrograde perfusion. Subsequent experiments employed the portal perfusion method for isolation of the liver cells. No significant difference ($P > 0.05$)

TABLE 1
COMPARISON OF MOUSE LIVER CELL ISOLATION METHODS^a

Perfusion Method	Number of Determinations	Total Cell Yield (×10 ⁶)	Percent Viability	Yield of Viable Cells/Gram Body Weight (×10)
1. Hepatic portal vein perfusion				
A. Perfusate allowed to run to waste	15	22.80 ± 5.59	86.2 ± 6.2	0.61 ± 0.17
B. Intermittent clamping of inferior vena cava	50	77.39 ± 18.2	93.7 ± 1.9	2.30 ± 0.39
Best 20% of perfusions using 1B	(10)	98.02 ± 16.53	9.40 ± 1.8	2.90 ± 0.21
2. Retrograde perfusion through inferior vena cava				
A. Modification of Renton et al. (12) through thoracic inferior vena cava	30	69.31 ± 14.20	92.0 ± 2.3	2.13 ± 0.38
B. Best 20% of perfusions using 2A	(10)	86.79 ± 12.51	92.3 ± 2.4	2.39 ± 0.30

^a Values represent the mean ± 1 SD.

was noted in the number of viable cells isolated using the two media (data not shown). Leibovitz' L15 medium therefore was used as the perfusate in all subsequent isolations. Constant oxygenation of the Hanks' solution and the collagenase perfusate by bubbling a mixture of 95% O₂:5% CO₂ through the respective solutions also resulted in no observable difference in cell yield or viability (data not shown). In view of these results, oxygenation of the perfusion solutions was not performed in subsequent isolations.

A variable shown to have a direct influence on the yield of viable hepatocytes was the concentration of collagenase (Table 2). The results showed that 100 U/ml collagenase in the perfusate produced the maximum yield of viable hepatocytes. No significant difference, however, was noted in the percent of cell viability between the three collagenase concentrations.

The force of centrifugation of the isolated cell suspension also proved important with regard to cell yield and viability (Table 3). Centrifugation forces of both 50 and 100 ×g proved to be statistically significant ($P < 0.005$) to the other forces tested. No difference in cell yield or viability was noted between isolation with 50 or 100 ×g. Toluidine blue stained HRLM sections of cells

from the lower four centrifugation forces displayed no variation in hepatocyte morphology. Higher numbers of injured cells were seen at the highest centrifugation force (200 ×g). Nonparenchymal cells also were pelleted with the hepatocytes. The percentage of nonparenchymal cells at each centrifugation force is shown in Table 3. Increased centrifugation force was associated with higher percentages of isolated nonparenchymal cells. A centrifugation force of 50 ×g produced the highest yield of viable hepatocytes with a minimum of contaminating nonparenchymal cells. This centrifugation force (50 ×g) was used routinely in subsequent isolations. In an effort to reduce the percentage of nonparenchymal cells from the total yield, the pellet obtained after 50 ×g centrifugation was resuspended in medium and centrifuged at 50 ×g. This washing was done twice. Samples for morphologic analysis and cell yield were taken after each resuspension. These results are summarized in Table 3. A decrease of almost 50% was seen in the nonparenchymal cell composition after only one washing. The second washing increased the purity of hepatocytes very little. After both washings, a small but insignificant decrease in viable cell yield was observed. Subsequent liver cell isolations included a single

TABLE 2
EFFECT OF COLLAGENASE CONCENTRATION ON MOUSE LIVER CELL ISOLATION YIELD AND VIABILITY^a

Collagenase Concentration ^b	Total Number of Cells Isolated (×10 ⁶)	Percent of Viable Cells	Number of Viable Cells Isolated per Gram Body Weight (×10 ⁶)
200 U/ml L-15	11.30 ± 2.08	88.8 ± 3.6	0.33 ± 0.07
100 U/ml L-15 ^c	71.23 ± 4.50	92.0 ± 2.1	2.19 ± 0.15
50 U/ml L-15	17.02 ± 1.76	83.4 ± 2.5	0.48 ± 0.04

^a All values represent the mean ± 1 SD of 10 isolations.

^b Collagenase was Sigma Type I (165 U/mg).

^c 100 U/ml Collagenase concentration was statistically significant over both 200 and 50 U/ml collagenase ($P < 0.005$).

