**Preparation of Single-Cell Suspensions from Normal Liver, Regenerating Liver, and Morris Hepatomas 9121 and 5123tc**

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**SUMMARY**

Suspensions of single cells were prepared from normal adult rat liver either by mincing the tissue and incubating it in various concentrations of sodium tetraphenylborate, ethylenediaminetetraacetate, and 0.1% hyaluronidase plus 0.05% collagenase or by continuous recirculating perfusion of the liver with 0.1% hyaluronidase plus 0.05% collagenase. Only the latter method produced cells in good yield that were not stained with trypan blue. Incorporation of L-leucine-1-14C into protein was up to 60 times higher in cells prepared by continuous perfusion with hyaluronidase plus collagenase than in cells prepared by incubation of the minced tissue in sodium tetraphenylborate or in hyaluronidase plus collagenase. Liver cells prepared by continuous perfusion had a better preserved ultrastructure than cells obtained by the other methods.

In regenerating liver, cells that were not stained with trypan blue were obtained by the two methods with hyaluronidase plus collagenase, the yield being highest after continuous perfusion.

In Morris hepatomas 9121 and 5123tc, continuous perfusion could not be established. However, mincing the tissue and incubating it in hyaluronidase plus collagenase yielded cells with an intact ultrastructure. Those cells excluded trypan blue. The amount of the incorporation of L-leucine-1-14C into protein was about 300 times higher than in cells prepared by sodium tetraphenylborate.

**INTRODUCTION**

Mammalian cell cultures provide well-defined conditions for metabolic studies. However, it has not yet been possible to obtain cell cultures of normal adult parenchymal liver cells, whereas preparation of suspensions of single liver cells has been described repeatedly (e.g., Ref. 46).

In single cells from rat and mouse liver prepared by mechanical disruption with and without addition of calcium-chelating agents or acid salt solutions (1, 8, 15, 39, 46, 53, 54, 62, 68, 85), lack of aerobic glycolysis (3, 23, 57) and of endogenous respiration (52, 57, 76, 83); impairment of the synthesis of protein (25), cholesterol (56), and RNA (47); failure of enzyme induction by cortisol (85); leakage of enzymes (3, 16, 19, 23, 34, 50, 81, 82, 85, 92), potassium ions (23, 29, 51, 67), and NAD and ADP (28, 43); an increased permeability to calcium ions (3, 23) and to substances of low (48, 78) and high (77, 79) molecular weight; and staining with trypan blue (21, 60) were observed. Furthermore, during preparation, destruction of the plasma membrane and of other cell organelles was demonstrated (2, 7, 10, 13, 15, 26, 66, 76). Only in calcium-free media or in media with cofactors added, could respiration (3, 23, 33, 39, 43, 45, 51, 68, 69, 83, 84), synthesis of lipids and cholesterol (10, 41, 42, 60), high rates of protein (11, 60, 76) and RNA synthesis (76), and oxidation of glucose and fatty acids (21, 22) be observed. Dispersion of the cells in polyvinyl alcohol inhibited leakage of enzymes (19) but diminished the rate of respiration (81).

Other preparation methods were based on the proteolytic cleavage of the intercellular matrix. With trypsin, alteration of the cell surface (2, 55, 58), deformability of cells (90), increase of permeability (29), and adsorption of trypsin to the cell surface (70) were reported. However, with the use of hyaluronidase and collagenase, described previously (91), liver cells with intact ultrastructure were isolated from rats (38) and mice (20). They synthesized protein (9, 49), RNA (49), and lipids (18, 49), and respired (40, 49) at higher rates than mechanically prepared cells. Calcium ions stimulated the respiration (40) and the synthesis of protein and lipids (60). Dexamethasone induced tyrosine aminotransferase (32). The disadvantage of the new preparation procedure was the low yield, but continuous recirculating perfusion of the liver improved the recovery considerably (6, 44). Leakage of enzymes was lower than in cells prepared with other methods (19). Furthermore, glycolysis (6), gluconeogenesis (6, 80), and albumin synthesis (89) were demonstrated. Chylomicrons (24), ethanol metabolism (4, 5), and regulation of lipid synthesis (12) were studied. The only observed damage was a loss of potassium ions (6).

