THE ENZYMATIC PREPARATION OF ISOLATED INTACT PARENCHYMAL CELLS FROM RAT LIVER

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ABSTRACT

Suspensions of isolated parenchymal cells were prepared from rat liver by incubation with collagenase and hyaluronidase followed by mechanical treatment. Utilization of 0.15% collagenase together with 0.15% hyaluronidase yielded adequate numbers of cells for experimental purposes. As shown by light and electron microscopy, approximately 75% of the isolated cells retain their structural integrity. The cell suspensions are capable of maintaining endogenous respiration in the presence of 1% albumin for periods of time up to 8 hr. These cell preparations consist almost entirely of parenchymal cells and offer a unique tissue preparation for the study of hepatic metabolism.

INTRODUCTION

Various methods have been described for the preparation of isolated parenchymal cells from mammalian liver tissue. Most of these methods have utilized mechanical treatment of the tissue alone (1–5) or in combination with EDTA or citrate perfusion of the intact liver (6–10). Enzymatic techniques have been utilized for the preparation of isolated cells from a variety of tissues. However, these methods have not proven effective with adult liver tissue since they have produced a poor yield of cells (7) or very low endogenous respiratory rates (11).

The maintenance of intact morphology in liver cells prepared by the techniques described to date has been extremely variable. In most instances in which electron microscopy has been carried out on the preparations, there has been evidence of severe alteration in cellular structure. Cells isolated from mouse liver have revealed broken plasma membranes, changes in mitochondrial structure, and a transformation of the endoplasmic reticulum into isolated vesicles (12). Morphological studies of cells prepared by perfusion with chelating agents have likewise revealed extensive cellular damage (8, 9). The observation that respiratory rates in preparations of cells can be maintained for varying periods of time is a poor criterion for morphological integrity since damaged cells may continue respiring. Consequently, techniques for preparing isolated cells should be evaluated by a correlated morphological and functional study.

Structurally intact isolated liver cells would afford an extremely valuable tissue preparation for the study of complex intracellular biochemical processes. Therefore, the further development and refinement of techniques for obtaining such a cell preparation was undertaken.

The results presented herein show that, by utilizing carefully controlled conditions of enzyme concentration, time of exposure of the tissue to the enzyme, and mechanical treatment, isolated cells can be prepared which retain their morpho-

logical integrity and which maintain satisfactory rates of respiration for prolonged periods of time.

MATERIALS AND METHODS

Adult male rats of the Sprague Dawley strain, weighing 100–200 g, were used throughout. Collagenase (Type 1), hyaluronidase (Type 1) and crystalline bovine serum albumin (prepared free of fatty acid by chloroform-methanol extraction) were obtained from Sigma Chemical Co., St. Louis, Mo. All glassware was siliconized. The enzymes were dissolved in calcium-free Hanks' solution (13) since the yield of cells was significantly diminished by incubation in the presence of calcium. With this exception, calcium- and glucose-free Hanks' solution was used throughout. Oxygen uptake was measured in a Gilson respirometer (Gilson Medical Electronics, Middleton, Wis.).

Preparation of cell suspensions

The liver was removed under light ether anesthesia and perfused immediately with 10 ml of ice-cold enzyme solution. The tissue was then cut into slices with a Stadie-Riggs tissue slicer (Arthur H. Thomas Co., Philadelphia, Pa.). Approximately 1 g of tissue slices was placed in each of six 50 ml Erlenmeyer flasks containing 3 ml of the enzyme solution. All procedures to this point were carried out at 4°C. The flasks were then incubated at 37°C in an atmosphere of 95% O_2 and 5% CO_2, with constant shaking. At the end of the incubation period, 10 ml of cold calcium- and glucose-free Hanks' solution were added to each flask and the tissue was disrupted by gently drawing the suspension in and out of a pipette having a tip with an internal bore diameter of 2 mm. The resulting tissue suspension was filtered through a single layer of stocking nylon and then through nylon mesh of 61 μ pore size (Henry Simon Ltd., Cheadle Heath, Stockport, England). The cells were then sedimented for 1 min at 50 *g* in an International refrigerated centrifuge at 4°C. The cells were washed once by resuspending in 10 ml of medium followed by recentrifugation. The pellet was resuspended in fresh medium and cell counts were determined with a hemocytometer.

Light and Electron Microscopy

After sedimentation of the isolated cells, the pellet was resuspended in several volumes of cold fixative (3% glutaraldehyde, 3 mM $CaCl_2$ in 0.066 M cacodylate buffer, pH 7.4) by gentle pipetting. This suspension was kept on ice for 15 min, following which the cells were recentrifuged at 90 *g* for 10 min at 4°C. The supernatant was removed, replaced with fresh fixative, and allowed to stand for several more hours. The fixative was then replaced with 0.1 M sodium cacodylate buffer in 7.5% sucrose and refrigerated overnight. On the following day, the pellets were postfixed in buffered osmium tetroxide (1.3% in 0.066 M collidine buffer, pH 7.4, made up in 5% sucrose) for 2 hr, dehydrated in ethanol, and finally embedded in Epon. All steps through dehydration were carried out at 4°C, and during dehydration the pellet was cut up into smaller pieces. Sections were prepared for both light and electron microscopy. The thin sections were viewed in an RCA EMU-3F electron microscope.