TABLE 3

COMPARISON OF CENTRIFUGATION FORCE ON MOUSE LIVER CELL ISOLATION YIELD, VIABILITY, AND CELL COMPOSITION^{a,b}

Centrifugation Force ($\times g$)	Total Number of Cells Isolated ($\times 10^6$)	Percent of Viable Cells	Percent of Total Cells That Were Nonparenchymal ($\times 10^6$) ^c
1	6.66 \pm 1.09	87.7	2.3 \pm 0.15
25	11.09 \pm 1.52	85.3	2.6 \pm 0.21
50	13.03 \pm 0.35	91.2	6.3 \pm 0.25
100	13.37 \pm 1.84	92.0	10.7 \pm 0.21
200	11.67 \pm 1.23	69.2	13.7 \pm 0.43
50 ^d	13.61 \pm 0.25	92.1	6.1 \pm 0.19
50	12.52 \pm 0.31	93.1	3.6 \pm 0.25
1st washing			
50	12.02 \pm 0.24	92.8	3.2 \pm 0.21
2nd washing			

^a Values represent the mean \pm SD of 10 experiments.

^b All centrifugations were performed in an IEC refrigerated centrifuge at 10° C for 5 min.

^c One thousand cells were counted on toluidine blue stained plastic sections for each washing per experiment.

^d Comparison of repeated resuspension and pelleting of isolated mouse liver cells.

washing of the first pellet obtained. The 50 $\times g$ centrifugation force was used for all subsequent pelleting.

In lieu of the above observations, the liver cell isolation procedure used for morphologic evaluation consisted of Procedure 1B (Table 1) using 100 U/ml collagenase in L15 medium. Isolated cells after combing, repeated pipetting, and filtering were centrifuged for 5 min at 10° C at a force of 50 $\times g$. The resulting pellet was washed once in complete L15 medium.

Liver morphology during hepatic isolation. The morphology of the mouse liver, sampled prior to perfusion with the dissociating solutions, is shown in Figs. 1 and 2. The light and electron microscopy of the plastic embedded control liver of this study (0 time sampling) resembled that described by Trump et al. (16). The hepatocytes, by high resolution light microscopy (Fig. 1), were present in cords one cell thick separated by sinusoids. The sinusoids were lined by endothelial cells, Kupfer cells, and in the Space of Disse an occasional fat-storing cell of Ito was seen. Hepatocytes were polygonal in shape containing one or two centrally situated nuclei. The cytoplasm was filled with roundish, gray bodies representing mitochondria. Lipid appeared as large, round monochromatic bodies within the cytoplasm. Areas of translucent, unstained cytoplasm were thought to represent regions of glycogen that were removed during buffer washing of the specimens. At higher magnification, bile canaliculi were evident between adjacent hepatocytes. A typical electron micrograph of a

control mouse hepatocyte displayed a large, round nucleus containing a prominent nucleolus (Fig. 2). The cytoplasm was filled with numerous oval mitochondria as well as several rounded lipid droplets. Endoplasmic reticulum (both smooth and rough surfaced) was present in vesiculated and tubular forms. Interdigitation of cell membranes was evident between adjacent hepatocytes. Junctional complexes were seen frequently along adjoining membranes. Bile canaliculi also were seen.

Perfusion with Hanks' solution. Figure 3 shows a high resolution light micrograph of a toluidine blue stained HRLM section of the liver after the washout perfusion with Hanks' solution. Hepatic architecture showed little change from control morphology with the exception of the sinusoids that were cleared of blood cells and were swollen slightly from the perfusion. Hepatocytes remained tightly joined together. Sinusoidal lining cells were unaffected. An opaque amorphous material filling the sinusoidal lumen was seen occasionally. No change was noted in hepatocyte morphology.

With electron microscopy (Figs. 4 and 5), perfusion with Hanks' solution resulted in several distinct morphologic changes to the majority of hepatocytes examined. These were characteristic of early sublethal cell injury that included diffuse swelling of the endoplasmic reticulum throughout the hepatocyte cytoplasm. Mitochondria displayed condensed matrices whereas other cytoplasmic organelles seemed normal. Partial