Besides hyaluronidase and collagenase, other dissociating agents are still in use. EDTA plus lysozyme was reported to be more effective than other dissociating agents, e.g., hyaluronidase plus collagenase (36, 37). The previously described dispersion of rat liver cells in TPB2 (71-73) was claimed to be the only method producing cells that could be grown in culture (14, 27).

In this paper, the main methods used recently for preparation of isolated liver cells are compared on the basis of...
trypan blue exclusion, of incorporation of L-leucine-\(^{14}\)C into protein, and of preservation of the ultrastructure. Hyaluronidase plus collagenase were found to be superior to other dissociating agents. The procedure could also be applied successfully to regenerating rat liver and to transplantable hepatomas.

**MATERIALS AND METHODS**

**Chemicals and Isotopes.** Hyaluronidase (type I) and neuraminidase (types V and VI) were purchased from Sigma Chemical Company (St. Louis, Mo.), and collagenase was from Sigma or from Th. Schuchardt, Chemische Fabrik (München, Germany) (Table 1, No. 10, Morris hepatoma 5123tc, first experiment) or from Serva, Feinbiochemica GmbH and Co. (Heidelberg, Germany) (Table 1, No. 10, Morris hepatoma 9121, both experiments). TPB, analytical grade, was purchased from Sigma or Fluka AG, Chemische Fabrik (Buchs, Switzerland), and lysozyme was from Merck (Darmstadt, Germany). Bovine serum albumin, highest grade, was obtained from Behringwerke AG (Marburg/Lahn, Germany). L-Leucine-\(^{14}\)C, 62 mCi/mmole, was bought from the Radiochemical Centre (Amersham, England).

**Animals, Tumors, and Partial Hepatectomy.** ACI and Buffalo rats of both sexes were used, the body weights ranging from 180 to 300 g. Animals were kept as previously described (74). Source and retransplantation of tumors are published elsewhere (74). Morris 9121 hepatomas were in generations 41, 42, and 47 and 5123tc hepatomas were in generations 98, 102, and 110. Hepatoma cell suspensions were prepared from tumors 3 to 4 weeks after transplantation.

Partial hepatectomies were performed according to the method of Higgins and Anderson (35). Suspensions of regenerating liver cells were prepared 48 hr after partial hepatectomy.

**Media for Dissociation and Incubation.** The TPB dissociation medium was the same as that of Gerschenson and Casanello (27). Hyaluronidase, collagenase, neuraminidase, and EDTA with or without lysozyme were dissolved in a modified Hanks' solution (30) consisting of 138.9 mM NaCl, 5.37 mM KCl, 0.81 mM MgSO\(_4\), 0.337 mM Na\(_2\)HPO\(_4\), 0.441 mM KH\(_2\)PO\(_4\), and 5.5 mM glucose. To each 100 ml, 2.5 ml of a 153.6 mM NaHCO\(_3\) solution were added.

