

# RESPIRATORY ACTIVITY AND MAINTENANCE OF CELL SUSPENSIONS OF RAT LIVER

DEAN H. HAYEK and SAMUEL R. TIPTON

From the Department of Zoology and Entomology, University of Tennessee, Knoxville, Tennessee. Dr. Hayek's present address is Department of Physiology, Medical College of Virginia, Richmond, Virginia

## ABSTRACT

Previous experimentation involving the use of dispersed rat liver cells have utilized suspending media common to fractionation and slicing methods. Cells in these media have not remained viable for prolonged periods of time and they have resisted culturing techniques. Suspensions of dispersed parenchymal cells were prepared from rat livers which had been perfused in situ via the dorsal aorta with an EDTA-sucrose solution. The maintenance of surviving cells was attempted in three different media: sucrose buffered with Tris-HCl, Waymouth medium, and Waymouth medium supplemented with 30% calf serum. Cells suspended in sucrose and buffered with Tris-HCl oxidized citrate, succinate, and  $\alpha$ -ketoglutarate but did not respire in the presence of other citric acid cycle intermediates. When cells were suspended in Waymouth medium without glucose, they oxidized malate and glutamate plus the above-mentioned substrates. Glucose and pyruvate did not stimulate oxygen uptake in either medium. Cells exhibited respiratory activity for up to 8 hr when incubated in Waymouth medium supplemented with calf serum. Both the ability to oxidize succinate and the morphological integrity of the cells were retained for this period of time. When cells were incubated in Waymouth medium alone, the time interval was reduced to 6 hr. Sucrose-Tris-HCl in the presence of succinate was not satisfactory as an incubation medium, since many of the cells underwent breakdown.

## INTRODUCTION

The preparation of dispersed rat liver cells has been achieved by both mechanical (1-4) and a combination of mechanical and chemical means (5). Variations in metabolic activities, dependent upon the method of dispersion and the choice of suspending medium, have been reported for dispersed cells isolated from rat and mouse liver. Kalant and Young (6) have ascribed glycolytic activity to dispersed cells, although others (7, 8) consider this phenomenon unlikely. Dispersed cells are capable of oxidizing citric acid cycle intermediates (9, 10), but are not capable of metabolizing carbohydrate substrates. Endogenous activity of cells dispersed in Krebs'-Ringer-

phosphate has been reported by Gibbons and Rienits (11). Berry (7) reported that endogenous activity was exhibited only by dispersed cells suspended in sucrose-Tris<sup>1</sup>-HCl.

Previously, the principal suspending media used have been Krebs'-Ringer, buffered with either phosphate or bicarbonate, and sucrose-Tris-HCl. The former is commonly used in experiments involving tissue slices, brei, and subcellular fractions. It was thought that the addition of

<sup>1</sup>The following abbreviations have been used: Tris = tris(hydroxymethyl)aminomethane; EDTA = ethylenediaminetetraacetic acid.

organic metabolites, such as protein, or amino acids, with less salts might give a cell suspension retaining more functional properties and surviving longer. As evidenced by a comparison of respiratory rates, cells suspended in a more elaborate medium would presumably possess greater oxidative capabilities than cells suspended in simpler media. Also, the morphological integrity of cells in suspension would be expected to be retained for longer time intervals in the more complex medium.

#### MATERIALS AND METHODS

Male albino rats from our own colony of Rockland rats, weighing 150 to 300 g, were used. Animals were maintained on Purina Laboratory chow and water ad libitum.

Animals were sacrificed by decapitation. The liver was perfused in situ with cold (0°–4°C) 0.001 M EDTA in 0.25 M sucrose. When the liver had completely blanched, it was excised, washed with the perfusion medium, blotted dry with filter paper, and minced with a double-edged razor blade. The subsequent preparation of dispersed cells from the mince was achieved by modifications of the method of Jacob and Bhargava (3). Following dispersion in the perfusion medium, the cells were filtered through two layers of nylon bolting cloth, in order to remove clumps of cells and connective tissue. The filtrate was then centrifuged in a refrigerated (0°–4°C) International Centrifuge (PR-2), head No. 169, at 250 to 300 RPM, 50 g, for 5 min. The supernatant was removed by suction and the pellet of hepatic cells was resuspended by adding a volume of either Waymouth medium (12) or sucrose-EDTA which was equal to two times that of the packed cell volume. Redispersion of the pellet was done either by gently swirling the contents of the tube until a homogeneous suspension of isolated cells was obtained or by syringing the suspension through successively smaller hypodermic needles (18-gauge and 22-gauge, respectively).