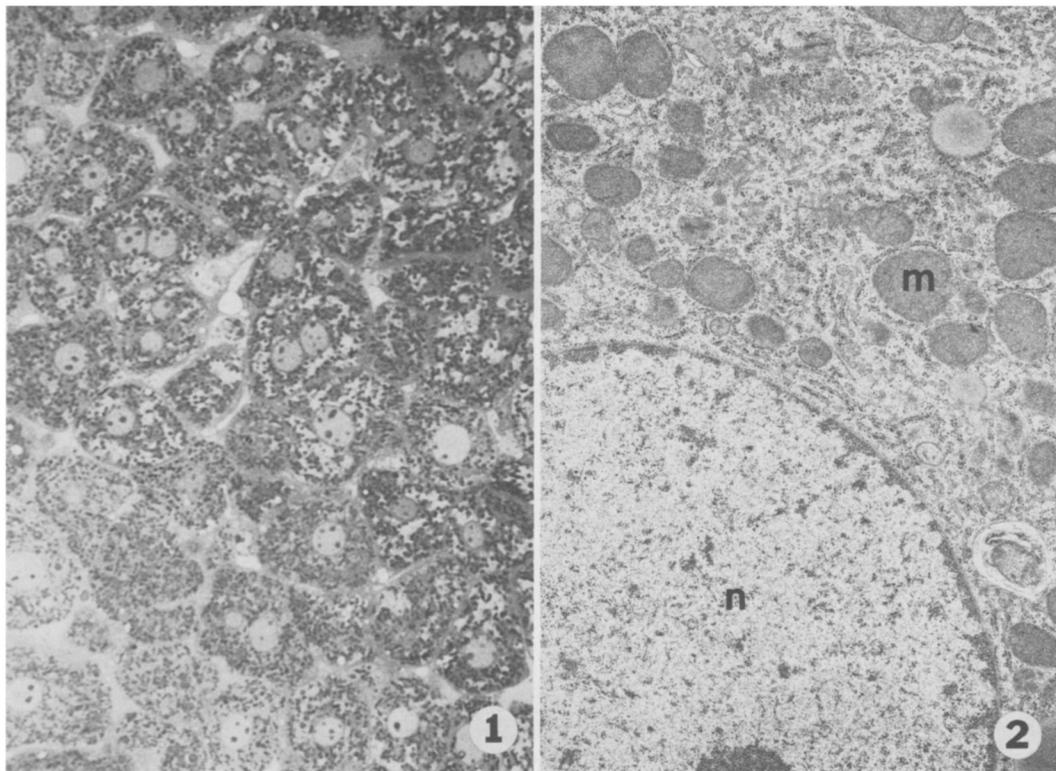


FIG. 1. High resolution light micrograph of liver sampled prior to perfusion. $\times 450$.

FIG. 2. Electron micrograph of a portion of a hepatocyte sampled prior to perfusion with dissociating solutions. *n*, Nucleus; *m*, mitochondrion. $\times 10,000$.

separation of hepatocytes was seen. Intercellular space between adjacent hepatocytes was more apparent than in the controls and cytoplasmic interdigitations with adjacent hepatocytes were pulled apart. The tight junctions between adjacent hepatocytes in all cases examined remained completely intact. Sinusoidal lining cells were either absent or maintained minimal contact with hepatic parenchymal cells.

In sites where sinusoidal lining cells were denuded, hepatocytes retained their cytoplasmic fingerlike projections into the former Space of Disse. Of the remaining sinusoidal lining cells, the majority were irreversibly injured. Clumps of necrotic lining cells occasionally were seen filling the sinusoidal space. These masses of cell debris represented the amorphous material seen by light microscopy. The alterations following Hanks' perfusion were generally diffuse throughout the liver.

Perfusion with collagenase. Following collagenase perfusion, liver morphology displayed several striking changes as seen by light micros-

copy (Fig. 6). The architectural pattern of the cords of hepatocytes separated by sinusoids was less apparent. A large amount of extracellular space was present between cells. Sinusoidal lining cells were seen rarely and, when found, they appeared to be closely associated with one or more hepatocytes. Thin strands of cell debris were found between hepatocytes.

Electron microscopy confirmed the general disruption of the classic hepatic architecture (Figs. 7 and 8). Hepatocytes were either totally separated from adjacent cells (approximately 20% of the cells seen), in which case no junctional complexes connecting cells were evident, or, in the majority of cases, were arranged in small groups of up to 10 cells. In this case contact was maintained between individual cells of the group by tight junctions at the bile canaliculi. Desmosomes were broken frequently, forming hemidesmosomes in dissociated cells. Microvilluslike projections were apparent on the outer cytoplasmic membrane of all hepatocytes, possibly reflecting their previous appearance in the Space of Disse (Fig. 8).

