THE FINE STRUCTURE, POTASSIUM CONTENT, AND RESPIRATORY ACTIVITY OF ISOLATED RAT LIVER PARENCHYMAL CELLS PREPARED BY IMPROVED ENZYMATIC TECHNIQUES

ROGER B. HOWARD, JOSEPH C. LEE, and LEROY A. PESCH

From the Departments of Medicine and Anatomy, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14214 and Michael Reese Hospital and Medical Center, Chicago, Illinois 60616. Drs. Howard and Pesch are presently at the latter address.

ABSTRACT

Further modifications of the enzymatic technique for the preparation of isolated, intact, parenchymal cells from rat liver as previously described by this laboratory are presented together with a detailed account of several critical factors involved during the procedure. In addition, the fine structure of the cells as revealed by electron microscopy and the characteristics of their respiratory activity in different media and with several added substrates are described. It is shown that cells obtained by adding calcium during the preparative procedure retain approximately 34% more potassium than cells prepared solely in a calcium-free medium. The former cells also demonstrate a higher respiratory activity, which is not due to uncoupling of respiration. Electron microscopy reveals that the cells have an intact plasma membrane and well-preserved intracellular organelles. Glycogen particles are observed in all cells and are particularly abundant when either 20 mM pyruvate is added during the preparation or Eagle's Minimum Essential Medium is employed.

INTRODUCTION

In 1967, this laboratory introduced the first successful enzymatic technique for the preparation of intact isolated parenchymal cells from rat liver (16). Since then we have been continually investigating possible modifications in the preparative technique in order to improve the morphology and metabolic activity of these cells. We have already reported some modifications of our original technique. These resulted in the production of suspensions containing a high percentage of intact and viable cells so that calcium ions stimulated oxygen uptake (17) instead of depressing it as had occurred with other liver cell preparations (2, 19).

In this communication two further modifications are described. The first involves a stricter control of the pH of the incubation medium, which has resulted in the better preservation of the intracellular matrix and organelles of the cells and also a higher cellular respiratory activity.

The second modification involves the addition of calcium to a previously calcium-free medium at an appropriate time during the preparative procedure. This procedure was developed as a result of our concern that cells prepared by our previous technique might exhibit some anomalies in structure or metabolic activity, owing to the medium employed during their preparation being deficient in calcium. In particular, we wondered whether

this deficiency might account for the low amount of potassium reported in these cells (3). It is shown, herein, that the second modification not only results in a greater retention of potassium in the cells, but also further improves their intracellular structure and further enhances their respiratory activity.

We have noted that our previously described procedure (16, 17) in the hands of some workers has resulted in the preparation of damaged cells (4, 10, 15, 20). Since there are many critical factors involved in obtaining intact, isolated liver parenchymal cells, we are describing them in detail together with a complete account of our latest modified procedure. In addition, we are presenting some of the metabolic characteristics of these cells, particularly with respect to the effect of added substrates on respiration. It will be seen that the effect can depend on the nature of the incubation medium employed and that, besides affecting respiration, added substrates can also produce loss of cell viability and can result in a considerable alteration of the pH of the medium which bathes the cells.

EXPERIMENTAL PROCEDURE

Materials

Adult male rats of the Sprague-Dawley strain weighing from 200 to 250 g were used throughout. Crude collagenase was obtained from Worthington Biochemical Corp., Freehold, N. J. Hyaluronidase (type 1), crystallized and lyophilized bovine albumin, and sodium pyruvate were obtained from Sigma Chemical Co., St. Louis, Mo. Deionized double quartz distilled water was used throughout. Solutions of all substrates were adjusted to pH 7.4 before use when necessary. All glassware was siliconized. The Stadie-Riggs tissue slicer was purchased from Arthur H. Thomas Co., Philadelphia, Pa. and the nylon mesh of 61 μm pore size from Henry Simon, Ltd., Cheadle Heath, Stockport, England. Media used were Hanks solution (13), Krebs-Ringer phosphate solution (5), and calcium-free Minimum Essential Medium based on Hanks solution (Microbiological Associates, Inc., Bethesda, Md.) of the formulation of Eagle (6).

Preparation of Cell Suspensions

An entire rat liver was removed under ether anesthesia, rinsed with ice-cold calcium-free Hanks solution, and perfused (*a*) immediately through the portal vein with 15 ml of ice-cold enzyme solution (*b*). The enzyme solution was 0.05% collagenase (*c*) and 0.10% hyaluronidase dissolved in calcium-free Hanks solution (*d*). The tissue was then cut into slices, by hand (*e*), with a Stadie-Riggs tissue slicer. Slices, 2 or 3 g, were placed into each of three 250-ml Erlenmeyer flasks containing 10 ml of enzyme solution (*f*). All procedures to this point were carried out at 4°C.

The flasks were then incubated at 37°C in an atmosphere of 100% oxygen (*g*) and shaken at a rate of 90 oscillations per minute. Incubation was carried out either for 70 min in the calcium-free medium (method A) or for 55 min, 0.1 ml of 1.41% $CaCl_2$ solution having been added to each flask at 35 min (method B). Other variations of these methods are described in the Results. The pH of the enzyme solution was periodically adjusted during the incubation. This was performed by the dropwise addition of 0.1 *N* NaOH until the color of phenol red indicated the reaction mixture to be approximately pH 7.4. These adjustments were generally made after 20 and 40 min of incubation. At the end of the incubation period, the contents of the flasks were filtered through a single layer of stocking nylon and then through nylon mesh of 61 μm pore size (*h*).

The filtration was aided by the addition of ice-cold calcium- and glucose-free Hanks solution (*j*) to a final volume of approximately 80 ml. This was transferred to two 50-ml centrifuge tubes and the cells were sedimented for 1 min at 50 *g* (*k*) in a Sorvall GLC–1 centrifuge. The supernatants were removed with a pipette and the cells were washed twice in 10 ml of ice-cold calcium- and glucose-free Hanks solution. The cells were dispersed in the wash solution by blowing the medium onto the pellet from a pipette. After each washing, the cells were sedimented by centrifugation at 20 *g* for 1 min (1). After the final centrifugation the cells were resuspended in the incubation medium and used immediately (*m*).

It is important to stress that the cells are easily damaged by mechanical abrasion and so must be handled gently throughout the preparative procedure (*n*). Yields ranging from 2 to 8 million cells per gram wet weight of liver can be obtained. The preparation consists almost entirely of parenchymal cells, of which 85–98% are viable. The viability and appearance of the cells (*o*) should be noted at the beginning of every experiment.

Footnotes to Cell Preparation

(*a*): The perfusion of the enzyme solution into the liver is a critical part of the preparative procedure. It not only gets rid of blood cells but also makes the enzymes readily available to all of the tissue. Perfusion was carried out by gently injecting the enzyme solution into the liver with a syringe. The needle of the syringe is inserted in turn into each of the primary branches of the portal vein immediately where they enter each lobe of the liver. The liver should become

completely blanched. To ensure this, a greater volume of enzyme may be used for the perfusion. In addition, the needle of the syringe can actually be inserted into different parts of the liver or into blood vessels other than the portal vein. It is important that the perfusion is not forceful, as this will damage the cells.

(*b*): The liver perfuses more easily with warm enzyme solution. However, an ice-cold solution is preferred by the present workers, as this cools down the liver more rapidly. Liver slices are generally prepared at cold temperatures so as to slow down the metabolism of the cells and so reduce the requirement for oxygen.

(*c*): Commercial collagenase preparations can vary considerably. On occasions this laboratory has encountered preparations with which very low yields of cells were obtained or the cells were not exhibiting their normal metabolic characteristics. Therefore, it is advisable to test a small quantity of the enzyme before ordering a large batch.

(*d*): The choice of medium for dissolving the enzymes is important. The use of complete Hanks solution or Krebs-Ringer phosphate solution (with or without calcium) results in very low yields of cells (less than 1×10^6 cells per gram of liver).

(*e*): The preparation of slices is designed to facilitate adequate oxygenation of the tissue during the incubation with enzymes, and so the slices should be made as thin as possible. Thick slices also do not disperse as readily into isolated cells. In the present work the slices were approximately 0.3 mm–0.4 mm thick.

