A RAPID METHOD FOR THE ISOLATION OF LARGE QUANTITIES OF RAT LIVER PARENCHYMAL CELLS WITH HIGH ANABOLIC RATES

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SUMMARY

A rapid method for the isolation of rat liver parenchymal cells using in vitro liver perfusion is described. The procedure requires 40-50 min from hepatectomy to cell isolation with exposure to collagenase (20 mg/100 ml) for 10-15 min. Under optimal conditions 4.5 to 6.0 x 10^8 cells (3.0 g of cells) were isolated from the liver of a 200 g rat. Ninety to 95% of the cells isolated by this method excluded a vital stain and were undamaged when viewed with light microscopy. These cells actively incorporate $NaHCl^{4}O_3$ into glucose and protein in the presence of lactate, alanine and pyruvate. Glucose production from gluconeogenic precursors was equivalent to that observed with the isolated perfused liver. Unlike liver slices or the isolated perfused liver, these cells show increasing gluconeogenic rates with increasing lactate concentrations above 20 mM. A concentration of 80 mM stimulated glucose production to 80-90% of the calculated in vivo gluconeogenic rate.

Several methods have been described for the isolation of rat liver parenchymal cells (1-6). Earlier methods relied upon chelating agents in conjunction with mechanical disruption (1-4). More recently, enzymatic procedures using high concentrations of collagenase and hyaluronidase have been described (5-6). These studies have dealt primarily with morphological description and respiratory capacity. The only study dealing with gluconeogenesis in isolated liver cells showed much lower rates of glucose production than we report here (7). In this communication we wish to report

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a rapid method for obtaining large quantities of viable hepatocytes with gluconeogenic rates comparable with that of the isolated perfused liver. These studies show for the first time an in vitro system which is capable of attaining 80-90% of the calculated in vivo gluconeogenic rate.

MATERIALS AND METHODS

Male, Cox rats fasted 18-24 hr (180-220 g) were used except where indicated. All buffers used were equilibrated with 95% O_2 and 5% CO_2. All reagents were purchased from Sigma Chemical Co. except where indicated. All procedures used for isolation and incubation of liver cells were carried out in plastic ware.

Cell preparation: Rats were anesthetized with Na-pentobarbital (60 mg/kg) (Abbott) and the portal vein was cannulated and perfused with a Ca^{++} and glucose free (25 mM $NaHCO_3$) modified Hanks buffer (8). Following the cannulation of the inferior vena cava the liver was removed and placed in a Miller recirculating perfusion apparatus and perfused for 15 min with 100 ml of modified Hanks buffer containing 1.5 g bovine serum albumin (Fraction V) and 10 mg streptomycin sulfate (Pfizer). Following this pre-perfusion period, 10 or 20 mg of collagenase (Type I, 125 units/mg) was added and the perfusion was continued for 10-15 min. The liver was then removed, placed in a beaker containing 30 ml of modified Hanks buffer, finely minced with scissors and then gently bubbled with 95% O_2 and 5% CO_2 for 2 min. The resulting tissue suspension was centrifuged at low speed (600-800 rpm) in an International Clinical centrifuge (Model CL) for 15-30 sec. The supernatant was discarded and the cells were resuspended in modified Hanks buffer (37°C) by gently stirring with a small glass rod. The tissue suspension was allowed to stand for approximately 30 sec or until larger particles had settled. The cells were carefully poured off, centrifuged and were resuspended in fresh modified Hanks. The above washings were repeated two times with an Umbreit Ringer 25 mM $NaHCO_3$ buffer (23-24°C). The final suspension was diluted to a volume of 30 ml. These isolated

cells were used immediately in all the biochemical studies reported here.

An aliquot of the final suspension (1 ml, 50-100 mg of cells wet wt containing 140,000 cells/mg) was incubated in 3 ml of Umbreit Ringer 25 mM $NaHCO_3$ buffer in stoppered 1.0 oz vials (Nalgene #2002) with various substrates at 37°C for 1 hr at 90 oscillations/min in a Dubnoff metabolic incubator. Vials were gassed with 95% O_2 and 5% CO_2 for 5 min. At the end of 1 hr of incubation, the vial contents were placed in iced centrifuge tubes and centrifuged in an International centrifuge at full speed for 5 min. Aliquots of the supernatant were taken for analysis. Glucose was estimated by the glucose oxidase method (9). Incorporation of C^{14} into glucose was estimated by isolating glucose as the phenyl osazone (10). Tissue proteins were isolated by trichloroacetic acid precipitation followed by extraction with hot trichloroacetic acid, performate digestion and reprecipitation according to the method of Manchester and Krahl (11). The protein was dissolved in Soluene 100 (Packard Instrument Co.), placed in liquid scintillation fluid and counted in a Packard scintillation counter. Cell viability was initially checked by using trypan blue (.2%) (6). Cell counts were carried out using a hemocytometer. All values are reported as mean $\pm$ S.E. of the mean.