RESULTS

Effect of Enzymes

Table I summarizes the methods of procedure found to be effective in producing a significant number of isolated cells. The amount of mechanical treatment necessary in these methods depended on the nature of the enzyme solution and on the period of contact of the slices with the enzyme. Hyaluronidase by itself failed to break up the slices into isolated cells, even when the enzyme concentration was increased to 0.5% or when the incubation time was increased to 2 hr; so considerable mechanical treatment was necessary to obtain isolated cells. Collagenase by itself caused some fragmentation of the slices which could be increased by prolonging the incubation time. However, this enzyme was more effective when used in combination with hyaluronidase. Thus, when slices were incubated with 0.15%

TABLE I

Details of the Methods Employed for the Preparation of Isolated Liver Cells

Method No.	Enzyme solution	Time of incubation of tissue with the enzyme	Mechanical treatment index*
		min	
1	0.15% hyaluronidase	15	20
2	0.05% collagenase	30	10
3	0.15% collagenase + 0.15% hyaluronidase	60	2
4	0.025% collagenase + 0.15% hyaluronidase	60	5

* Mechanical treatment index refers to the number of times the tissue suspension is passed through the 2-mm bore of a pipette after incubation in the enzyme solution.

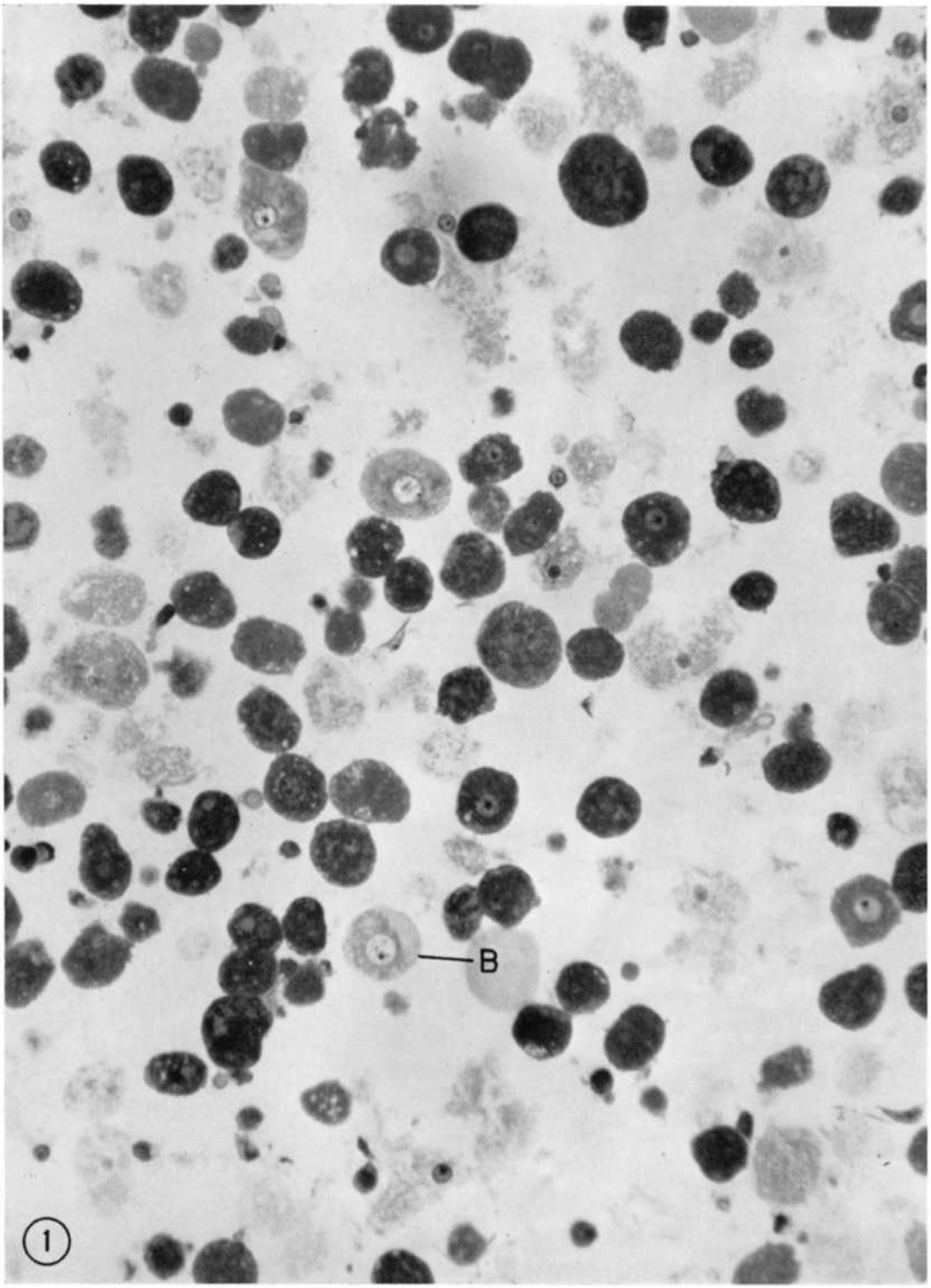

FIGURE 1 Light micrograph of isolated liver cells prepared by method 3. The dark cells are generally well preserved, while the light cells have suffered various degrees of degenerative change. Note the bleb (*B*) on one of the cells. Thick Epon section of material prepared for electron microscopy. × 460.