A new method for obtaining purified homogeneous suspensions of isolated cells has been attempted, using the zonal density gradient centrifuge and rotor. Following the centrifugation in the International Refrigerated Centrifuge, the cells were suspended in 10% colloidal silica solution (Ludox, courtesy of E. I. Dupont de Nemours Co., Wilmington, Delaware) and centrifuged through a continuous density gradient running from 10 to 30% Ludox as a cushion at the periphery of the rotor. The cells were centrifuged at 15,000 RPM for 1 hr. The centrifuge was unloaded, while still running, by collecting 40-ml aliquots of the 1,200 ml capacity gradient.

Cells which were suspended in the perfusion medium were buffered with Tris-HCl, pH 7.3.

Glucose was omitted from the Waymouth medium when this was used as the suspending medium.

Respiratory activity was determined in conventional Warburg apparatus. All suspensions were incubated at 37°C and were allowed to equilibrate for 15 min. Cells suspended in the perfusion medium were gassed with O<sub>2</sub>, and the oxygen uptake was determined by the direct method of Warburg (13). Cells suspended in Waymouth medium were gassed with 5% CO<sub>2</sub>–95% O<sub>2</sub>, and the oxygen uptake was determined by the indirect method of Warburg (14).

Oxygen uptake is expressed by:

$$q_{O_2}^{\text{prot}} = \mu\text{l O}_2 \text{ per mg of protein per hr.}$$

Prolonged incubation of cell suspensions was attempted in three series of experiments. In the first case, cells were suspended in the perfusion medium with succinate added (final concentration: 0.033 M) and buffered with Tris-HCl. Waymouth medium was utilized as the suspending medium in the second case. Thirdly, a mixture of Waymouth medium and calf serum (70 to 30%) was used.

In the first experiment, 1 ml each of suspension, 0.1 M succinate, and Tris-HCl was added to the incubation flasks. In the second and third experiments, 3 ml of cell suspension was added to each flask.

Twenty-five-ml Erlenmeyer flasks served as the incubation containers. These were stoppered with a two-hole rubber stopper. A gas inlet and outlet tube provided access and escape for the gas phase. Flasks were shaken in a gyrotory shaker (New Brunswick Corp., New Brunswick, New Jersey) at a speed of 120 RPM and at a temperature of 37°C.

The protein content of the cell suspensions was determined by the method of Lowry et al. (15).

#### RESULTS

The appearance of the cell suspensions in phase microscopy was that of a relatively homogeneous population of hepatic parenchymal cells. Clumps of cells (5 to 10 cells per clump) comprised no more than 5% of the total cell population. Nuclei, which clump together in sucrose solutions, and a small amount of cellular debris were present.

Cells from the zonal density gradient centrifuge runs had sought their own density in the gradient and were collected from the second through fifth fractions. A relative absence of contamination was noted in these fractions. There was little debris, and nuclei, either isolated or clumped, were not detected. In addition, the cell clumps were of small size, having only 2 to 4 cells per clump. Fractions of higher density contained clumped nuclei and cellular debris.

The majority of cells from the five fractions appeared to possess intact, uninterrupted cell membranes when viewed under phase contrast. Small blebs were evident on several of the cells. Analyses of the 5 cell-containing fractions showed that the specific gravity of these fractions ranged from 1.045 to 1.060. Seventy % of the original cell preparation was recovered in these 5 fractions.

Cells suspended in sucrose-EDTA and buffered with Tris-HCl exhibited significantly increased respiratory activity when they were incubated with succinate or  $\alpha$ -ketoglutarate (Table I). Glutamate, glucose, malate, and pyruvate were without effect on the respiratory activity of the cells. Citrate significantly depressed the oxygen uptake of the cells.