For staining, cells were suspended in a 5.2 mM solution of trypan blue in modified Waymouth medium (88) consisting of 103 mM NaCl, 2 mM KCl, 0.82 mM CaCl\(_2\), 1.18 mM MgCl\(_2\), 0.81 mM MgSO\(_4\), 2.11 mM Na\(_2\)HPO\(_4\), 0.59 mM KH\(_2\)PO\(_4\), 26.7 mM NaHCO\(_3\), 27.8 mM glucose, 0.1 mM ascorbic acid, 0.05 mM glutathione, 1.8 mM choline-HCl, 0.18 mM hypoxanthine, 0.03 mM thiamine-HCl, 0.002 mM calcium pantothenate, 0.003 mM riboflavin, 0.003 mM pyridoxine-HCl, 0.0008 mM folic acid, 0.00008 mM biotin, 0.005 mM myo-inositol, 0.008 mM nicotinamide, 0.00015 mM vitamin B\(_12\), 0.57 mM cystine-HCl, 2.38 mM glutamine, 1.42 mM L-lysine-HCl, 0.8 mM L-histidine-HCl, 1.02 mM L-glutamic acid, 0.64 mM L-threonine, 0.36 mM L-arginine-HCl, 0.55 mM L-valine, 0.46 mM L-aspartic acid, 0.66 mM glycine, 0.44 mM L-proline, 0.34 mM L-methionine, 0.3 mM L-phenylalanine, 0.22 mM L-tyrosine, 0.2 mM L-tryptophan, 0.19 mM L-isoleucine, 0.006 mM L-cystine, 0.56 mM alanine, 0.21 mM serine, 10% calf serum, 0.00288% sodium penicillin G, 0.00012% potassium penicillin G, and 0.01% streptomycin. For studies on protein synthesis and on ultrastructure, cells were suspended in nonmodified Hanks' solution (30) containing the amino acids according to the listing of Waymouth (88), 0.02% heparin, 2.5% bovine serum albumin, and antibiotics as described above. The pH was adjusted to 7.4 unless stated otherwise.

**Preparation of Liver Cell Suspensions by Shaking.** After the abdomen and chest of the rat were opened under ether anesthesia, the liver was perfused for about 1 min via the thoracic portion of the inferior vena cava with 20 ml of dissociation medium at 37\(^\circ\)C. The liver was excised, weighed, and minced with scissors in 10 ml of ice-cold dissociation medium into pieces of about 10 cu mm. After being drained on a plastic tea sieve, the pieces of tissue were resuspended in 50 ml of cold, fresh dissociation medium and placed on ice until used. Suspensions were incubated at 37\(^\circ\)C under an atmosphere of 95% O\(_2\) and 5% CO\(_2\) with continuous shaking. After 20 min, nondissociated pieces of tissue were separated by passing the suspension through a tea sieve and incubated in 50 ml of fresh dissociation medium. This was repeated 3 times. Cells were collected from each filtrate by centrifuging for 10 min at 30 to 70 X g. For washing, the pellet was resuspended in 10 ml of incubation medium, centrifuged again, and resuspended in 50 ml of incubation medium to give the final cell suspensions.

**Preparation of Liver Cell Suspensions by Continuous Recirculating Perfusion.** The technique of Berry and Friend (6) with the modifications described previously (89) was used. Cells were washed once with incubation medium.

**Preparation of Tumor Cells.** Under ether anesthesia, the skin of both hind legs and of the lower abdomen was removed and the abdomen was opened. The arteriae and venae iliacae were carefully isolated from the surrounding tissue, and the arteries were held by threads. A 12-gauge cannula was inserted into the femoral vein. The arteriae and venae iliacae were clamped above the holding thread. The arteriae iliacae were lifted by the thread and opened, and perfusion was started through the inserted cannula. We used 20 ml of 37\(^\circ\)C dissociating medium for a perfusion of about 1 min. In most cases, it was not possible to obtain a homogeneous perfusion of all parts of the tumor. After perfusion, the tumor was excised and further dissociation was performed by shaking as described for normal and regenerating liver.

**Cell Yield and Trypan Blue Exclusion.** Five aliquots of 0.5 ml of the final suspensions were added to 25 ml of the filtered solution of trypan blue. After 1 hr incubation at 37\(^\circ\)C under 95% O\(_2\) plus 5% CO\(_2\), cells were counted in a Neubauer hemocytometer. Counting was repeated on 8 separate aliquots per experiment. The average number of cells per counting was 45 ± 5.

**Incorporation of L-Leucine-\(^{14}\)C into Protein.** The suspensions containing L-leucine-\(^{14}\)C were incubated at 37\(^\circ\)C under 95% O\(_2\) plus 5% CO\(_2\). Radioactive protein was isolated according to the method of Mans and Novelli (63, 64). Radioactivity was determined as described previously (75).
Electron Microscopy. Samples for the electron microscopy were prepared as described elsewhere (86).