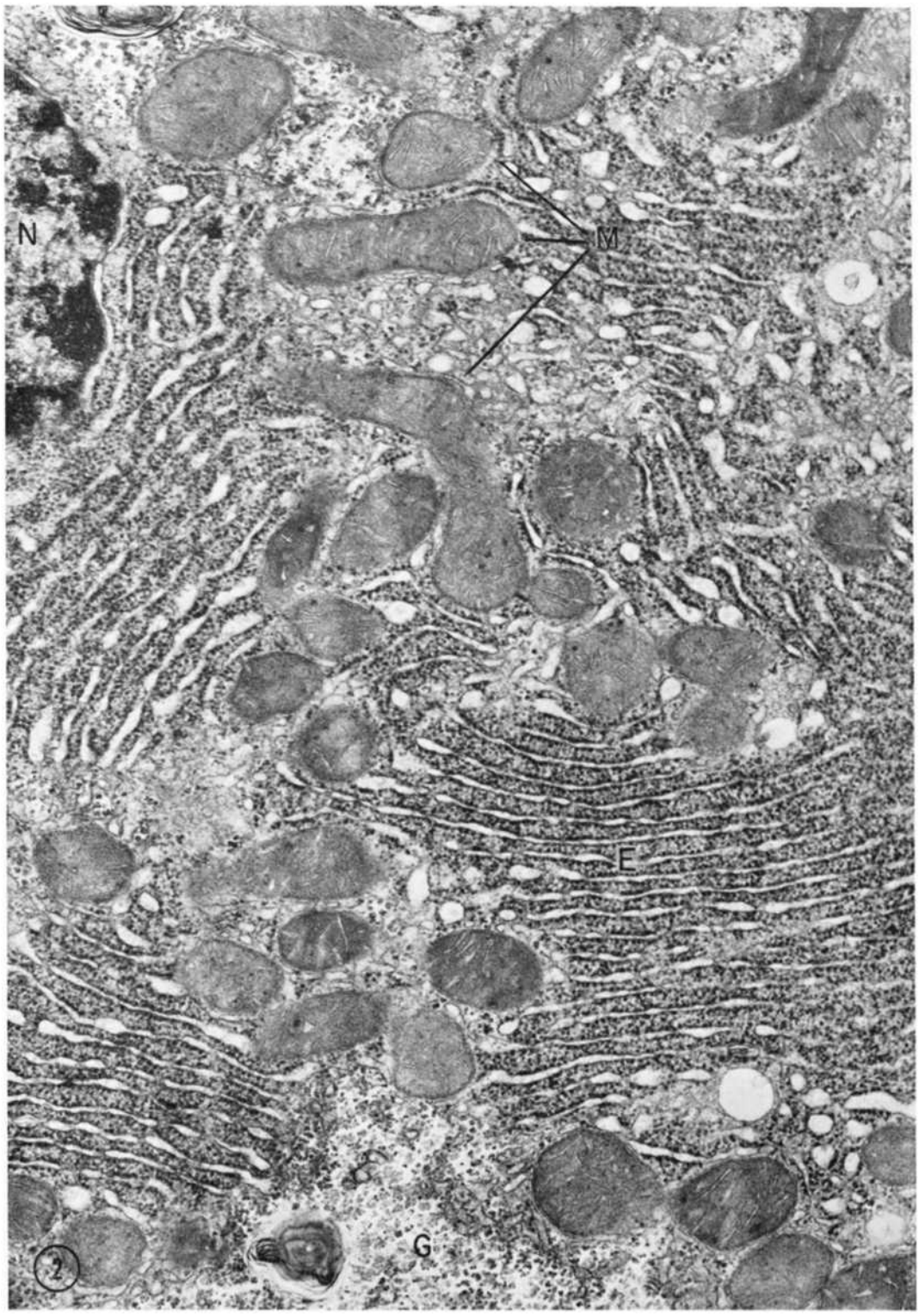

FIGURE 2 Electron micrograph of part of an intact liver cell obtained by method 3. Shows normal mitochondria (*M*), endoplasmic reticulum (*E*), nucleus (*N*), and areas of glycogen deposition (G). × 31,000.