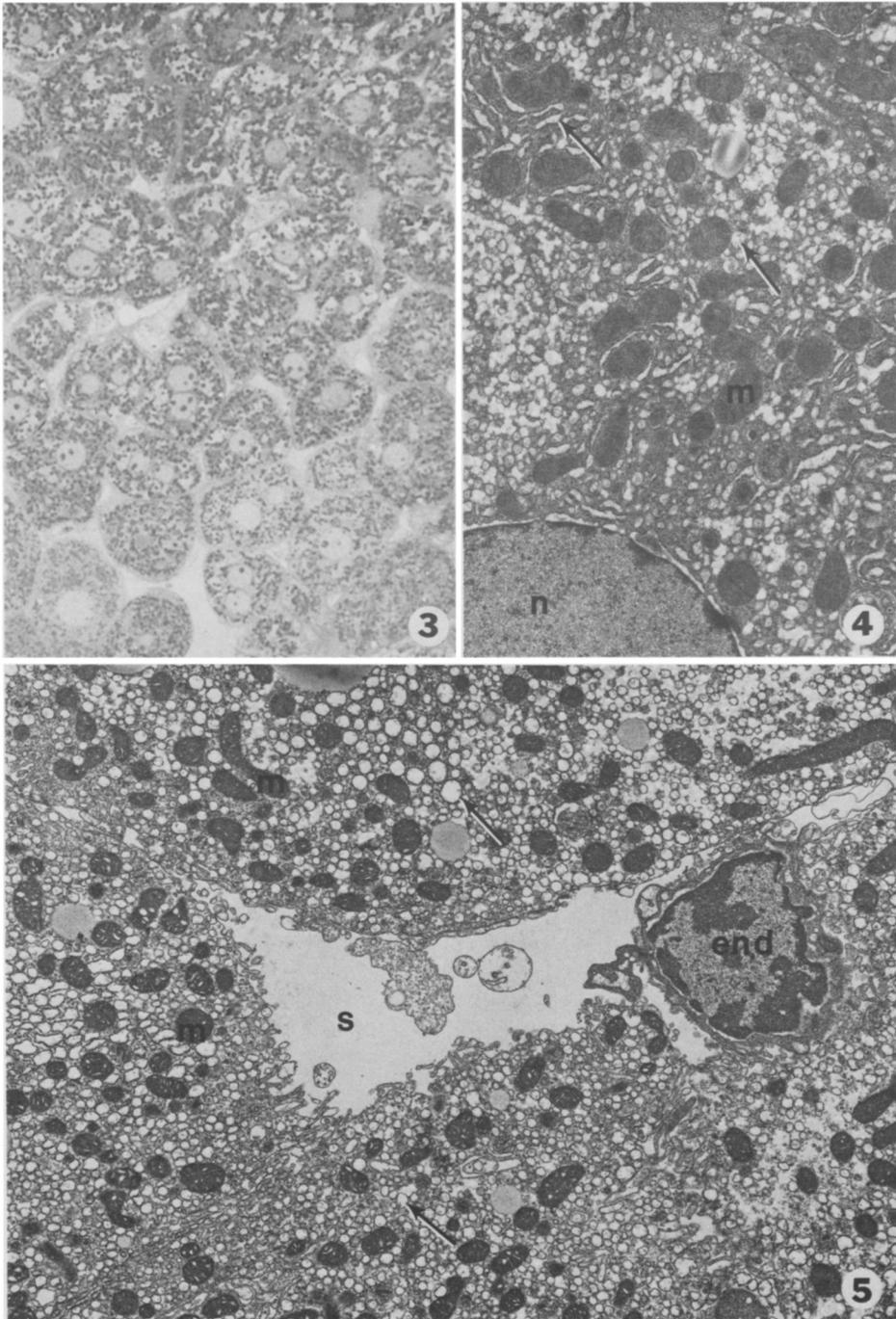


FIG. 3. Light micrograph of liver after perfusion with Hanks' solution. Sinusoids between hepatocytes are slightly swollen. $\times 400$.

FIG. 4. Electron micrograph of hepatocyte after perfusion with Hanks' solution. Profiles of smooth and rough endoplasmic reticulum (*arrows*) are slightly swollen indicating early reversible cell injury. *n*, Nucleus; *m*, mitochondrion. $\times 8800$.

FIG. 5. Electron micrograph of several hepatocytes surrounding a sinusoid(s) after perfusion with Hanks' solution. Hepatocyte endoplasmic reticulum are swollen (*arrows*), and mitochondria have condensed matrices indicative of early cell injury. Extracellular space between hepatocytes also is increased. *end*, Endothelial cell lining sinusoid. $\times 4400$.

The strands of cellular material seen by light microscopy were shown to represent dead sinusoidal lining cells that were sloughed away from hepatocytes and existed in groups of two to four cells. Cell debris found between hepatocytes represented necrotic sinusoidal lining cells and an occasional necrotic hepatocyte.

Residual liver plug. The white, residual plug of material that remained after combing of livers consisted of fibrous tissue and portal tract material (Fig. 9). Occasionally, apparently viable hepatocytes were present but they represented a minority of the cell types observed.