The use of the Stadie-Riggs tissue slicer is highly recommended. Attempts in this laboratory to obtain reasonable yields of cells from slices obtained with an automatic tissue chopper (McIlwain Tissue Chopper, The Mickle Laboratory Engineering Co., Gomshall, Surrey, England) were unsuccessful. It appears that the force of the blade damages a high percentage of the cells during the preparation of the slices.

(*f*): The volume of enzyme solution and the size of the flasks were chosen so as to provide enough motion of the contents to disperse the slices into isolated cells without any further mechanical treatment. A larger volume of solution also increases the depth of the medium over the slices. This greater depth would hinder the availability of oxygen to the slices (25).

(*g*): The oxygen is first saturated with water by bubbling it through water at 37°C, and then blown constantly over the enzyme solution in the flasks. It is felt that this facilitates the oxygenation of the slices better than the technique of initially gassing the flasks and stoppering them up. This laboratory has found that low yields of poor quality cells are obtained when air is used as the gas phase. This is not surprising since it has been reported that a high oxygen tension is necessary for the inner cells of liver slices to survive (9). Thus, for adequate oxygen to reach the inner cells of slices, approximately 0.35 mm thick, its tension needs to be 1 atm.

The use of 100% oxygen is preferred to the mixture of 95% oxygen and 5% CO_2 previously described (16, 17). The reason for this is that it is easier to maintain a pH close to 7.4. As the slices disperse, the enzyme solution becomes more acid. In fact, it was found that when 95% O_2 and 5% CO_2 was used the enzyme solution quickly dropped to pH 6.6. The use of 100% oxygen counteracts this. However, it is still necessary at times to readjust the pH of the enzyme solution. The addition of drops of 0.1 *N* NaOH does not appear to do any harm. Adjustments to the pH are least required when 3 g of slices are present in the flasks.

(*h*): The use of this mesh is to separate out clumps of cells. It is rare to find other than single or paired cells in the filtrate. The contents of the flasks should be gently filtered, preferably directly into small beakers, so that the cells do not have to drop from a great height.

(*j*): Cold calcium- and glucose-free Hanks solution for aiding filtration and also washing was chosen because in several experiments the addition of calcium or glucose was being studied. Krebs-Ringer phosphate solution was not used in the washing procedure. This solution occasionally contains a precipitate which would accumulate with the cells during centrifugation.

(*k*): The centrifugation speed and time should be strictly adhered to. Faster speeds result in the sedimentation of broken cells and debris. These can be observed as a white fluffy layer on top of the cell pellet and should be removed, if present. However, at a faster speed nonviable cells also mix in with the cell pellet and are difficult to eliminate.

(*l*): The lower *g* force assists in eliminating damaged and nonviable cells. Therefore, it is important not to spin faster.

(*m*): It has been reported that liver slices take up water, calcium, and sodium ions while losing potassium and magnesium ions at a low temperature (14, 27). Such changes in the ionic environment may be harmful to the cells. In fact, the percentage of viable cells obtained in the preparation decreases the longer they are stored at 4°C.

(*n*): It has been emphasized previously (16, 17) that mechanical force damages liver cells. The studies of Berry and Friend (3) indicate clearly why this is so. Thus, although the enzymes loosen the cells, they are still attached at tight and gap junctions. An intact cell when it separates has a part of the plasma membrane of an adjacent cell adhered to it. It is clear that mechanical treatment must be kept to a minimum so as to avoid tearing of the plasma membrane of all cells. The gentle shaking of liver slices as described in

this paper, appears to be the best way to accomplish the separation of the loosened cells.

(*o*): It is important to check the appearance and viability of the cells for each experiment. The cells should be round and have a well-defined plasma membrane (17). Viability was determined in this work with trypan blue. It must be stressed that viable cells do not take up any trypan blue. Damaged cells are identified by a darkly-stained nucleus and a lightly-stained cytoplasm.

Measurement of Potassium

Potassium was measured in a flame photometer (Baird Atomic, Inc., Bedford, Mass.) using standard techniques. The cells were washed twice with an ice-cold solution consisting of 5 parts of 0.9% NaCl and 1 part of sodium phosphate buffer (0.1 M, pH 7.4), instead of with calcium-free Hanks solution, and finally suspended in deionized double quartz distilled water. They were then disrupted by sonification. The concentration of potassium for both isolated cells and homogenates of liver was based on protein, which was measured by the method of Lowry et al. (22).

Respiration Studies

Isolated cells were incubated in 3 ml of Krebs-Ringer phosphate solution, pH 7.4, except where otherwise noted. Oxygen uptake was measured in a Gilson respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.) as previously described (17). Carbon dioxide production was determined by omitting KOH from the center wells of flasks and subtracting the gaseous change from that observed for oxygen uptake. Rates of oxygen uptake are expressed as microliters per hour per million cells. $Q_{O_2}{}^{prot}$ values can be calculated from these rates ($Q_{O_2}{}^{prot}$ = microliters of oxygen uptake per mg protein per hour). It was previously found that one million cells is equivalent to 2.3 mg of protein (17). The viability of liver cells was estimated by 0.2% trypan blue staining and observed by light microscopy. This was carried out at the beginning and at the end of incubations. Cell counts were determined with a hemocytometer. The pH of solutions was determined with a Corning Model 12 pH meter (Corning Glass Works, Science Products Div., Corning, N. Y.).

Electron Microscopy

After sedimentation of the washed cells, the pellet was resuspended in 2 ml of cold 4% glutaraldehyde in phosphate buffer by gentle pipetting. This suspension was kept on ice for 15 min at 4°C. The supernatant was removed, replaced with fresh fixative, and allowed to stand for several hours. The sediment was trimmed into 1 mm^3 pieces. These pieces were post-fixed in 1% phosphate-buffered osmium tetroxide, dehydrated in graded ethanols, and embedded in Maraglas mixture. Thin sections were stained with lead citrate for electron microscope examinations.

RESULTS

The Preparation of Isolated Liver Parenchymal Cells

Effect of Calcium on Cell Yield

Table I shows the effect of calcium on the yield of isolated liver parenchymal cells obtained by the enzymatic technique fully described in the Experimental Procedure. It can be seen that the yield of cells is considerably lower when calcium is present throughout the entire incubation or added after 10 min than when it is completely omitted from the medium. The lower yields of cells are due to the failure of the liver slices to disperse rather than to the enzymes employed attacking cells that have already been isolated. Concentrations of calcium as low as one third of that used in the experiment shown in Table I also hindered dispersion of the slices when present during the entire preparation. It is also seen in Table I that if calcium is added after 20 min or longer, the yield of cells is as great as or greater than that obtained when no calcium

Table I

Effect of Adding Calcium to Liver Slices on the Production of Isolated Liver Cells

Two rat livers were each perfused with 30 ml of enzyme solution (0.05% collagenase and 0.1% hyaluronidase in calcium-free Hanks solution). Slices from these livers were evenly distributed among six preparative flasks and incubated in 10 ml of enzyme solution for a total of 60 min. 0.1 ml of 1.41% $CaCl_2$ solution was added to the preparative flasks at the times indicated. This addition results in a final concentration of calcium of 1.26 mM as found in complete Hanks solution.

Time at which calcium was added	Wet weight of tissue per flask	Number of cells obtained per gram wet weight of tissue
min	*g*	10^{-6}
0	3.2	1.0
10	2.6	1.8
20	2.3	4.2
30	3.0	5.4
40	3.5	4.0
No calcium added	2.6	4.0

TABLE II

Average Potassium Content of Liver Cells Prepared in Different Media

The livers were perfused with the same medium as that used for the incubation of slices. All media contained 0.05% collagenase and 0.1% hyaluronidase. Slices from the perfused livers were incubated for 60 min. 0.1 ml of 1.41% $CaCl_2$ solution was added where indicated. All measurements were from populations containing over 90% of viable cells. The average potassium content for liver homogenates was 489 ± 9 m-equiv/kg protein (30 experiments).

Experiment number	Medium in which enzymes were dissolved	Potassium content of isolated cells
		m-equiv/kg protein
1	Calcium-free Hanks solution	225 ± 16* (9)‡
2	Calcium-free Hanks solution but calcium added after 35 min of incubation	302 ± 13 (11)
3	Calcium-free Hanks solution + 20 mM pyruvate but calcium added after 35 min of incubation	362 ± 22 (7)
4	Calcium-free Hanks solution + 1% albumin	178 ± 24 (3)

* Mean value ± standard error of mean.