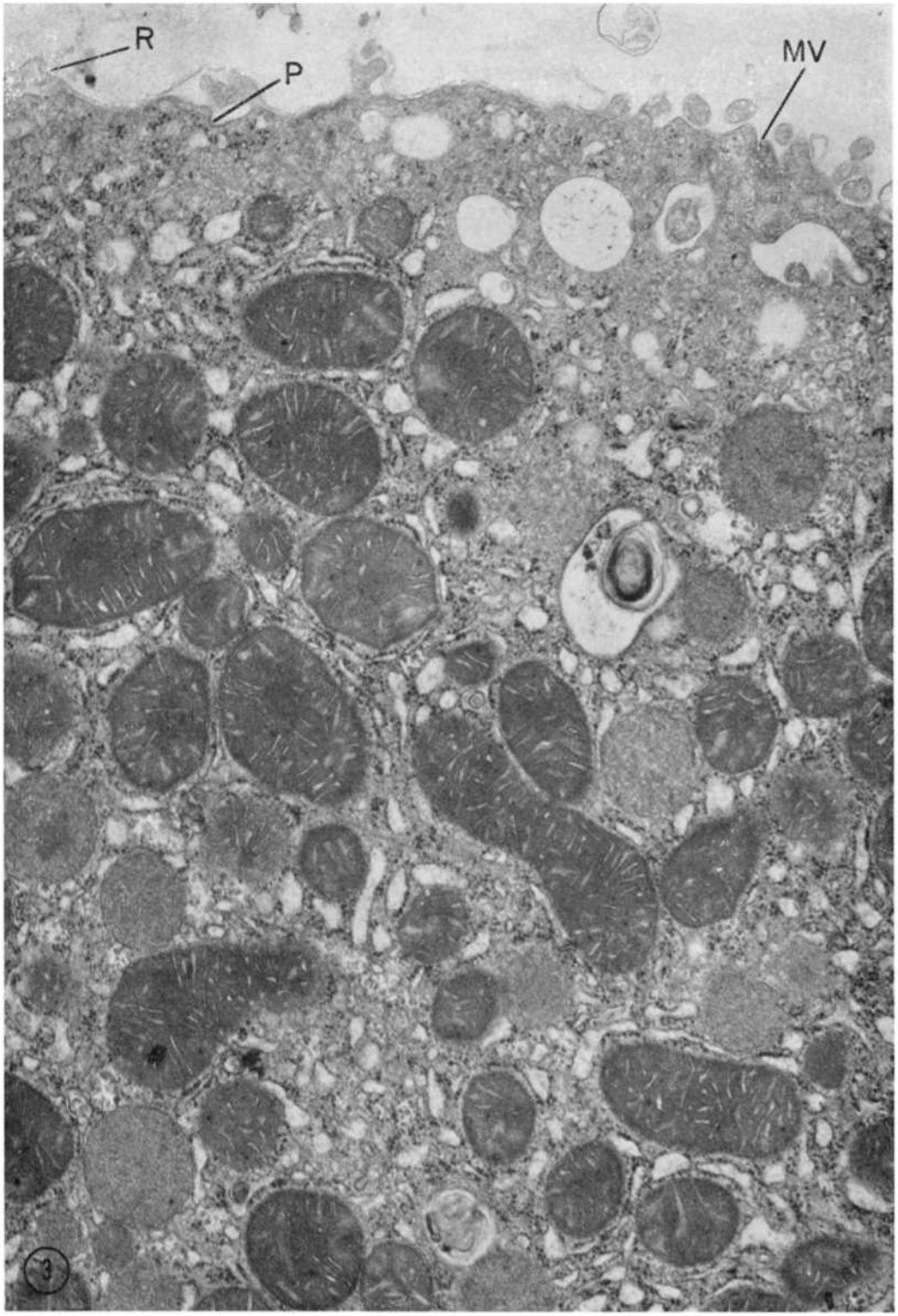

FIGURE 3 Intact liver cell obtained by method 3, showing the plasma membrane (*P*), microvilli (*MV*), and remnants of the plasma membrane of an adjacent cell (*R*). The organelles appear essentially normal, except for slight swelling of the endoplasmic reticulum. × 27,000.