Isolated cells. Isolated liver cells observed by phase contrast microscopy were predominantly hepatocytes (96 to 98%). Hepatocytes were spherical and their outer cell membrane contained multiple microvilluslike projections. Membrane blebbing was seen rarely. The majority of hepatocytes (80 to 85%) were binucleated. Viability (trypan blue dye exclusion) of isolated cells was consistently 93 to 95%. Hepatocytes generally were present as individual cells (85 to 90%). The remaining 10 to 15% appeared as small groups of two to five cells in number.

Representative isolated liver cells stained for HRLM are shown in Fig. 10. The majority of cells appeared spherical with prominent interdigitations and evaginations of the plasma membrane. Centrally located within a basophilic cytoplasm were one or two round nuclei. The majority of cells were binucleated. Nucleoli were discernible easily as one or two darkly stained bodies within the lighter staining nucleoplasm. The cytoplasm routinely contained patches of unstained areas considered to be sites of glycogen removal, and several lipid droplets. Cell debris and non-parenchymal cells were observed occasionally between the isolated hepatocytes.

Ultrastructurally, isolated hepatocytes appeared spherical (Fig. 11). Multiple evaginations of the plasma membrane were present around the entire cell periphery. The nucleus (or nuclei in binucleated cells) was located centrally within the cytoplasm. Heterochromatin was located peripherally along the inner nuclear membrane. One or two eccentrically located nucleoli were evident within the nucleoplasm. The isolated hepatocyte cytoplasm contained an identical organelle composition to that seen in hepatocytes of intact liver. Numerous mitochondria were seen throughout the cytoplasm. These were present in both elongated and oval configurations. Parallel stacks of rough endoplasmic reticulum cisternae also

were diffusely distributed. Other cytoplasmic constituents included Golgi, smooth endoplasmic reticulum, lipid droplets, and diffuse areas of glycogen (much of which was removed during electron microscopic processing). Occasionally hemidesmosomes were present on outer surfaces of hepatocyte plasma membranes. These were interpreted as the remains of previous desmosomes broken during the isolation. Blebbing of plasma membranes was an infrequent finding.

Supernatant morphology. Phase contrast, HRLM, and electron microscopic examination of the centrifugation supernatant fluid after pelleting of the liver cells was performed also. Material consisted largely of cell debris, including isolated nuclei, lipid droplets, and mitochondria. A few structurally intact hepatocytes were seen.

DISCUSSION

Two approaches to the isolation of high yields of viable mouse hepatocytes were utilized in this study. Both techniques involved the initial perfusion through the liver of a calcium chelating solution, which was followed by a collagenase containing medium. One procedure mimicked the hepatic portal perfusion commonly used for rat liver cell isolation; the second duplicated the retrograde perfusion via the thoracic inferior vena cava first reported by Renton et al. (12). In the present study, both methods resulted in relatively high yields of viable hepatocytes. In our hands the portal perfusion procedure produced consistently higher yields than perfusion through the thoracic inferior vena cava. The observed differences in cell yield between the two procedures may be due in part to our prior experience with the portal perfusion. Further trials with the thoracic inferior vena cava perfusion therefore may increase the yield of liver cells. No difference was noted (by light microscopy) between the morphologic character of cells isolated by the two methods. However, this point needs to be investigated further.

These two methods of perfusion reflect two entirely opposite movements of perfusate through the hepatic lobule. In the retrograde vena cava perfusion, the perfusate infiltrates the hepatic lobule from the central vein through the sinusoids to the portal tracts. The portal perfusion method follows the normal flow of portal blood, traversing the lobule from the portal tracts to central veins. Hepatocytes comprising the liver lobule have been shown to be heterogeneous in function