‡ Number of experiments.

is added. We observed during this work that cells incubated for longer than 30 min with calcium tended to assume a ragged appearance although they did not take up trypan blue. In practice, we find that the highest quality cells are obtained when calcium is added after 25–35 min with a subsequent incubation period of 20–25 min. In this way, yields ranging from four to six million cells per gram wet weight of liver are routinely obtained with 85–98% of the cells viable. These cells are round and have a well-defined plasma membrane (see reference 17).

EFFECT OF CALCIUM ON POTASSIUM CONTENT

Table II presents the potassium content of isolated cells prepared in several different media. Since it is impossible to assess the extent of damage in nonviable cells and consequently how much potassium has leaked out from them, the average potassium content was measured in only those preparations containing over 90% of viable cells. It can be seen that cells prepared in calcium-free Hanks solution contain about 40% of the potassium found in liver homogenates. This is an agreement with the results of Berry and Friend (3). Murthy and Petering (23) reported much lower values for cells prepared by the method of Howard and Pesch (17) but they did not state the percentage of viable cells present in their preparations.

Table II shows that the addition of calcium during the preparation of the liver cells has a marked effect on their potassium content. Thus, they contain 34% more potassium than cells prepared solely in a calcium-free medium. This is not the result of the cells prepared with calcium having less protein since the amount of protein per million cells was found to be the same for cells obtained from both preparative procedures. The amount of potassium in the liver cells prepared with the addition of calcium is even higher when pyruvate is present in the incubation medium, but is still only 74% of that found for liver homogenates (Table II). This effect of pyruvate was not observed when calcium was omitted during the entire procedure.

Crystalline bovine albumin (1%) does not increase the potassium content of the cells (Table II). Similar results were obtained with concentrations as high as 4%. Albumin also did not further increase the potassium content when calcium was added during the preparation. Cells prepared with albumin tended to have a ragged appearance although they did not take up trypan blue.

Table III shows that the potassium content of liver cells is higher when calcium is added during the preparative procedure to other media based on sodium chloride. In Experiment 2, the liver was kept at 37°C throughout and the cells were washed with NaCl-phosphate buffer solution at the same temperature. Cells isolated in this way together with exposure to calcium during the preparative procedure are still partially deficient in potassium, and those cells prepared solely in the calcium-free medium still contain considerably less potassium than when calcium is added. It is clear, therefore,

TABLE III

Effect of Calcium on the Potassium Content of Isolated Liver Cells Prepared in Different Media

A different liver was used for each experiment. The livers were perfused with the same medium as that used for the incubation of slices. All media contained 0.05% collagenase and 0.1% hyaluronidase. Slices from the perfused livers were incubated for 60 min. 0.1 ml of 1.41% $CaCl_2$ solution was added after 35 min where indicated.

Experiment number	Incubation medium	Potassium content of isolated cells	
		No calcium added	Calcium added during preparation
		m-equiv/kg protein	
1	Calcium-free Hanks solution	191 (90)*	289 (94)
2	Calcium-free Hanks solution (warm preparation)‡	211 (85)	366 (98)
3	Calcium-free Krebs-Ringer phosphate solution	250 (95)	363 (97)
4	Calcium-free Eagle's Minimum Essential Medium	210 (82)	333 (96)

* Percentage of viable cells.

‡ All solutions were at 37°C throughout, and slices were cut at room temperature.

that the presence of calcium plays a more important part than temperature in the retention of potassium in these cells.

Electron Microscopy Studies

Fig. 1 shows the fine structure of a parenchymal liver cell which is representative of the cell population obtained by incubating liver slices with collagenase and hyaluronidase in calcium-free Hanks solution (method A described in Experimental Procedure). The plasma membrane is intact with numerous microvilli projecting from the surface. In general, the mitochondria are intact except that many do not show the presence of cristae. The nucleus and endoplasmic reticulum are well preserved. The slight swelling of the endoplasmic reticulum which was observed in the cells prepared by the original technique of Howard et al. (16) is not evident. Darkly-stained particles of glycogen are scattered throughout the cell.

There are some signs of damage, however. The saccules of the Golgi apparatus are slightly dilated, and small vacuoles are present in a few areas. There are no microbodies present in Fig. 1, although some were observed in other electron micrographs. Nevertheless, the number of microbodies, myelin figures, lysosomes, and vacuoles is considerably less than in those cells prepared by Howard et al. (16).

Fig. 2 reveals the fine structure of a typical cell obtained by adding calcium during the preparative procedure (method B described in Experimental Procedure). The plasma membrane, nucleus, mitochondria, endoplasmic reticulum, and glycogen are well preserved. Two improvements in these cells over the cells prepared in a calcium-free medium were noted. First, the cristae are well defined in practically all of the mitochondria. Second, none of the cells with an intact plasma membrane reveals a swollen Golgi apparatus.

The presence of 20 mM pyruvate in the incubation flasks further increases the potassium content of the cells when calcium is added during the preparative procedure (Table II). The fine structure of cells so prepared is extremely well preserved. Fig. 3 represents part of a typical cell. In the areas shown there are no signs of degeneration. Intact mitochondria and stacks of undilated saccules of Golgi are present. The particulate glycogen is abundant. Overall, more glycogen was observed in these cells than in those prepared in Hanks solution in which glucose is the sole substrate.

Fig. 4 shows part of a typical cell obtained by method B, except that calcium-free Eagle's Minimum Essential Medium was used for dissolving the enzymes. The fine structure of these cells is extremely well preserved and glycogen is exceptionally abundant.

Respiratory Characteristics of Isolated Liver Parenchymal Cells

ENDOGENOUS RESPIRATION

The endogenous rate of respiration of cells obtained by adding calcium during the preparation (method B) is higher than that of cells prepared in