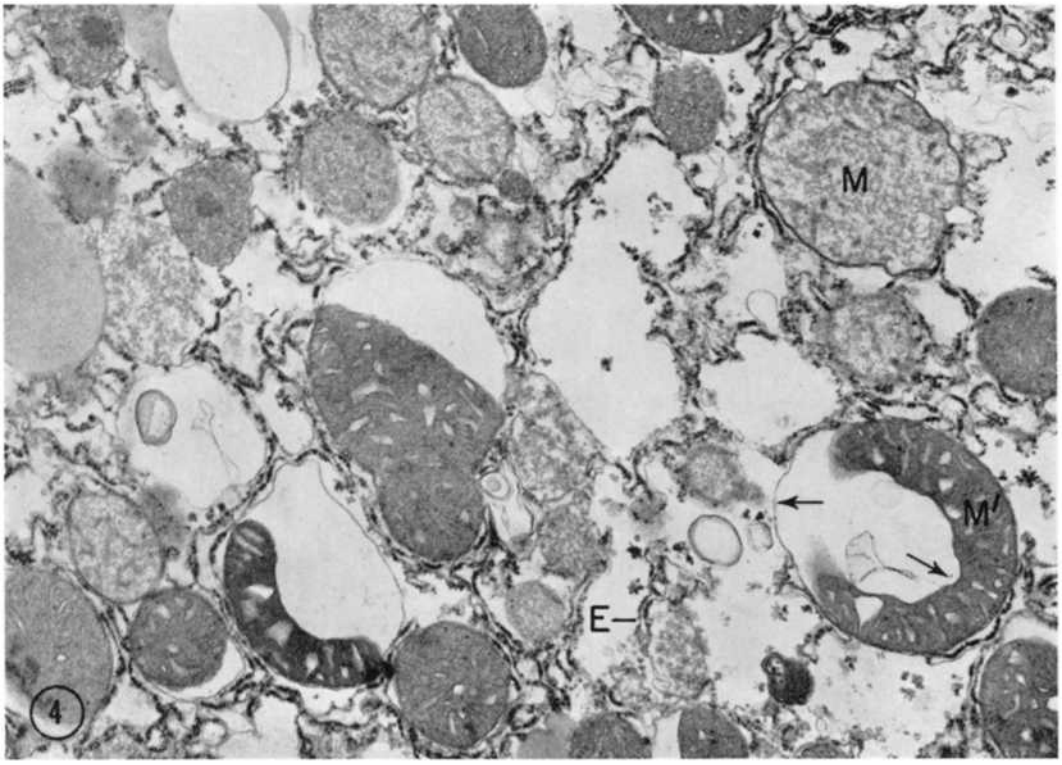

FIGURE 4 Damaged liver cell prepared by method 3. In some mitochondria (*M*) the matrix appears to have swollen while in others (*M'*) the outer compartment of the mitochondrion, between the inner and outer membranes (arrows), is vacuolated, and the matrix may have undergone some shrinkage. The endoplasmic reticulum (*E*) is in disarray. × 23,000.

collagenase and 0.15% hyaluronidase for 1 hr, only small fragments of tissue remained. Many cells were free in the medium, but the yield could be considerably increased by mild mechanical treatment.

The concentration of collagenase had a marked effect on the plasma membrane. Thus, widespread formation of blebs was noted at an enzyme concentration of 0.25% but this decreased significantly at 0.15% and was rarely observed at 0.025%.

Morphology of the Isolated Cells

Fig. 1 is a light micrograph of a thick Epon section of cells prepared for electron microscopy. A mixture of dark and light cells can be seen. The appearance of the dark cells as revealed by electron microscopy is shown in Figs. 2 and 3. The nucleus, the mitochondria, and endoplasmic reticulum all appear essentially normal. The plasma membrane of these cells is found to be intact, with the microvilli well preserved. A portion of a cell showing these features is seen in Fig. 3. In contrast, the light cells have an altered fine structure as shown in Figs. 4 and 5. It can readily be seen that severe alteration of the organelles has occurred. The endoplasmic reticulum is in disarray and has rounded up into vesicles. The mitochondria seem to have reacted to the trauma in one of two ways: either the matrix swelled uniformly (Fig. 4, *M*), or the outer compartment, between the outer and inner mitochondrial membranes, swelled, with shrinkage of the matrix (Figs. 4 and 5, *M'*). These changes in mitochondrial structure were similar to those observed by Berry and Simpson (12) in damaged mouse hepatic cells. The nuclei of these light cells were badly swollen and showed degenerative changes. In most cases in which internal damage