The effect of Waymouth medium (without glucose) on respiratory activity is presented in Table I. The same substrates were utilized in this medium as in the sucrose-EDTA medium. Glucose (final concentration: 0.033 M) was also used, but it did not enhance respiratory activity. Succinate, malate, glutamate, citrate, and  $\alpha$ -ketoglutarate significantly increased oxygen uptake. In two extended studies, using succinate as the substrate, the  $q_{O_2}^{prot}$  was maintained at a constant level for at least 3 hr when the experiments were terminated. Pyruvate again lowered the respiratory activity, although not significantly.

The use of 70% Waymouth medium plus 30% calf serum as a suspending medium indicated that this was an acceptable medium for maintenance studies of more than 6 hr. Cells serially removed from the incubation flasks and resuspended in Waymouth medium without glucose were examined under phase contrast. Nuclei were distinct, vacuolization was not seen, and no significant membrane blebbing had occurred. The morphology of the cells was maintained, according to these criteria, for up to at least 8 hr. The  $q_{O_2}^{prot}$  (succinate), measured by direct manometry, decreased from 13.9 to 10.0, not a significant difference, over a period of 8 hr, whereas the endogenous activity remained practically the same, 2.6 as compared to 2.8. The degree of cell reaggregation, which began almost immediately, increased with the length of the incubation period, and large quantities of cells adhered to the walls of the flasks. The pH decreased from 7.4 to 7.3 over this time interval. When the incubation time exceeded 8½ hr, the cells began to degenerate, the pH decreased to less than 7.2, and the morphological appearance of the cells deteriorated.

TABLE I  
*Mean Respiratory Activity of Dispersed Rat Liver Cells in 0.25 M Sucrose-0.001 M EDTA and Waymouth Medium\* in the Presence of Substrates*

Substrate	$q_{O_2}^{prot}$	
	Sucrose-EDTA	Waymouth medium
None	1.2 ± 0.2	2.7 ± 4.9 (12)
Citrate	0.4 ± 0.3‡	40.5 ± 10.8§ (4)
Succinate	10.0 ± 3.5§	29.1 ± 6.2§ (6)
Glutamate	2.2 ± 1.1	10.8 ± 3.4§ (6)
$\alpha$ -Ketoglutarate	2.8 ± 0.1§	11.7 ± 8.6‡ (5)
Glucose	1.3 ± 0.2	—
Malate	1.6 ± 1.5	29.9 ± 7.7§ (4)
Pyruvate	0.8 ± 0.4	-0.5 ± 3.8 (6)

Warburg flasks contained 1 ml of dispersed cells suspended in 0.25 M sucrose-0.001 M EDTA containing 0.02 M Tris-HCl buffer. Final substrate concentrations, 0.033 M. Substrate volume, 1 ml. 0.2 ml KOH in center well. Total volume, 2.2 ml. Gas phase, O<sub>2</sub>. Incubation time, 1 hr. Temperature, 37°C. Flasks containing dispersed cells suspended in Waymouth medium (without glucose) were incubated for 1 hr. Final substrate concentration, 0.033 M. Gas phase, 5% CO<sub>2</sub>-95% O<sub>2</sub>. Temperature, 37°C. Values for  $q_{O_2}^{prot}$  represent the mean ± SD of duplicate determinations on 4 animals (sucrose-EDTA medium) and on the number of animals shown in parentheses for each group (Waymouth medium). Statistical analyses by the *t* distribution test; \* Without glucose

‡ = P < 0.05.

§ = P < 0.01.

One of the first indications of deterioration appears to be the disappearance of the formerly prominent nucleus. Associated with this, but at a somewhat later time, is the appearance of many vacuoles within the cytoplasm. The plasma membrane, during this time, still appeared intact and unbroken, although considerably more blebbing was seen.

When Waymouth medium was used as the suspending medium, the cells began to deteriorate and the pH decreased to less than 7.2 after 6 hr. Cells suspended in sucrose-EDTA with succinate

and Tris-HCl added did not survive incubation for longer than 3 hr. Considerable cell breakage was observed after this time.