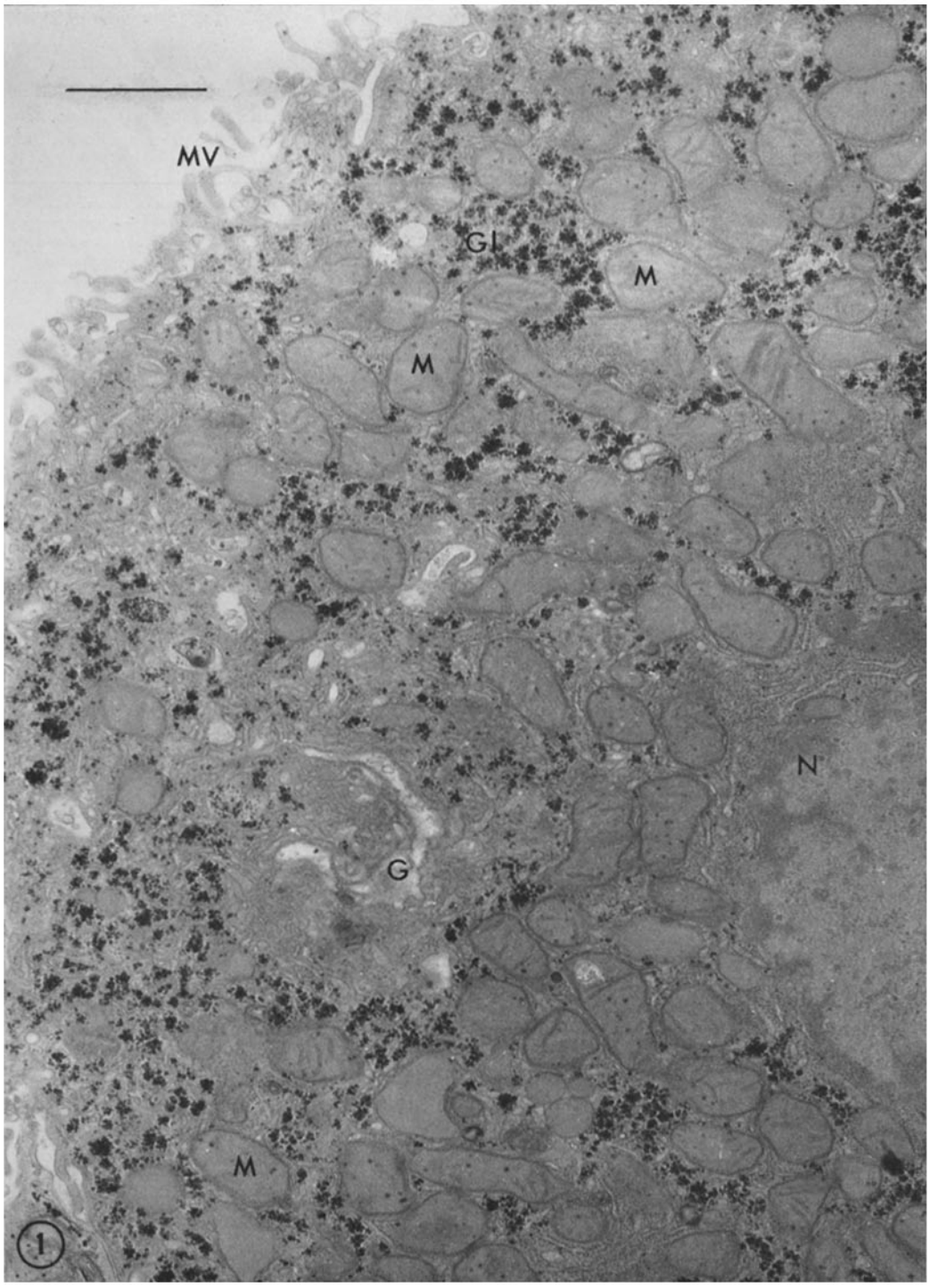

FIGURE 1 Part of an isolated parenchymal cell prepared from liver slices incubated with enzymes dissolved in calcium-free Hanks solution for 60 min. The plasma membrane with numerous microvilli (*MV*), the nucleus (*N*), and the endoplasmic reticulum appear normal. Some mitochondria (*M*) have lost their cristae and the saccules of the Golgi apparatus (*G*) are swollen. Particles of glycogen (*Gl*) are scattered among the organelles. Scale bar, 1 μm. × 20,250.

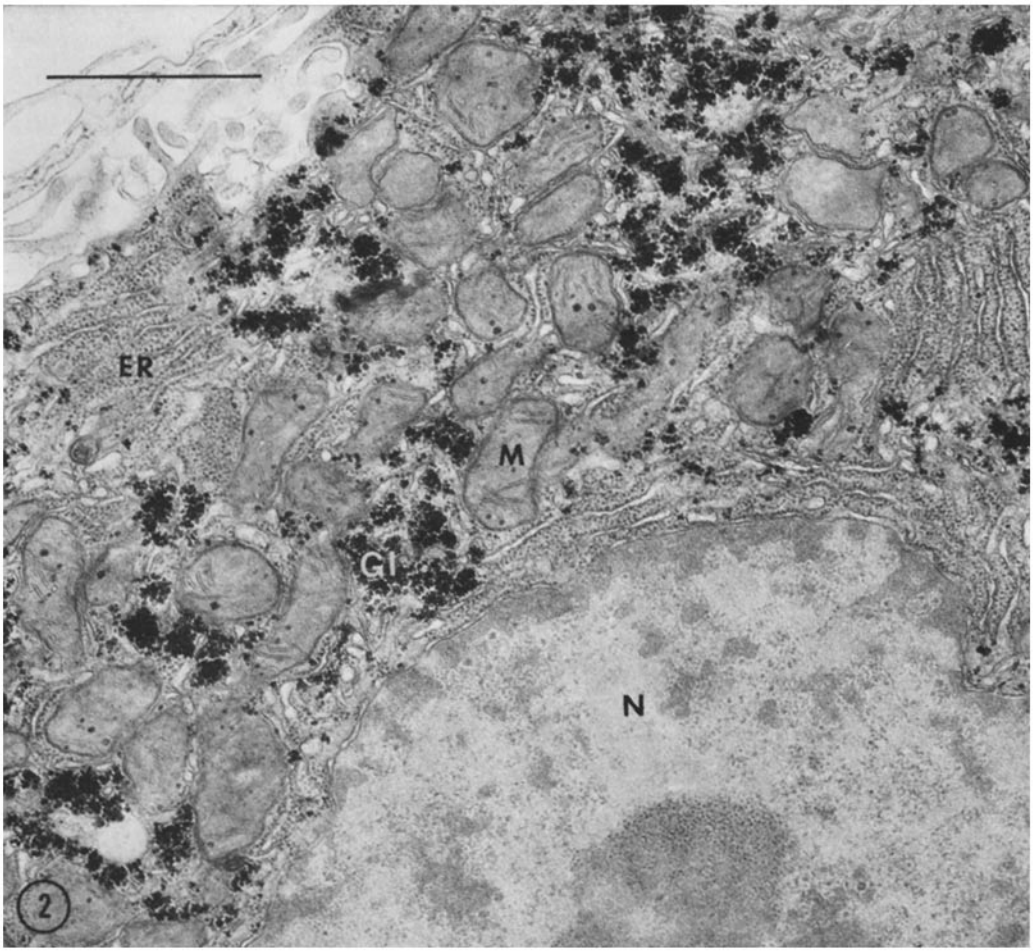

FIGURE 2 Isolated parenchymal cell prepared from liver slices incubated with enzymes dissolved in calcium-free Hanks solution for 35 min and then for a further 25 min with added calcium. The granular endoplasmic reticulum (*ER*) is often arranged in parallel stacks of cisternae, and the mitochondria (*M*) have well-preserved cristae. *Gl*, glycogen; *N*, nucleus. Scale bar, 1.05 μm. × 27,000.

a calcium-free medium (method A but with 55 min allowed for incubation). In 11 paired experiments, in which cells were prepared from the same liver for each experiment with or without the addition of calcium, the average oxygen uptake of the former in Krebs-Ringer phosphate solution was 30.0 ± 0.98 μl/hr per 10^6 cells, ($Q_{O_2}{}^{prot}$ = 13.0) whereas that of the latter was 25.2 ± 1.20 μl/hr per 10^6 cells ($Q_{O_2}{}^{prot}$ = 11.0). The standard error of the average mean difference in oxygen uptake, as determined by paired data analysis, was ±1.13 and is highly significant ($p < 0.005$). These rates were maintained for 60–120 min with a subsequent gradual decline. The higher rates of oxygen uptake in cells prepared with the addition of calcium is not the result of respiration being uncoupled, since the addition of 2,4-dinitrophenol greatly stimulated oxygen uptake in these cells (Fig. 5).

Although suspensions containing a high percentage of viable cells could occasionally be obtained by carrying out the whole preparative procedure at 37°C, these cells had considerably lower rates of respiration than cells which had been exposed to ice-cold temperatures (method B employed). The best rate obtained was 14.0 $\mu l O_2$/h per one million cells. Electron microscopy revealed that these cells contained many vacuoles and that numerous mitochondria had lost their cristae.

The respiratory characteristics which follow were carried out with cells prepared in a calcium-