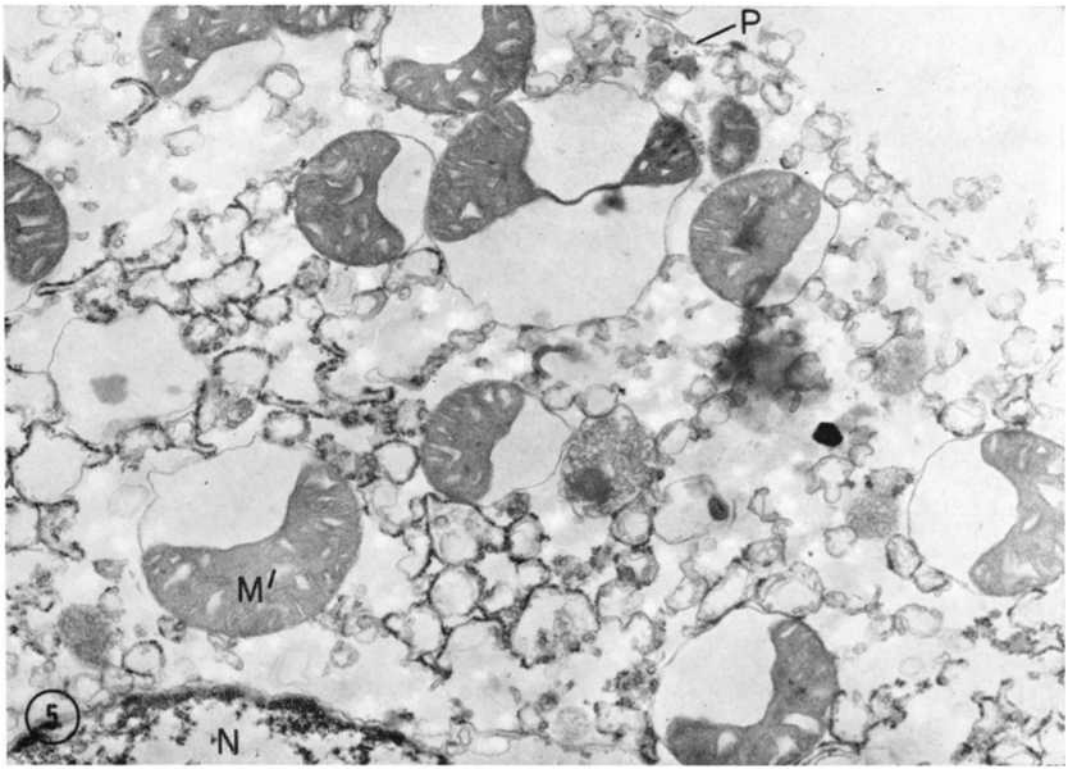

FIGURE 5 Damaged liver cell prepared by method 1. The plasma membrane (*P*) is disrupted, and the nucleus (*N*) is disarranged. The mitochondria (*M'*) show a swelling of the outer compartment like that seen in the previous figure. × 19,000.

is observed, the plasma membrane or the microvilli were not intact.

The percentages of dark cells obtained by the four preparative methods described in Table I are shown in Table II. It can be seen that the method involving the least mechanical treatment gives by far the highest percentage of intact cells.

On the basis of the electron microscopic studies, the structurally intact cells could readily be identified under the light microscope. These were more compact, tended to round up, and appeared to have a more prominent cell outline (Fig. 6). On the other hand, damaged cells were larger and polygonal in shape (Fig. 7).

Almost all of the cells in the light and electron micrographs of this study can be identified as parenchymal cells. No Kupffer cells were recognized, although a few other cell types were observed. With two washings, the preparations were even cleaner than that shown in Fig. 1.

TABLE II

Yield of Structurally Intact Parenchymal Liver Cells as a Function of Enzymatic and Mechanical Treatment

Method No.*	Total yield of cells (10^{-6}/g wet tissue)	Structurally intact (dark) cells
		%
1	5	0
2	7	20
3	7	77
4	14	12

* Method No. refers to the methods of preparation described in Table I.

Respiratory Activity

The respiratory activity of the cell preparation containing the highest percentage of intact cells (method 3, Table I) is shown in Table III. There was a significant rate of endogenous

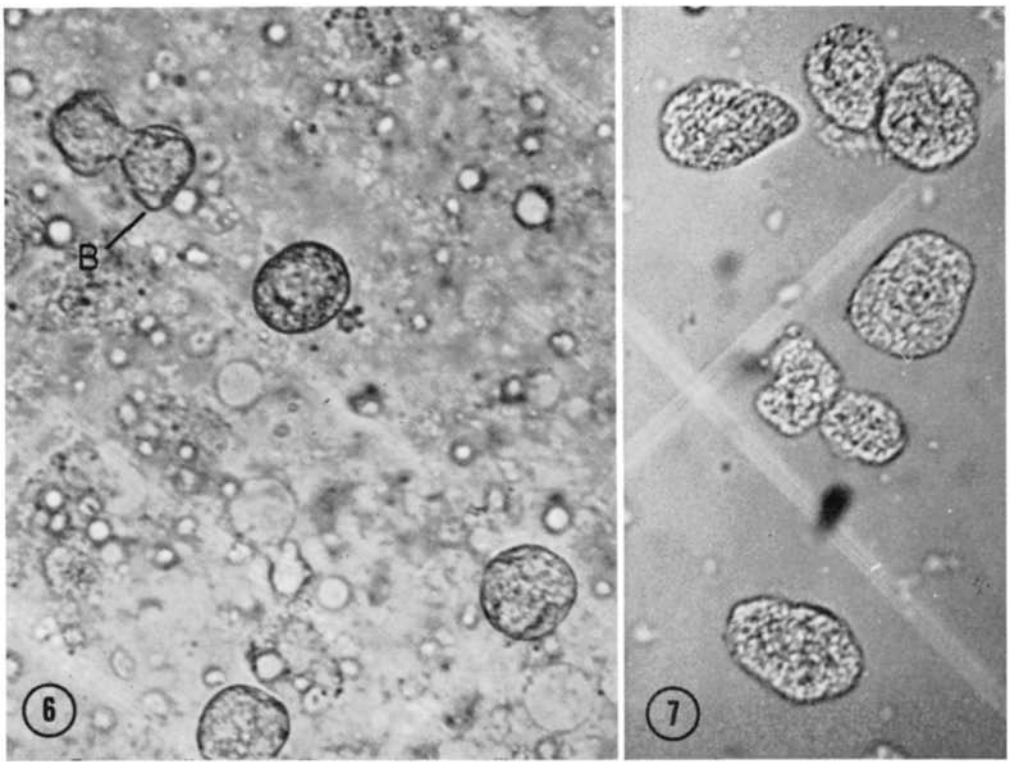

FIGURE 6 Liver cells obtained by incubating liver slices in 0.15% collagenase and 0.15% hyaluronidase prior to mechanical treatment. Contamination is present since the suspension has not been purified by centrifugation. Note that the cells are spherical in shape and have a smooth outline which is well defined. One cell shows blebbing (*B*) of the plasma membrane. × 500.