RESULTS

Cell suspensions were prepared by shaking the minced tissue in dissociation medium or by continuous perfusion of the liver with dissociation medium. Dissociating agents were TPB, EDTA, hyaluronidase, neuraminidase, and collagenase. Total yield and percentage of unstained cells after 1 hr of incubation in trypan blue are given in Table 1. Two experiments were performed for each dissociation technique. In some experiments, when the tissue was dispersed in hyaluronidase plus collagenase, bovine serum albumin was added to the dissociation medium. No consistent effect of bovine serum albumin on total yield and proportion of unstained cells was observed.

Normal Liver. Unstained cells were obtained only after dissociation with hyaluronidase plus collagenase. Collagenase could not be replaced by neuraminidase. When the continuous recirculating perfusion was used, the proportion of unstained cells was higher than for the best version of the shaking method.

Incorporation of L-leucine-\(^{14}\)C into protein of liver cells as a function of time is shown in Chart 1. Cells prepared by continuous perfusion incorporated 60 times more L-leucine-\(^{14}\)C into protein than cells prepared by shaking in hyaluronidase plus collagenase or by shaking in TPB.

The majority of the cells prepared by continuous perfusion \textit{in situ} with hyaluronidase plus collagenase had a well-preserved ultrastructure with an intact plasma membrane, parallel stacks of rough endoplasmic reticulum, and glycogen particles (Fig. 1). The only difference from the aspect of a normal hepatocyte in tissue was the loss of the polyhedral shape and the very small number of microvilli (Fig. 1, \textit{b} and \textit{c}). Broken cells and large intracellular vacuoles (Fig. 1a) were seldom observed. In contrast to continuous perfusion, shaking of the minced tissue in TPB yielded swollen cells with a disrupted plasma membrane, a vesiculated endoplasmic reticulum, and disintegrated mitochondria, which had lost most of their cristae (Fig. 2, \textit{a} and \textit{b}). Shaking of the minced tissue in hyaluronidase plus collagenase yielded a homogenate with many isolated vesicles, pieces of swollen endoplasmic reticulum, inflated or washed out mitochondria (Fig. 3b), and badly preserved nuclei. A few cells were also obtained (Fig. 3c). They contained large cytoplasmic vesicles, condensed mitochondria, and a relatively electron-transparent nucleoplasm. Some cells were partly surrounded by a 2nd membrane presumably originating from adjacent cells (Fig. 3a).

Regenerating Liver. Only enzymatic dispersion was used to obtain single cells from regenerating liver. With the shaking procedure, 9 to 12 million cells were obtained from 1 g of liver. From 10 to 32% of those cells were not stained with

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\( ^a \) Each value is the mean of 8 countings.

\( ^b \) Values are obtained from 2 separate experiments.
Single-Cell Suspensions from Liver and Hepatomas

trypan blue. Continuous perfusion yielded 5 to 7 million cells from 1 g of liver with 28 to 52% unstained cells.

**Morris Hepatomas 9121 and 5123tc.** It was not possible to establish continuous recirculation in hepatomas. However, unstained cells from the hepatomas were obtained in the same yield by the shaking technique as by the continuous recirculation technique for normal and regenerating liver. In contrast to normal and regenerating liver, unstained cells were also observed after treatment with EDTA (Table 1).

Incorporation of L-leucine-1-14C into protein of cells obtained from Morris hepatoma 5123tc is described as a function of time in Chart 2. In contrast to normal liver, dissociation of the minced tissue by hyaluronidase plus collagenase produced cells that incorporated about 300 times more L-leucine-1-14C into protein than cells obtained with TPB.

Tumor cells prepared by dissociation with hyaluronidase plus collagenase showed a well-preserved ultrastructure, which was comparable to that of Morris hepatoma cells in situ. Rough endoplasmic reticulum and nuclear envelopes were dilated (Fig. 4, a and b). In cells prepared by TPB, the ultrastructure was severely altered (Fig. 5), as described above for liver cells (Fig. 2, a and b).