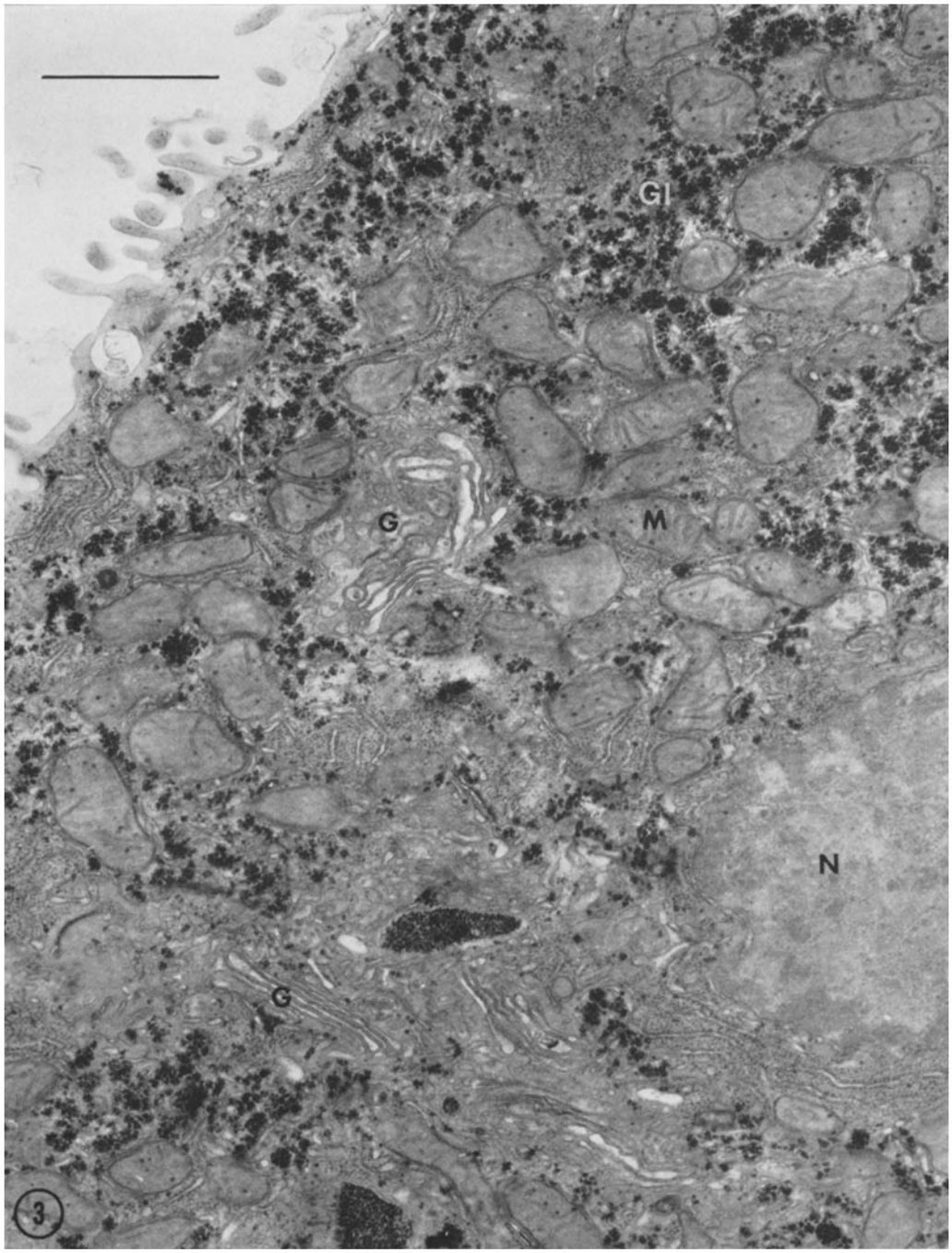

FIGURE 3 Cells prepared as in Fig. 2, but 20 mM pyruvate was present throughout. All organelles, including the nucleus (*N*), mitochondria (*M*), endoplasmic reticulum, and Golgi apparatus (*G*), are well preserved. Darkly-stained particles of glycogen (*Gl*) are very prominent. Scale bar, 1 μm. × 25,000.

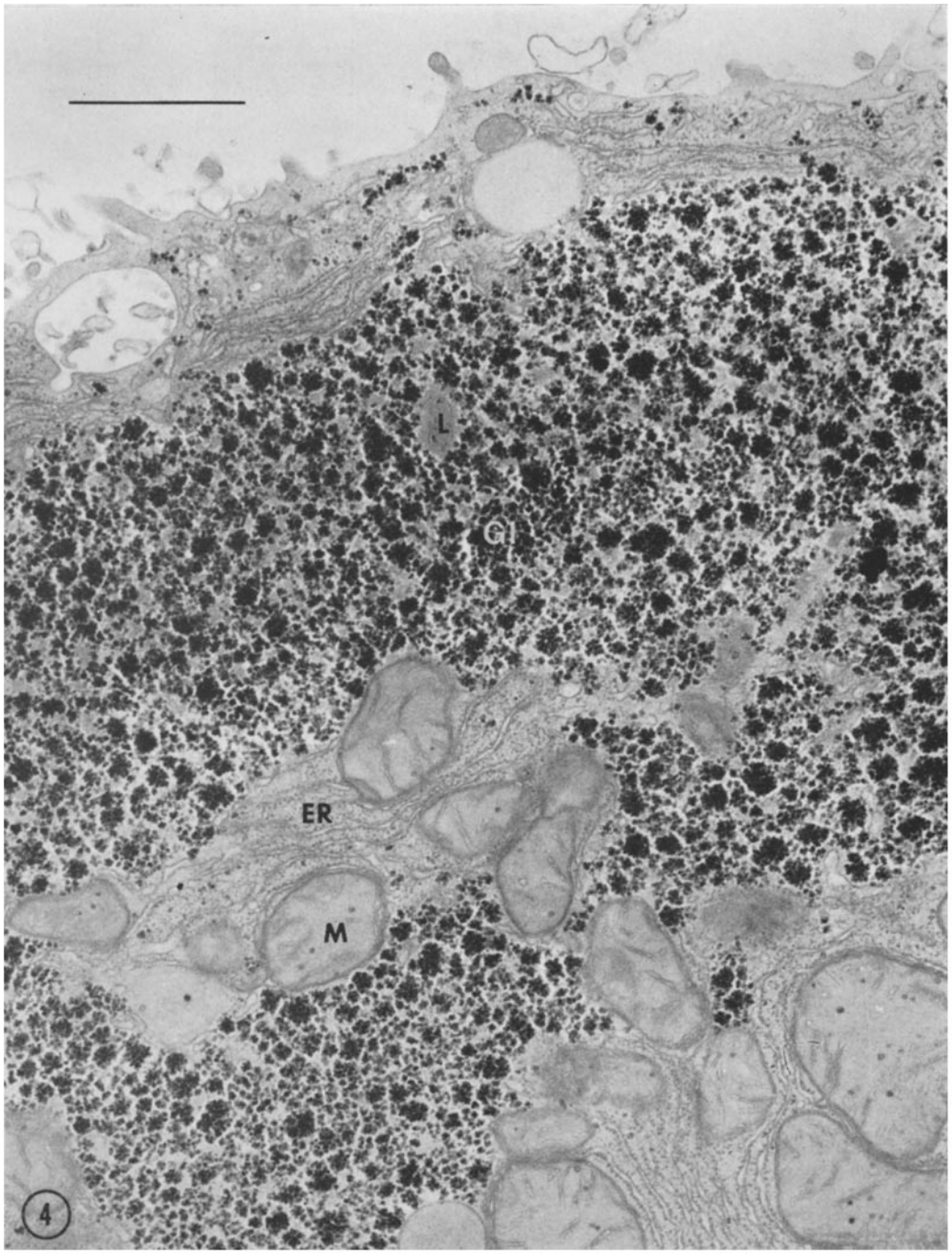

FIGURE 4 Isolated parenchymal cell prepared from liver slices incubated with enzymes dissolved in calcium-free Eagle's Minimum Essential Medium for 35 min and then for a further 25 min with added calcium. Mitochondria (*M*) are surrounded by granular endoplasmic reticulum (*ER*) arranged in parallel cisternae. Glycogen particles (*Gl*) are abundant, and a lysosome-like body (*L*) is also present. Scale bar, 0.76 μm. × 33,000.

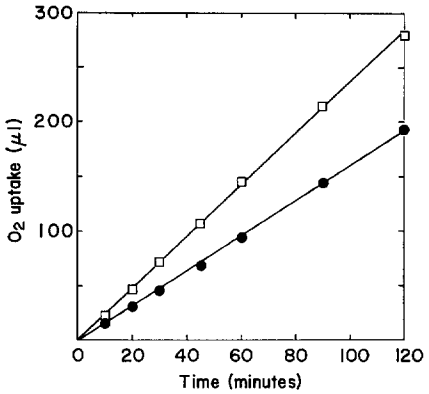

FIGURE 5 Effect of 2,4-dinitrophenol on oxygen uptake. The cells were prepared in calcium-free Hanks solution for 35 min and then in the complete medium for another 20 min (method B). Each incubation flask contained 3.9 × 10^6 cells suspended in 3 ml of Krebs-Ringer phosphate solution for measurement of oxygen uptake. 90% of the cells were viable at the beginning of the incubation. ●——●, no addition; □——□, 100 μm 2,4-dinitrophenol.