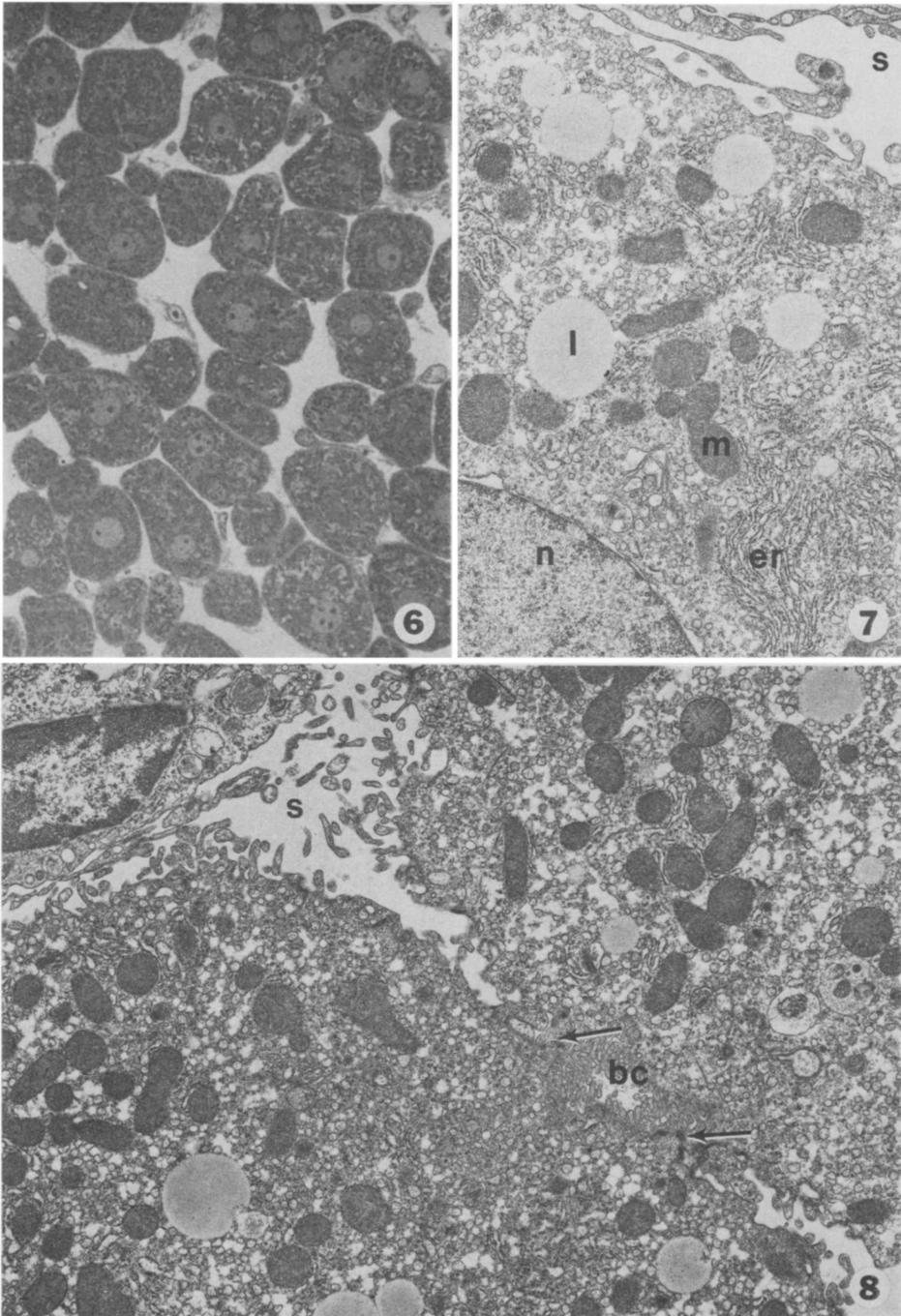


FIG. 6. Light micrograph of liver after perfusion with collagenase. Sinusoidal spaces between hepatocytes are highly swollen, but hepatocytes appear to remain complexed together. $\times 400$.

FIG. 7. Electron micrograph of a portion of a hepatocyte sampled after perfusion with collagenase. Cytoplasm appears normal. Portions of a living cell in the sinusoidal space(s) is apparent. *n*, Nucleus; *l*, lipid; *m*, mitochondrion; *er*, endoplasmic reticulum. $\times 8800$.

FIG. 8. Electron micrograph of two hepatocytes bordering a sinusoidal space(s) after perfusion with collagenase. Cells are still connected at the bile canalculus (*bc*) by the maintenance of tight junctions (*arrows*). $\times 8800$.