Liver perfusion: Livers were removed and perfused as indicated above except that Umbreit Ringer 25 mM $NaHCO_3$ buffer was used throughout. Livers were allowed to equilibrate for 30 min before the addition of substrate (10 mM lactate, alanine or pyruvate). Glucose production was followed by taking aliquots of the perfusate at 30 min intervals for 90 min.

RESULTS AND DISCUSSION

Table I indicates the cell yields obtained under various conditions. In the presence of 20 mg/100 ml of collagenase and Umbreit Ringer buffer as the perfusion medium the cell yield was 0.24 g/100 g body wt. Under similar conditions in a Ca^{++} free Hanks buffer the cell yield was increased to 1.23 g/100 g body wt. Although the use of either 10 or 20 mg/100 ml of

TABLE I

Cell Yield Under Various Experimental Conditions

g of cells/100 g body wt

Animal	Fed			Starved 24 hr
Buffer	Ca^{++} Umbreit Ringer	Ca^{++} Free Hanks		
Amount of Collagenase	20 mg/100 ml		10 mg/100	20 mg/100
Cell Yield (N)	0.24+0.04 (8)	1.23+0.08 (8)	1.07+0.01 (4)	1.59+0.1 (6)

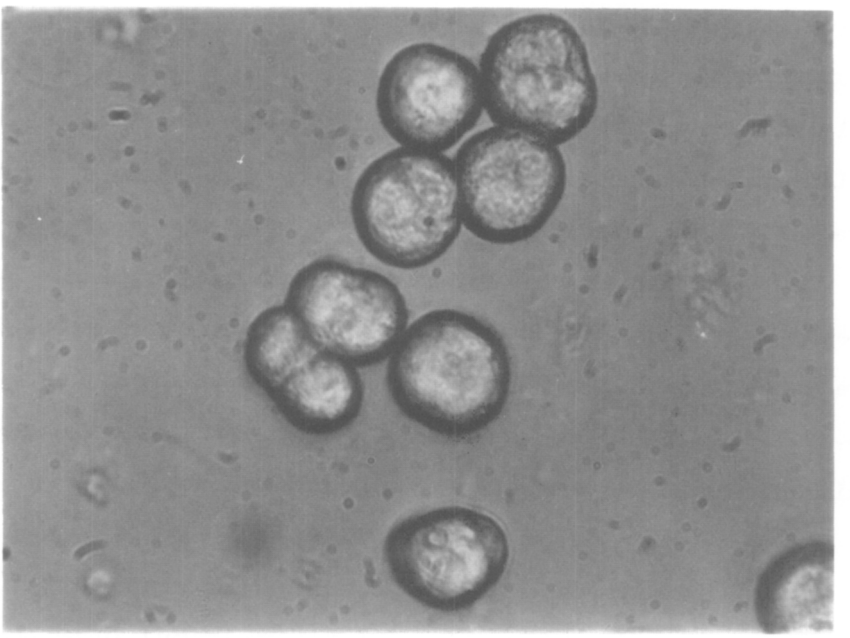

Figure 1

Light micrograph of hepatocytes during final stage of isolation (600 x magnification).

collagenase in the perfusion media results in adequate cell yields, 20 mg/100 ml of collagenase produced a more rapid disruption of the liver. Higher concentrations of collagenase (> 40 mg/100 ml) resulted in a reduction of perfusion rates. This same phenomenon was also observed if less than 10-15 min was allowed for equilibration before the addition of 20 mg of collagenase. Earlier reports have used a minimum of 50 mg/100 ml of collagenase in conjunction with high concentrations of hyaluronidase (> 100 mg/100 ml). Hyaluronidase was not required with our method. Under ideal conditions 2.3 to 3.0 x 10^8 cells were isolated per 100 g body wt, a 10-fold increase over that reported by other authors (6,12,13).