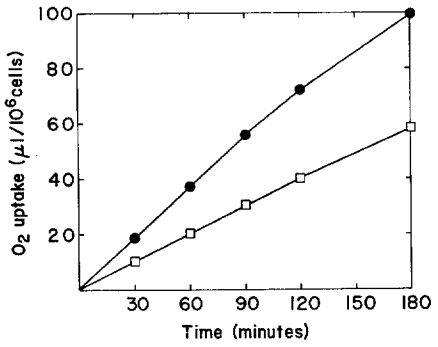

FIGURE 6 Oxygen uptake of liver cells. □——□, cells incubated in Hanks solution (6.6 × 10^6 cells/flask); ●——●, cells incubated in serum from a fed rat (8.5 × 10^6 cells/flask). The cells were from the same liver prepared by method A. 85% of the cells were viable at the beginning of the incubation.

free medium (method A) since the experiments were carried out in our early studies. The average rate of endogenous respiration in Krebs-Ringer phosphate solution of these cells was 21.1 $\mu l O_2$/h per one million cells (21 experiments). This rate is lower than that described for method A above. However, incubation of the tissue during the preparative procedure was for 70 instead of 55 min.

Cells prepared by method A respire in 100% rat serum at a rate approximately twice that in Hanks solution (Fig. 6). The initial rate of oxygen uptake

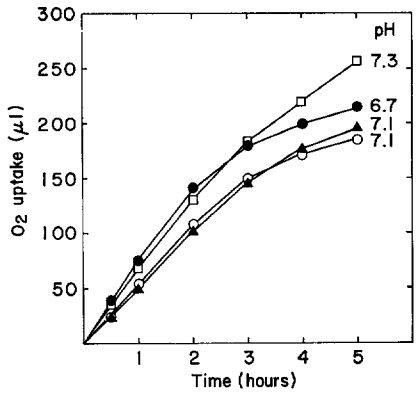

FIGURE 7 Effect of added substrates on the respiratory activity of liver cells. ○——○, no addition; ▲——▲, addition of 10 mM glucose; ●——●, addition of 10 mM fructose; □——□, addition of 20 mM pyruvate. The pH values are those of the extracellular medium after the cells had been incubated for 5 h with the different substrates. The incubation flasks contained 2.4 × 10^6 cells suspended in 3 ml of Krebs-Ringer phosphate solution. 85% of the cells were viable at the beginning of the incubation and were prepared by method A.

of 37.5 μl/h per one million cells ($Q_{O_2}^{prot}$ = 16.3) in rat serum probably represents a more accurate measurement of the rate of respiration of parenchymal cells in vivo since this is the fluid which normally bathes the cells. Serum, like Hanks solution, is buffered by bicarbonate and so a high concentration of cells is necessary to maintain the pH of the medium at about 7.4 (see reference 17).

EFFECT OF CARBOHYDRATE SUBSTRATES ON RESPIRATORY ACTIVITY: Oxygen uptake is stimulated by fructose and pyruvate but not by glucose (Fig. 7). In fact, glucose slightly depresses the initial respiratory rate. The respiration rate declines more rapidly with fructose than with pyruvate or glucose. This can be associated with the greater acidity of the incubation medium which develops when fructose is added. Usually, pyruvate has the opposite effect. Fig. 7 shows that in the presence of pyruvate, the pH of the incubation medium is higher than when no substrate is added. In another experiment, oxygen uptake was almost linear for 5 h with added pyruvate, there was little loss of cell viability and the pH of the incubation medium rose to 7.9. By contrast the pH of the medium, when no substrate is added or with the addition of 10 mm glucose, always falls below 7.4. The incubation medium becomes more acidic with 50 mM glucose than with 10 mM glu-

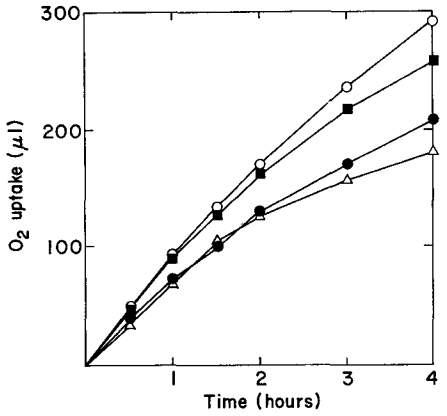

FIGURE 8 Effect of glucose and pyruvate on the respiratory activity of liver cells. △——△, no addition; ●——●, addition of 50 mM glucose; ■——■, addition of 20 mM pyruvate; ○——○, addition of 50 mM glucose and 20 mM pyruvate. The incubation flasks contained 3.0 × 10^6 cells incubated in 3 ml of Krebs-Ringer phosphate solution. 85% of the cells were viable at the beginning of the incubation and were prepared by method A.

cose but oxygen uptake is still no higher than with endogenous substrates.

Of all the substrates utilized in our work to date, pyruvate has been the best for maintaining both respiratory activity and cell viability. This latter parameter is important as seen from the results of succinate-supported respiration later in this paper. Oxygen uptake is even better maintained over a long time period when glucose is added with pyruvate (Fig. 8).

EFFECT OF INTERMEDIATES OF THE TRICARBOXYLIC ACID CYCLE ON RESPIRATORY ACTIVITY: Table IV shows that the various intermediates of the tricarboxylic acid cycle produce different degrees of stimulation to the initial rate of oxygen uptake. Succinate more than doubles the rate and this is maintained for the full 2 h of incubation. Oxalacetate is the only other intermediate which produces a large stimulation, but this high rate is not maintained. Citrate does not stimulate oxygen uptake, and α-ketoglutarate and malate only produce a slight stimulation.

The rapid decline in the rate of oxygen uptake with added oxalacetate can be associated with the pH of the incubation medium becoming alkaline (Table IV). This rise in pH is more rapid than that observed with pyruvate. At a pH of 8.5 all of the cells are nonviable. Only with added citrate is the original pH of the medium maintained. In all other cases it is lowered to the same value of 6.9.

The addition of the intermediates of the tricarboxylic acid cycle results in a more rapid loss of cell viability (Table IV). The percentage of viable cells was difficult to determine when citrate had been added to the incubation medium as the cells had all clumped together. This may have been due to the calcium in the medium complexing with citrate.

EFFECT OF CALCIUM AND SUCCINATE ON RESPIRATORY ACTIVITY

The effects of calcium and succinate on the respiratory activity of liver cells are shown in Table V. It is seen that the endogenous rate of oxygen uptake is lower when calcium is omitted from either Krebs-Ringer phosphate solution or glucose-free Hanks solution than when present. This effect is more dramatic with the latter medium. It should be noted that the endogenous rate of oxygen uptake falls more rapidly when calcium is omitted from both media. The rate of respiration also falls off more rapidly in glucose-free Hanks solution (calcium present or absent) than in Krebs-Ringer phosphate solution. The faster decline in rate in the former medium can be associated with its alkaline pH. This process has been described in detail previously (17).

Succinate-supported respiration differs considerably in the four media shown in Table V. Thus, the initial rate in glucose-free Hanks solution is four times that in the calcium-free medium and approximately twice that in Krebs-Ringer phosphate solution. Another difference is that oxygen uptake is greater in calcium-free Krebs-Ringer phosphate solution than in the complete medium, whereas it is greater in glucose-free Hanks solution than in the calcium-free medium.

All cells incubated with succinate in the four media shown in Table V were nonviable after 4 h of incubation. At this stage, the pH of glucose-free Hanks solution (calcium present or absent) had risen to 8.7 and so the cells exhibit negligible oxygen uptake (Table V). In contrast, the pH of Krebs-Ringer phosphate solution (calcium present or absent) had fallen to 7.1. At this pH, the cells incubated with succinate still actively consume oxygen despite complete loss of viability.

Unlike its effect on oxygen uptake, succinate does not greatly stimulate carbon dioxide production so that the RQ for the cells is reduced (Table

TABLE IV

Effect of Intermediates of the Tricarboxylic Acid Cycle on the Respiratory Activity and Viability of Liver Cells

The cells were suspended in 3 ml of Krebs-Ringer phosphate solution. All substrates were 20 mM. Cells were prepared by method A.