FIGURE 7 Liver cells obtained by incubation of liver slices in 0.15% hyaluronidase for 15 min followed by extensive mechanical treatment (method 1). In contrast to the cells in Fig. 6, these cells are not spherical and the cell outline is less clear. × 500.

respiration, which was not depressed by the presence of 1.26 mM calcium ions. This rate fell off dramatically after 30 min. Respiration was significantly stimulated by the addition of malate or succinate. However, even though substrate was present in excess, the rates of respiration still declined.

It can be seen in Fig. 8 that the presence of 1% albumin not only stimulated the endogenous respiration to substrate-supported levels, but also sustained respiratory activity for a prolonged period of time. When malate was present the addition of albumin did not cause any significant stimulation of the initial respiratory rate. The data shown here indicate rates observed over a period of 3½ hr. In other experiments, significant oxygen uptake continued for as long as 8 hr. The stimulation of endogenous respiration by albumin was observed in the cell preparations obtained by other methods described in Table I. However, with these preparations, calcium ions depressed the endogenous rates.

DISCUSSION

In the development of this technique for the isolation of intact parenchymal cells from rat liver, two principles need emphasis. First, the preparation of slices is designed to facilitate adequate oxygenation of the tissue during the incubation with enzymes. Second, the perfusion of the entire organ with the enzyme solution prior to slicing disperses the enzyme throughout the tissue and facilitates enzymatic activity.

Collagenase has previously been used in the

TABLE III

Respiratory Activity of Isolated Liver Cells Prepared by Method 3

Additions	Oxygen uptake (μliters/10^6 cells)	
	0–30 min	30–60 min
None	3.8	1.2
Succinate (20 mM)	8.2	2.5
Malate (20 mM)	7.7	2.9
Calcium (1.26 mM)	3.8	1.5
Albumin (1%)	7.9	2.9

The results represent the average of three experiments, each being carried out on a single preparation. The incubation flasks contained 2–6 × 10^6 cells in a total volume of 3 ml of calcium and glucose-free Hanks' solution. The center wells of the flasks contained 0.2 ml of 10% KOH. All reactions were carried out at 37°C in an atmosphere of air. The flasks were shaken at 120 oscillations per minute and equilibrated for 10 min. Malate and succinate solutions were added as the sodium salts at pH 7.4.

preparation of cells from adipose tissue (14) and from embryonic heart (15). Recently, hyaluronidase has been employed in the preparation of epithelial cells from rat intestine (16). In the present studies, this enzyme by itself did not cause any fragmentation of the liver slices, and the obtaining of cells by method 1 was probably due more to the mechanical treatment than to the enzyme. However, hyaluronidase was effective when used in combination with collagenase.

A recent report (17) which appeared while the present work was in progress indicates that this combination of enzymes is also effective in the preparation of isolated Ehrlich ascites carcinoma cells. The nature of this synergistic effect is not clear. A similar effect of hyaluronidase in combination with trypsin has also been reported by Mateyko and Kopac (4). These workers suggested that one enzyme aided the permeation of the other by unmasking reactive groups.

Our observations with the light and electron microscope reveal that the procedure involving the least mechanical treatment yields the highest

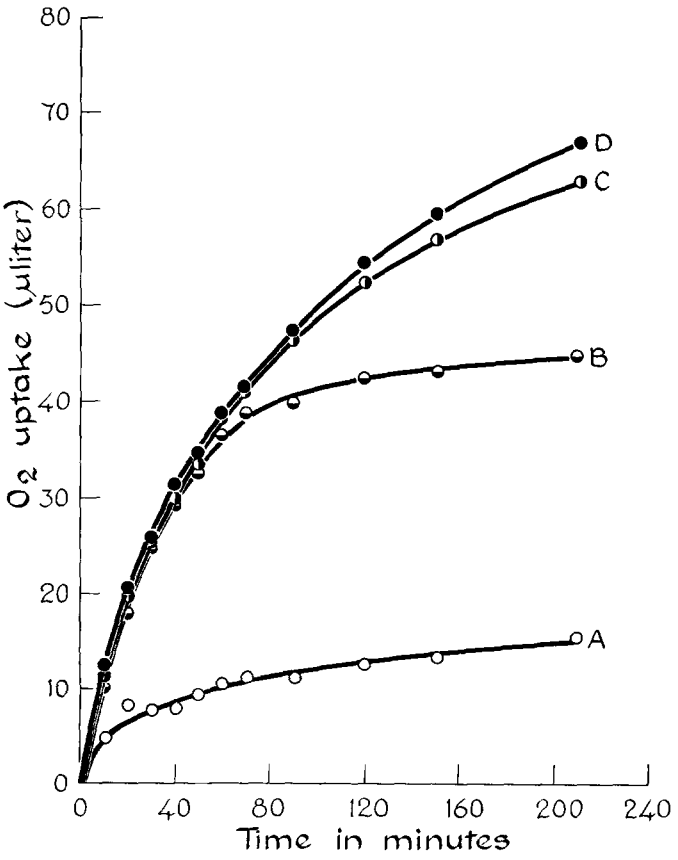

FIGURE 8. Oxygen uptake by intact liver cells prepared by method 3. Conditions are the same as those described in Table III. Each vessel contains 2 × 10^6 cells. Curve *A* represents endogenous respiratory activity; curve *B*, addition of 20 mM malate; curve *C*, addition of 1% albumin; and curve *D*, addition of 20 mM malate in the presence of 1% albumin.