**DISCUSSION**

We could not confirm that EDTA plus lysozyme was superior to hyaluronidase plus collagenase for the preparation of single rat liver cells, as claimed by Hommes et al. (36, 37). After dispersion in EDTA with or without lysozyme, all cells stained with trypan blue. This is in agreement with the reported alteration of the permeability (28, 29, 51, 59, 85). Furthermore, a low rate of respiration in calcium-containing media in cells prepared with EDTA plus lysozyme was described (36). Destruction of the ultrastructure by EDTA (17, 31, 36, 65, 66) and a loss of enzymes after treatment with lysozyme (19) were also reported.

Like other authors (26, 32, 60), we were not able to obtain unstained cells with the concentrations of TPB published by Rappaport and Howze (71–73). Our results are in agreement with the previously reported impairment of protein synthesis (25, 60, 61) and with the observed morphological alteration of cell organelles (26, 31, 60, 66). In mitochondria 0.08 to 0.2 mM TPB had an uncoupling effect on respiration and oxidative phosphorylation (87). Impairment of enzyme induction (25, 32), leakage of enzymes (26, 81), low endogenous respiration (81), and a lack of the synthesis of lipids (60, 61) after treatment with TPB were also described. In liver slices, uptake of oxygen and retention of potassium ions were remarkably decreased by 3 mM TPB (67).

Preparation of single cells with hyaluronidase plus collagenase was also feasible for regenerating rat liver. Furthermore, unstained cells with intact ultrastructure and with a high rate of protein synthesis could be isolated from transplantable hepatomas only with hyaluronidase plus collagenase.

**ACKNOWLEDGMENTS**

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Single-Cell Suspensions from Liver and Hepatomas


Figs. 1 to 5. Electron micrographs of glutaraldehyde-fixed cells postfixed with osmium tetroxide. Sections were stained with uranyl acetate and lead citrate. ER, endoplasmic reticulum; sER, smooth ER; rER, rough ER; G, glycogen; J, junctional complex; L, lipid droplet; Ly., lysosome; M, mitochondrion; MB, microbody; N, nucleus; PC, perinuclear cisterna.

Fig. 1. Cells prepared from normal rat liver by continuous perfusion with hyaluronidase plus collagenase. a, single isolated cell. X 7,000. b, parallel stacks of rough endoplasmic reticulum, vesicles of smooth endoplasmic reticulum, and glycogen particles (arrows). X 30,000. c, well-preserved cell organelles and an intact plasma membrane. X 22,000. d to f, normal (d) and condensed (e) mitochondria and both types in immediate vicinity (f). X 30,000; X 18,000; X 28,000, respectively.

Fig. 2. Parts of cells prepared from normal rat liver by shaking of the minced tissue in TPB. All cell organelles are swollen or disintegrated. The plasma membrane is disrupted at many sites (arrows). a, X 7,000; b, X 33,000.

Fig. 3. Parts of cells prepared from normal rat liver by shaking of the minced tissue in hyaluronidase plus collagenase. a, condensed mitochondria, few single cisternae of rough endoplasmic reticulum, and electron-transparent nucleoplasm. The cell is partly surrounded by a 2nd membrane (arrows) presumably originating from adjacent cells. X 18,000. b, homogenate-like aspect. Most mitochondria appear inflated or leaked out. Irregularly shaped cytoplasmic vesicles, lipid droplets, and single cisternae of endoplasmic reticulum can be seen. X 16,000.
Fig. 4. Cells prepared from Morris hepatoma 5123tc by shaking of the minced tissue in hyaluronidase plus collagenase. a, single isolated cell. × 9,000. b, section of a. Well-preserved cell organelles, intact plasma membrane with microvilli-like structures, and many free ribosomes. The single cisternae of the endoplasmic reticulum and the nuclear space are dilated. × 20,000.

Fig. 5. Part of a cell prepared from Morris hepatoma 5123tc by shaking of the minced tissue in TPB. Swollen and disrupted organelles. Discontinuity in the plasma membrane (arrow). × 9,000.
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