## DISCUSSION

The results show that mammalian liver cells have an active respiratory response to many Krebs' cycle substrates when they are dispersed in a medium containing many organic and ionic forms similar to those found in extracellular fluid. This response is considerably depressed when they are dispersed in a sucrose-EDTA-Tris-HCl medium. It is probable that surviving cells require media of different properties and components than do growing cells. Actively growing cells must have active respiration and utilization of substrates from the citric acid cycle in order to obtain energy for synthetic processes such as protein synthesis. Waymouth medium allows both, but sucrose medium does not support respiration and probably does not support protein synthesis or mitotic increase.

At present, no data are available on the osmotic pressure of rat liver cells in suspension. Opie (16) has shown that liver slices are isotonic with NaCl solutions having two times the concentration of physiological salt solutions. This would indicate, then, that slices are isotonic with 0.35 M (0.25 to 0.4 M) NaCl which is equivalent to 17.39 atmospheres (12.79 atm to 20.46 atm). Olmstead and Granum (17) reported that the oxygen uptake of rat liver slices in hypertonic or hypotonic salt solutions is not significantly different from that in isotonic salt solution. In this instance, the isotonic salt concentration was 320 milliosmols or 0.16 M NaCl, which is equivalent to 8.05 atmospheres. Berry and Simpson (18) indicated that the morphology of dispersed cells is better maintained with 0.4 M sucrose than it is at lower concentrations. This is equivalent to an osmotic pressure of 10.12 atmospheres. Regardless of the actual osmotic pressure of dispersed rat liver cells, that of sucrose-EDTA medium is only 6.34 atm, while that of the Waymouth medium is considerably greater (i.e. greater than 9.00, considering a cryoscopic constant of at least 2.0 for the entire medium).

Berry (7) reported that aldolase and lactic acid dehydrogenase were leached from his preparations and that ketogenesis was associated with endogenous respiration. Exton (10) confirmed this latter finding in rat liver cells and, in addition, reported a greatly impaired glycolytic activity in such cells.

The rapid oxidation of succinate is clear evidence that cytochrome *c* is not leached from the cells. The ability of cells to metabolize other citric acid cycle intermediates reflects the continued presence of such cofactors as NAD, NADP, and flavoproteins and supports the view that mitochondrial stability has been retained to a significant degree. This does not exclude the possibility of some other enzyme and cofactor leakage from the cells.

The inability of pyruvate to stimulate the respiratory activity of cells suspended in either sucrose or Waymouth medium is rather surprising, since others (7, 10, 19) have reported increased respiratory activity upon the addition of pyruvate to cell suspensions. It is believed that the reaction involves the formation of acetylated lipoic acid and subsequently of acetyl-CoA. Since several soluble compounds (lipoic acid, TPP, CoA, and NAD) and magnesium are required, it may be that one or more factors, in significant concentration and combination, have been lost to the medium, resulting in a decreased ability to oxidize this acid. It is also possible that pyruvate plus malate may have been effective if oxaloacetate were in low or limiting concentration.

The effects of using a chelating agent such as EDTA have been indirectly questioned by several workers. Leeson and Kalant (20) showed that the only observable effect of EDTA, and, therefore, of the presumed removal of all divalent ions, in a perfusion medium was a separation of cells at their interfaces. Structural alteration of the hepatic cell membrane was not noted. Zimmerman et al. (8) also reported that cell membranes appeared intact in their preparations. On the other hand, Kalant et al. (21) have reported a significant increase in leakage of "basic protein 4" from rat liver slices incubated *in vitro* with 0.5% EDTA. It would appear, therefore, that results obtained from tissues prepared from solutions containing EDTA should be viewed with some caution until the effects of such treatments are definitely known.

The endogenous respiratory activity of dispersed rat hepatic cells in this study has been shown to compare favorably with earlier reports (7, 22). However, the respiratory activity of such cells has been shown to exhibit a greater response to certain citric acid cycle intermediates, except pyruvate, when incubated in a synthetic medium than has

been previously reported. The  $q_{O_2}^{prot}$  increased 15-fold upon the addition of citrate, 10-fold upon the addition of either malate or succinate, and 4-fold upon the addition of either  $\alpha$ -ketoglutarate or glutamate. In addition, the use of such a medium has increased the survival time of such cells by several hours.

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