	Experiment 1 (2.2×10^6 cells/flask; Initial viability = 80%)			Experiment 2 (2.8×10^6 cells/flask; Initial viability = 90%)			
Period of incubation	None	α-keto-glutarate	Succinate	None	Malate	Oxalacetate	Citrate
min				*μl/h per 10^6 cells*			
Oxygen uptake							
0–30	23.8	25.0	51.2	21.1	22.6	30.0	21.1
30–60	23.8	22.6	50.6	21.1	19.5	26.3	15.8
60–90	23.8	21.4	50.0	21.1	19.5	17.3	15.8
90–120	17.9	20.3	50.0	18.0	18.0	6.8	13.5
pH of incubation medium							
120	6.9	6.9	6.9	6.9	6.9	8.5	7.4
% of viable cells							
120	76	50	53	60	47	0	42*

* The cells clumped, therefore, cell viability could not be accurately determined.

TABLE V

Effect of Calcium and Succinate on the Respiratory Activity of Liver Cells

The cells were obtained from the same preparation by Method A. Succinate was 20 mM; 80% of the freshly prepared cells were viable.

	Oxygen uptake in Krebs-Ringer phosphate solution (2.3×10^6 cells/flask)				Oxygen uptake in glucose-free Hanks solution (4.3×10^6 cells/flask)			
Period of incubation	No addition	Calcium deleted	Succinate added	Calcium deleted and succinate added	No addition	Calcium deleted	Succinate added	Calcium deleted and succinate added
min				*μl/h per 10^6 cells*				
0–30	16.2	12.5	46.7	58.9	17.4	7.4	84.2	22.4
30–60	13.3	12.5	46.7	58.9	12.8	2.9	43.2	6.2
60–90	11.8	8.8	46.7	58.9	9.7	2.3	20.9	3.1
90–120	10.3	6.6	46.7	58.9	6.6	1.0	12.0	1.5
120–180	8.8	5.2	46.5	43.4	3.4	0.8	4.1	0.9
180–240	5.9	2.2	41.2	30.2	1.9	0.5	1.3	0.9
pH of incubation medium								
240	7.1	7.1	7.1	7.1	8.7	8.7	8.7	8.7

VI). Similar observations have been made with liver slices (7, 29). It has also been demonstrated that most of the added succinate accumulates as malate or fumarate (29). Thus, the increase in oxygen uptake produced by succinate in these cells appears to be due to the direct transfer of electrons from this substrate to the electron transport chain rather than to an acceleration of the tricarboxylic acid cycle.

DISCUSSION

Throughout this work we have shown that calcium is very important for the maintenance of both the structural and metabolic integrity of liver paren-

TABLE VI

Oxygen Uptake and Carbon Dioxide Production by Liver Cells

The cells were incubated in 3 ml of Krebs-Ringer phosphate solution for 30 min. Each flask contained 2.6×10^6 cells in Experiment 1 and 3.3×10^6 cells in Experiment 2. Succinate was 20 mM. Cells were prepared by method A.

Experiment number	Period of incubation	Additions	Oxygen uptake	Apparent* carbon dioxide production	Apparent RQ‡
	min		*μl*	*μl*	
1	0–30	None	23	19	0.83
		Succinate	68	21	0.31
2	0–30	None	34	17.5	0.51
		Succinate	95	21	0.22

* A formula has been presented for converting the apparent to real output of CO_2 in constant-pressure respirometers (12). This is difficult to apply in these studies as the pH of the medium changes during incubations and this affects the amount of CO_2 retained by the medium (26). In the present experiments it is sufficient to record the apparent volume since the pH of the medium is the same whether succinate is present or not.

† RQ is the respiratory quotient and represents the ratio of CO_2 produced/oxygen consumed.

chymal cells. Ideally, one would like to expose the liver to calcium throughout the entire preparative procedure. However, it is apparent from our results that a calcium-free medium must be employed during part of the procedure in order to obtain adequate yields of cells. This may be the result of one or more of the contaminating enzymes in crude collagenase being more active in a calcium-free medium. On the other hand, it is well established that the removal of calcium from the liver by perfusion with chelating agents loosens cell adhesion (1). Therefore, perfusion, and incubation of the liver with a calcium-free medium may partially promote this loosening.

We have shown that more potassium is retained in cells which are exposed to calcium during the preparative procedure. Geyer et al. (11) have reported similar findings with rat liver slices incubated in both aerobic and anerobic conditions. Although the present results show that the addition of calcium to calcium-free Hanks solution more than doubles the rate of oxygen uptake of isolated liver cells, its effect on potassium retention does not appear to be related to a higher respiratory activity since albumin, which also stimulates oxygen uptake (16), does not increase the potassium content of the cells. Therefore, the effect of calcium on potassium retention may be at the plasma membrane of the cells as suggested by Geyer et al. (11).

We have also shown that cells exposed to calcium during their isolation exhibit higher rates of oxygen uptake, which are not due to uncoupling of respiration. This may possibly be associated with the better preservation of the mitochondria within the cells. Thus, we noted a loss of some mitochondrial cristae in cells prepared without calcium (Fig. 1).

This laboratory now routinely prepares cells using the technique of adding calcium during the preparative procedure (method B). The present studies have shown that the addition of 20 mM pyruvate during the preparative procedure further increases the potassium content of these cells. Cells prepared with pyruvate are extremely well preserved (Fig. 3). However, this laboratory is still rather cautious about adopting the procedure of adding 20 mM pyruvate during the preparation of the cells, since the liver is not normally exposed to such a high concentration of this substrate in vivo. From the results detailing the potassium content of the cells and our electron microscope studies, the use of Eagle's Minimum Essential Medium during the preparative procedure is more attractive. This medium contains amino acids and vitamins. Possibly, an even more supplemented medium than this might be considered for future work.

Some excellent modifications of our original enzymatic technique have been made by Berry and Friend (3). They obtain high yields of viable cells by employing a continuous recirculation perfusion

of the rat liver in vivo before shaking the liver in buffer in vitro. Other methods have been developed from this (4, 18, 21). The technique of adding calcium during the preparative procedure can obviously be applied to these methods. Although the method described in this paper produces less cells, it is simpler to use. Thus, the preparation can be accomplished by one person, no operative skill is required, and the apparatus is less elaborate. Enough cells can be obtained from one or two rat livers for most experiments. This is because only 2–3 million cells per flask are needed for metabolic studies.

Another advantage of our technique is that glycogen is extremely well preserved in the cells. In contrast, cells prepared from the isolated cyclically-perfused liver reveal only faint areas of glycogen deposition (3). This may be the result of either degradation or alteration in structure of the glycogen. Since the maintenance of glycogen is dependent on oxygen (9), cells in the isolated cyclically-perfused liver may not receive enough oxygen, possibly as a result of the lowering of oxygen tension as the perfusion medium circulates through the liver.

The fine structure of the cells presented in this paper is representative of that of most of the cells observed in the sections prepared for electron microscopy. When compared with the electron micrographs of cells obtained by the original method of Howard et al. (16), the improvement in quality of the cells is quite considerable. Our findings of improved cellular morphology are supported by our results of the endogenous respiratory activity of the cells. There is a clear correlation between these two parameters, as evidenced by the continual rise in the rate of endogenous respiration as the quality of the cell suspensions has improved (cf. references 16 and 17).

It is important to stress that sole measurement of the percentage of viable cells in a suspension does not necessarily correlate with the rate of endogenous oxygen uptake. Thus, in this paper we described that suspensions with a high percentage of viable cells could be obtained when the entire preparative procedure was carried out at 37°C. However, such cells revealed much vacuolation and loss of mitochondrial cristae. The highest rate of endogenous respiration observed with such preparations was 14.0 μl O_2/h per one million cells. Therefore, the high maintained rates of endogenous respiration of cells prepared by methods A and B in Krebs-Ringer phosphate solution as described in this work emphasize not only the intactness of the plasma membrane but also the intactness of the intracellular organelles of the cells.