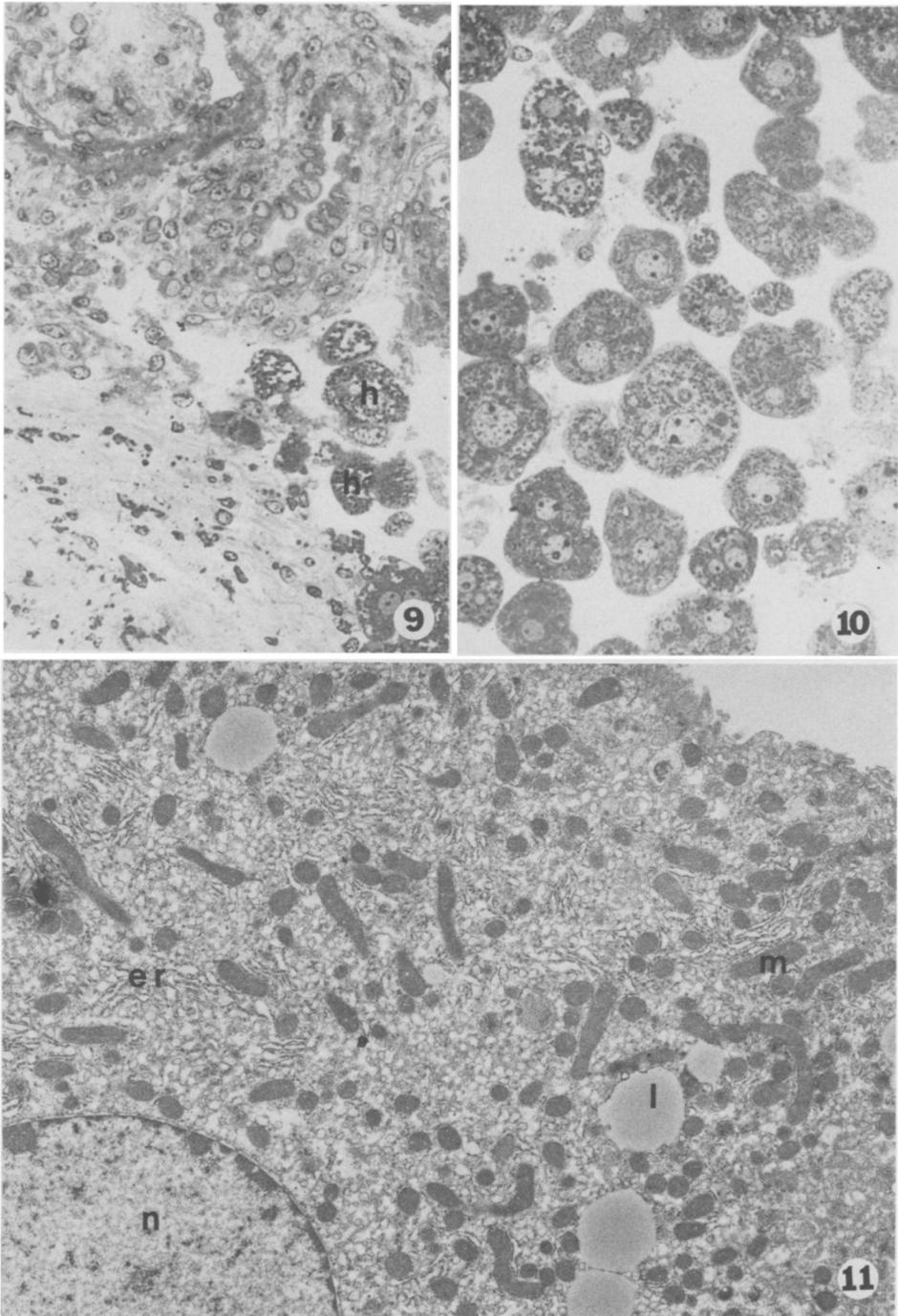


FIG. 9. High resolution light micrograph of residual fibrous plug that remained after combing. Several hepatocytes (*h*) are still present within the plug. $\times 400$.

FIG. 10. High resolution light micrograph of isolated hepatocytes. The majority of hepatocytes are individual and somewhat oval in shape. $\times 400$.

FIG. 11. Electron micrograph of a portion of an isolated hepatocyte. *n*, Nucleus; *m*, mitochondrion; *l*, lipid; *er*, endoplasmic reticulum. $\times 4400$.

and morphology. The method of perfusion, therefore, may influence the type of hepatocyte that is isolated. It was reported that in hepatocellular carcinoma, blood flow through the hepatic portal vein was decreased from normal (17). In such a situation, perfusion via the inferior vena cava may prove superior. In practice, this has been true as shown by our own experience in isolating cells from neoplastic mouse liver (unpublished observations).

Williams et al. (4) reported total hepatocyte recoveries of 57% of that theoretically possible in the rat liver. This was based upon a previously reported hepatocyte number of 630×10^6 cells/100 g of rat body weight (6). In an effort to compare our yields with those reported by Williams et al. (4) the number of hepatocytes present in the BALB/c mouse liver was calculated using established morphometric analysis techniques (18) on $0.5 \mu\text{m}$ toluidine blue stained sections of 3% glutaraldehyde fixed mouse liver. The number of hepatocytes per unit volume (cubic centimeter) of mouse liver (N_{ah}) was calculated by determining the number of hepatocyte nuclear profiles (N_{sh}) and hepatocyte volume fraction (V_{vh}) per unit area. The livers of five mice were examined. Ten slides from each liver were prepared and ten random fields per slide were counted. The number of hepatocyte nuclei (N_{vh}) was calculated using the following equation:

$$N_{vh} = \frac{1}{B} \cdot \frac{N_{sh}^{2/3}}{V_{vh}^{1/2}} \cdot K \quad (19,20) \quad (1)$$

where K is a coefficient of size distribution [assumed to be 1.03 for liver (20)] and B is the shape and size coefficient [assumed to be 1.38 for liver (20)]. Using this formula the number of hepatocyte nuclei per cubic centimeter was calculated to be 9.1×10^7 . Assuming one nuclei per hepatocyte, this would equal 9.1×10^7 hepatocytes per cubic centimeter. Mean volume displacement for the entire liver was experimentally determined to be 1.30 cm^3 . The total number of hepatocytes per liver therefore equals 1.18×10^8 ($1.30 \text{ cm}^3 \times 9.1 \times 10^7$ hepatocytes per cubic centimeter). The total number of hepatocytes per gram body weight was determined to be 5.90×10^6 (assuming one nuclei per hepatocyte). Using the value for the viable cell yield of the 20% best hepatic portal perfusion (Table 1B), the viable cell yield of mouse hepatocytes per gram body weight would represent 49% of that theoretically

possible (assuming no hepatocyte binucleation). This correlates well with the 50% viable cell yield reported by Williams et al. (4) for the rat. However, a high percentage (80 to 85%) of isolated mouse hepatocytes were binucleate when viewed with phase contrast microscopy. Therefore, the theoretical number of hepatocytes per gram body weight was calculated for 50% (4.43×10^6 hepatocytes/g body wt) and 75% (3.54×10^6 hepatocytes/g body wt) hepatocyte binucleation. Both of these percentages, we feel, are conservative estimates of the true number of binucleated cells. Assuming 50% binucleation, and using the 20% best hepatic portal perfusions, the total cell yield would represent 69% of that theoretically possible. The viable cell yield would represent 65% of that possible. Assuming 75% binucleation, the total and viable cell yield would represent 87 and 82% of that possible, respectively. These values are significantly greater than the percentage yields previously reported for the rat (4).

Viability of the mouse liver cell isolates in both the present study and that reported by Renton et al. (12) were consistently greater than 93%. In comparison, the viabilities of isolated rat liver cells usually have averaged in the 80 to 85% range. Mouse liver cells may be more resistant during the isolation procedure to the effects of the chelating agents and the collagenase. However, this speculation lacks experimental evidence at present.

The influence of the concentration of collagenase in the perfusate has been shown to affect the yield and viability of isolated rat liver cells (3-5). The present report revealed a similar relationship for the mouse. Most investigators isolating rat liver cells have used a crude collagenase preparation containing 50 mg/100 ml of perfusate. However, the specific activity of collagenases varies from lot to lot. Williams et al. (4) expressed the collagenase concentration on a unit per milliliter basis rather than milligram percent and routinely used 100 U/ml of perfusate. For the mouse, a standard of 100 U/ml also was found to be the most effective for cell dispersion using the perfusion procedures described above.

The relationship between the force of centrifugation and viable cell yield of the isolated liver cell suspension was investigated previously in part in the rat (4). The force of $50 \times g$ has been used routinely in rat liver cell isolation and appeared with the mouse to provide the greatest yield of viable liver cells with the lowest contamination of nonparenchymal elements. A single washing of

the pellet reduced the nonparenchymal cells to less than 4%.

Morphologic changes of the liver during the two-step portal perfusion were of interest. The early reversible cell injury to many hepatocytes after Hanks' calcium- and magnesium-free salt solution containing 0.5 mM EDTA was suggestive of damage resulting from liver cell isolation by chelators (9-11) and mechanical means (7,8). In the present report, the damage appeared to be reversible following perfusion with the collagenase perfusate. The Hanks' solution appeared to exert its major effect on the disruption and separation of the sinusoidal lining cells although some hepatocyte desmosomes were separated. After collagenase perfusion, most hepatocytes remained connected by tight junctions. Williams et al. (4) reported significant increases in viable cell yield by employing combing and multiple pipetting of the perfused rat liver in fresh collagenase solution. This reinforces the importance of some type of mechanical dispersion of the collagenase perfused liver.

The present report describes the methodology for the isolation of high yields of viable mouse liver cells. Isolated rat liver cells have been used in biochemical (21-24), toxicity (5-6), and carcinogenicity (26-28) studies. The advantages of using liver cells for short-term in vitro toxicity and carcinogenicity testing have been noted previously (5,9,30). The development of a reproducible method for the isolation of mouse liver cells permits the use of these cells in similar studies.

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