percentage of intact cells. This is not surprising since mechanical treatment would most likely damage cells by abrasion of the plasma membrane, breaking of the microvilli, or by tearing the plasma membrane at the junctional complex between adjacent cells. Breaks in the plasma membrane would allow the external medium to readily enter the cell and so disrupt the organelles. However, it is possible that breaks in the plasma membrane might occur as a result of cellular swelling.

It is difficult to be certain to what extent the techniques of fixation for electron microscopy may have contributed to the morphological differences between the cells. Some of the dark cells may have shrunken to some extent during preservation, while other cells may have swollen. However, it is highly improbable that the preparation for electron microscopy would improve the cellular morphology. So the main point of the paper, that a high percentage of cells remain intact after a suitable isolation procedure, would seem to be unaffected by any question of artifacts of electron microscopy.

The most interesting aspect of the respiratory studies is the observation that the endogenous rate of respiration is greatly stimulated by the presence of 1% albumin. This effect has previously been reported with liver cells (18) and intestinal epithelial cells (16). The mechanism of the stimulation is not clear. It is possible that the albumin binds to the plasma membrane, stabilizes it in some way, and so prevents the escape of small molecules and cofactors from the cell or prevents the external medium from entering. An alternative explanation is that the albumin binds with some metabolic inhibitor. If this is the case, then the present work suggests that the inhibitor exerts its effect on the breakdown of substrates before entry into the Krebs cycle. This is based on two observations: first, albumin stimulates the endogenous respiration up to the same rate as that attained by malate stimulation; second, albumin does not further increase the initial rate of malate-stimulated respiration.

The stimulation of respiration by malate and succinate in isolated liver cells has previously been observed (3, 19). In contrast to that of the cell preparations of other workers (3, 19, 20), the endogenous respiration of the cells prepared by method 3 was not depressed by calcium ions. However, this depression of respiration was observed when the cells were not structurally intact.

The structurally intact liver parenchymal cells obtained in high per cent by method 3 provide an excellent preparation for the study of hepatic metabolism. Approximately 75% of the cells so obtained have essentially normal morphology. Finally it is important to emphasize that this preparation consists almost entirely of parenchymal cells and that the contribution of other cell types to the total cell volume of the preparations is negligible.

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BIBLIOGRAPHY

1. KALTENBACH, J. P. 1952. *Federation Proc.* **11**:237.
2. BRANSTER, M. V., and R. K. MORTON. 1957. *Nature.* **180**:1283.
3. BERRY, M. N. 1962. *J. Cell Biol.* **15**:1.
4. MATEYKO, G. M., and M. J. KOPAC. 1963. *Ann. N.Y. Acad. Sci.* **105**:183.
5. HOWARD, R. B., and C. GREEN. 1965. *Biochem. J.* **96**:33P.
6. ANDERSON, N. G. 1953. *Science.* **117**:627.
7. JACOB, S. T., and P. M. BHARGAVA. 1962. *Exptl. Cell Res.* **27**:453.
8. CASTAGNA, M., and J. CHAVEAU. 1963. *Compt. Rend.* **257**:969.
9. MARSILII, G., and M. G. BUCCIOLINI. 1963. *Sperimentale.* **113**:119.
10. TAKEDA, Y., A. ICHIHARA, H. TANIOKA, and H. INOUÉ. 1964. *J. Biol. Chem.* **239**:3590.
11. SCARPINATO, B. 1963. *Sperimentale.* **113**:251.
12. BERRY, M. N., and F. O. SIMPSON. 1962. *J. Cell Biol.* **15**:9.
13. HANKS, J. H., and R. E. WALLACE. 1949. *Proc. Soc. Exptl. Biol. Med.* **71**:196.
14. RODBELL, M. 1964. *J. Biol. Chem.* **239**:375.
15. CAVANAUGH, D. J., W. D. BERNDT, and T. E. SMITH. 1963. *Nature.* **200**:261.
16. PERRIS, A. D. 1966. *Can. J. Biochem.* **44**:687.
17. YAMADA, T., and E. J. AMBROSE. 1966. *Exptl. Cell Res.* **44**:634.
18. ICHIHARA, A., H. TANIOKA, and Y. TAKEDA. 1965. *Biochim. Biophys. Acta.* **97**:1.
19. IYPE, P. T., and P. M. BHARGAVA. 1965. *Biochem. J.* **94**:284.
20. ONTKO, J. A. 1967. *Biochim. Biophys. Acta.* **137**:13.