In this paper we have determined some of the respiratory characteristics of isolated liver parenchymal cells when incubated with added substrates and in different media. Several interesting observations on the viability of cells and the pH of the extracellular medium were also made during these determinations. Further work is necessary in order to interpret many of these findings. The most important of these are as follows: (*a*) The rate of endogenous respiration of isolated cells is lowered when calcium is omitted from the incubation medium. Not only is the initial rate of oxygen uptake lowered, but also the fall-off in rate is more dramatic. Consequently, it would appear that calcium should not be omitted from media used for studies with isolated liver cells as has been the case in some laboratories (8, 20). (*b*) The effect of an added substrate can be dependent on the nature of the incubation medium. This was clearly shown in Table V where the degree of the stimulation of oxygen uptake by succinate was completely different in the four media employed. (*c*) The addition of some substrates can result in drastic changes in the pH of the extracellular medium so that the cells become nonviable with a resultant decrease in oxygen uptake. This was observed when fructose or oxalacetate was the substrate (Fig. 7, Table IV). (*d*) The viability of cells can be lowered by the addition of certain substrates which do not produce a nonphysiological extracellular pH. Thus we observed a lowering of cell viability when any tricarboxylic acid cycle intermediate was added (Table IV). (*e*) Nonviable cells still respire at high rates with added succinate. From these observations we recommend that during long-term incubations, which are necessary for some metabolic experiments, a careful watch should be maintained over the pH of the incubation medium and that cell viability also be measured, otherwise some anomalies with respect to the interpretation of data could arise. These measurements might also be an aid in determining some metabolic processes.

As summarized by Sandström (24), trypan blue stains nonviable cells because of increased permeability of the plasma membrane. Therefore, it appears from the present studies that the addition of intermediates of the tricarboxylic acid cycle interferes with processes associated with the control of

the plasma membrane of isolated liver cells. This loss of control may be due to an insufficient supply of ATP at the plasma membrane. Thus, some of the energy produced in these conditions may be utilized for the movement of the carboxylate anions and accompanying cations in and out of the mitochondria. Such a mechanism has been proposed by van Rossum (28), who observed that there was no net increase in ATP production in the succinate-stimulated oxygen uptake of liver slices. It is interesting in this respect that over long periods of incubation we have found that succinate is the most effective substrate which produces a complete loss of cell viability at physiological pH. Although Table VI shows that succinate does not hinder the flow of the tricarboxylic acid cycle initially, other experiments in this laboratory have indicated that, as the period of incubation increases, the direct oxidation of succinate dominates and the flow of the tricarboxylic acid cycle is progressively slowed down. Presumably, most of the energy produced is now utilized for ionic transport in and out of the mitochondria so that none is available at the plasma membrane.

Since we first developed this enzymatic technique (16) we have constantly been searching for modifications so that the quality of the cells might be improved. The first objective was to ensure that a high percentage of the cells obtained had intact plasma membranes so that the intracellular organelles were protected from extracellular physiological saline media. After this, we desired to improve the quality of the intracellular organization of the cells. The present results have demonstrated our advances. Thus 85–98% of the cells obtained have intact plasma membranes, display a well-preserved intracellular structure, retain considerably more potassium than before, and respire at higher rates in physiological saline media. Other studies in this laboratory show that the cells synthesize DNA, RNA, protein, and glycogen and that they can be maintained in monolayer tissue culture for as long as 10 days.

The technical assistance of Dr. W. H. Amy Yu and Mr. John Worth in the electron microscopy studies is gratefully acknowledged.

This work was supported by Grant AM-10680 from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

Received for publication 26 May 1972, and in revised form 2 February 1973.

REFERENCES

1. ANDERSON, N. G. 1953. The mass isolation of whole cells from rat liver. *Science (Wash. D. C.).* **117**:627.
2. BERRY, M. N. 1962. Metabolic properties of cells isolated from adult mouse liver. *J. Cell Biol.* **15**:1.
3. BERRY, M. N., and D. S. FRIEND. 1969. A high-yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol.* **43**:506.
4. CRISP, D. M., and C. I. POGSON. 1972. Glycolytic and gluconeogenic enzyme activities in parenchymal and non-parenchymal cells from mouse liver. *Biochem. J.* **126**:1009.
5. DELUCA, H. F., and P. P. COHEN. 1964. Suspending media for animal tissues. *In* Manometric Techniques. W. W. Umbreit, R. H. Burris, and J. F. Stauffer, editors. Burgess Publishing Company, Minneapolis. 132.
6. EAGLE, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Wash. D. C.).* **130**:432.
7. ELLIOT, K. A. C., M. E. GREIG, and M. P. BENOY. 1937. The metabolism of lactic and pyruvic acids in normal and tumour tissues. III. Rat liver, brain and testis. *Biochem. J.* **31**: 1003.
8. FELTS, J. M., and M. N. BERRY. 1971. The metabolism of free fatty acids and chylomicron triglyceride fatty acids by isolated rat liver cells. *Biochim. Biophys. Acta.* **231**:1.
9. FIGUEROA, E., R. VALLEJOS, A. PFEIFER, and C. KAHLER. 1966. Effect of oxygen pressure on glycogen synthesis by rat-liver slices. *Biochem. J.* **98**:253.
10. GALLAI-HATCHARD, J. J., and G. M. GRAY. 1971. A method of obtaining a suspension of intact parenchymal cells from adult rat liver. *J. Cell Sci.* **8**:73.
11. GEYER, R. P., K. J. SCHOLTZ, and E. J. BOWIE. 1955. Influence of calcium on potassium concentration in rat liver in vitro. *Am. J. Physiol.* **182**:487.
12. GREGORY, K. F., and H. C. WINTER. 1965. Data reduction with constant-pressure respirometers. *Anal. Biochem.* **11**:519.
13. HANKS, J. H., and R. E. WALLACE. 1949. Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.* **71**:196.
14. HECKMANN, K. D., and D. S. PARSONS. 1959. Changes in the water and electrolyte content of rat-liver slices in vitro. *Biochim. Biophys. Acta.* **36**:203.
15. HOMMES, F. A., M. I. DRAISMA, and I. MOLENAAR. 1970. Preparation and some properties of

isolated rat liver cells. *Biochim. Biophys. Acta.* **222**:361.

16. HOWARD, R. B., A. K. CHRISTENSEN, F. A. GIBBS, and L. A. PESCH. 1967. The enzymatic preparation of isolated intact parenchymal cells from rat liver. *J. Cell Biol.* **35**:675.
17. HOWARD, R. B., and L. A. PESCH. 1968. Respiratory activity of intact, isolated parenchymal cells from rat liver. *J. Biol. Chem.* **243**:3105.
18. INGEBRETSON, JR., W. R., and S. R. WAGLE. 1972. A rapid method for the isolation of large quantities of rat liver parenchymal cells with high anabolic rates. *Biochem. Biophys. Res. Commun.* **47**:403.
19. IYPE, P. T., and P. M. BHARGAVA. 1965. The respiration of isolated rat-hepatic cells in suspension. *Biochem. J.* **94**:284.
20. JEZYK, P. F., and J. P. LIBERTI. 1969. Metabolic activities of mechanically and enzymatically prepared rat liver cells. *Arch. Biochem. Biophys.* **134**:442.
21. JOHNSON, M. E. M., N. M. DAS, F. R. BUTCHER, and J. N. FAIN. 1972. The regulation of gluconeogenesis in isolated rat liver cells by glucagon, insulin, dibutyryl cyclic adenosine monophosphate, and fatty acids. *J. Biol. Chem.* **247**:3229.
22. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265.
23. MURTHY, L., and H. G. PETERING. 1969. A comparison of some biochemical properties of dissociated liver cells and of slices. *Proc. Soc. Exp. Biol. Med.* **132**:931.
24. SANDSTRÖM, B. 1965. Studies on cells from liver tissue cultivated in vitro. 1. Influence of the culture method on cell morphology and growth pattern. *Exp. Cell Res.* **37**:552.
25. STEVENS, K. M. 1965. Oxygen requirements for liver cells in vitro. *Nature (Lond.).* **206**:199.
26. UMBREIT, W. W. 1964. Direct and indirect methods for carbon dioxide. *In* Manometric Techniques. W. W. Umbreit, R. H. Burris, and J. F. Stauffer, editors. Burgess Publishing Company, Minneapolis. 30.
27. VAN ROSSUM, G. D. V. 1970. Net movements of calcium and magnesium in slices of rat liver. *J. Gen. Physiol.* **55**:18.
28. VAN ROSSUM, G. D. V. 1969. The inhibition of active movements of Na^+ and K^+ in rat-liver slices by high concentrations of succinate and other carboxylate anions. *Arch. Biochem. Biophys.* **133**:385.
29. VAN ROSSUM, G. D. V. 1969. The oxidation of succinate and permeability of dicarboxylate anions in rat-liver slices. *Arch. Biochem. Biophys.* **133**:373.