TABLE II

Incorporation of $NaHC^{14}O_3$ into Protein and Glucose by Isolated Rat Liver Parenchymal Cells*

Substrate (10 mM)	Protein dpm/mg/hr	Glucose cpm/g/hr	$NaHC^{14}O_3$ into Glucose umoles/g/hr
L(+)-Lactate (N)	139 + 18.2 (5)	60,300+9,100 (5)	8.2 + 1.2 (5)
L-Alanine (N)	204 + 14.3 (5)	36,700+1,900 (5)	5.1 + 0.27 (5)
Na-Pyruvate (N)	128 + 12.2 (5)	85,800+7,400 (5)	11.7 + 1.0 (5)
No Substrate Added (N)	53 + 2.3 (3)	3,600+ 500 (4)	0.49 + 0.07 (4)

*50-100 mg of isolated rat liver parenchymal cells (140,000 cells/mg) were incubated in 3 ml of Umbreit Ringer 25 mM $NaHCO_3$ buffer containing 0.5 uCi of $NaHC^{14}O_3$ with various substrates on this and Table III.

Figure 1 shows the typical spherical shape of the isolated hepatocytes with intact cell membranes. Only 5 to 10% of the cells prepared by our method stained with trypan blue, demonstrating relatively low contamination with disrupted cells. Furthermore, these preparations displayed a high rate of glucose production. In a few preparations where greater than 40-50% of the cells stained with trypan blue very low gluconeogenic rates were observed.

The data showing the incorporation of $NaHC^{14}O_3$ into glucose and protein is summarized in Table II. The addition of lactate, pyruvate or alanine differentially stimulated incorporation of $NaHC^{14}O_3$ into both protein and glucose. These data show a 10-fold increase in the incorporation of CO_2 into protein and a 5-fold increase in the incorporation of $C^{14}O_2$ into glucose over the incorporation observed with liver slices (14,15).

TABLE III

Glucose Production in Various Rat Liver Preparations

(umoles glucose/g wet wt/hr)

Substrate (10 mM)	Liver Cells	Perfused Liver	Liver Slices*
L(+)-Lactate (N)	42.5+4.7 (5)	29.2+5.7 (4)	24.0+2.0 (6)
L-Alanine (N)	18.2+1.2 (5)	20.7+1.1 (5)	14.0+2.4 (6)
Na-Pyruvate (N)	35.2+3.2 (5)	32.8+6.6 (5)	16.0+1.5 (6)
No Substrate Added (N)	2.29+0.6 (4)	7.2+1.3 (14)	9.6+0.7 (6)

*Previously published data (16) included for comparative purposes only.

Alanine stimulated the incorporation of $NaHC^{14}O_3$ into protein to a greater degree than did either lactate or pyruvate. Lactate and pyruvate served as better precursors for CO_2 fixation into glucose than did alanine. Incorporation of $NaHC^{14}O_3$ into protein and glucose was linear for 120 min. Longer time periods were not studied.

Glucose production obtained from liver cells are compared with perfused liver and with liver slice data (16) in Table III. Isolated liver cells produced glucose at rates comparable with those of perfused liver. Furthermore, the cells isolated by our method responded to increasing lactate concentrations (Table IV) a phenomenon not observed in liver slices (16) or isolated perfused liver (17). Liver cells isolated by Berry and Friend (7) produced glucose at rates more comparable with those of liver slices than those of perfused liver.

TABLE IV

Net Glucose Production with Various Concentrations of L(+)-Lactate by Isolated Rat Liver Parenchymal Cells*

Substrate Concentration Lactate (mM)	(umoles glucose/g wet wt/hr
0	1.7 ± 0.7
0.5	15 ± 1.0
1.0	23 ± 1.8
5.0	38.3 ± 1.3
10	44.0 ± 2.2
20	57.6 ± 4.7
40	74.6 ± 6.7
60	84.7 ± 8.2
80	93.2 ± 6.6

*50-100 mg of isolated rat liver parenchymal cells (140,000 cells/mg) were incubated in 3 ml of Umbreit Ringer 25 mM $NaHCO_3$ buffer with various concentrations of lactate. N = 5.

Our laboratory has previously calculated by isotope dilution (16) that *in vivo* glucose production may approach 106 umoles/g/hr. In the present studies glucose production with various concentrations of lactate approached 80-90% of this *in vivo* gluconeogenic rate (Table IV). Similar results were not observed with pyruvate or alanine (data not shown).

Hems *et al.* (18) have suggested that the stringent test of metabolic integrity of liver cells is the ability to produce glucose from lactate, since this process requires energy as well as the cooperation and integrity of both mitochondrial and cytoplasmic compartments. The cells described here demonstrated a high degree of metabolic integrity by these standards as shown by the high glucose production in the presence of lactate. This method should prove to be a useful tool in studying the regulation of hepatic metabolism.

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