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Characterization of Gluconeogenesis in Enzymatically Isolated Parenchymal Cells of Rat Liver[1] (37778)

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Evaluation of hepatic parenchymal cell metabolic functions *in vitro* has been attempted utilizing a number of test systems, *viz.*, the perfused liver (1, 2) liver slices (3, 4), and liver cells isolated by a variety of techniques (5–15). While studies of the perfused rat liver have been extensive and successful, two major disadvantages of this system are (i) it is not exclusively a population of parenchymal cells, and (ii) the number of variables able to be studied simultaneously is limited. The value of liver slices is also restricted since they are a nonuniform and complex system in which oxygen diffusion to cells on the inside of the slice is limited and cells on the outer surface are mechanically damaged. A homogeneous preparation of intact parenchymal cells overcomes many of the disadvantages mentioned in the other systems and, in addition, permits the study of many variables while also providing simultaneous control samples.

The purpose of the present investigation was to characterize a method for the preparation of intact viable hepatocytes with particular emphasis on their application to the *in vitro* study of gluconeogenesis. The method employed was a modification of the enzymatic technique developed by Berry and Friend (6) and involved perfusion of isolated rat livers with media containing collagenase and hyaluronidase. The ability of the parenchymal cells isolated by this method to exclude vital dyes and to retain enzymes localized to the cytoplasmic, mitochondrial, and lysosomal portions of the cell was determined. These tests were deemed critical because mechanically isolated hepatocytes have been shown to be damaged sufficiently to take up trypan blue and to leak cytoplasmic enzymes (5, 9, 11). Since Krebs and coworkers (2) have stated that a stringent test of the metabolic integrity of the hepatocyte is its ability to synthesize glucose from lactate, the gluconeogenic capacity of the isolated cells was evaluated in the present study. Of special interest was the influence of fasting duration on hepatocyte gluconeogenesis in light of recent evidence identifying peripheral substrate release as the probable rate controlling factor in hepatic gluconeogenesis during prolonged fasting (16).

Materials and Methods. Animals. Male rats of the Holtzman strain (Holtzman Co., Madison, WI) weighing 280–310 g were fed Purina chow and water *ad libitum* in animal quarters maintained at 76–78° F and at an automatically regulated 12-hr light–dark cycle (7 AM–7 PM). Experiments commenced at 9 AM on rats which were fasted with access to water for 24, 48, 96, or 168 hr prior to use.

Liver isolation. Parenchymal cells were isolated from livers by a modification of the method of Berry and Friend (6). Rats were anesthetized with sodium pentobarbital at a dose of 30 mg/kg via the dorsal vein of the penis. A midline laporotomy was performed, the inferior vena cava and portal vein were isolated, and 500 USP units of heparin were injected iv. After ligating the abdominal vena cava above the renal veins, a PE 200 polyethylene catheter was inserted and secured in the portal vein. The liver was rapidly flushed using 15–20 ml of calcium and glucose-free

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Hanks' solution. The thorax was opened and a PE 240 polyethylene catheter was inserted and secured in the thoracic vena cava via penetration of the right atrium. The liver was rapidly excised, and placed in a modified Miller isolated liver-perfusion apparatus (Metalloglass, Inc., Boston, MS). The portal cannula was connected and perfusion initiated so that the elapsed time from ligation of the abdominal vena cava to maintenance of portal perfusion was less than 3 min.

Liver perfusion. The perfusion medium consisted of 0.05% collagenase (Sigma Type I) and 0.10% hyaluronidase (Sigma Type I) (Sigma Chemical Co., St. Louis, MO) in calcium and glucose-free Hanks' solution adjusted to pH 7.4. The livers were perfused at 25–30 ml/min and at a hydrostatic pressure of 10–12 cm H_2O. The medium was oxygenated with 100% O_2 and liver temperature was maintained at 37° as measured by a thermistor wedged between the lobes. After approximately 20 min of perfusion the liver exhibited marked breaks in the capsular surface. At this time the liver was removed from the perfusion apparatus, trimmed to remove the diaphragm and large blood vessels, and rapidly cooled in 50 ml of iced perfusion medium.

Cell separation. The digested liver tissue was gently dispersed by cutting connective tissue with a scissors. In order to disperse the remaining tissue aggregates, the liver cell suspension was incubated in two 250-ml Nalgene Erlenmeyer flasks at 37° under 100% O_2 in a Dubnoff metabolic shaker bath for 15 min at 100 oscillations/min. The hepatocyte suspension was then sequentially filtered through gauze and 100-mesh silk sieve cloth into iced 50-ml centrifuge tubes. Intact parenchymal cells were separated from cellular debris, damaged cells, and Kupffer cells by centrifuging at 60*g* for 2 min at 10°. The cell pellet was washed three times with calcium and glucose-free Hanks' solution. The isolated parenchymal cells were filtered again and finally suspended in glucose-free Krebs–Ringer bicarbonate buffer. Cell counts were made in a hemocytometer and viability estimated by 0.2% trypan blue exclusion (11). The cells were rejected if the initial viability was less than 85%.

In vitro *gluconeogenesis.* The hepatocytes were incubated at a concentration of 4×10^6 cells/ml as 3-ml samples in 25-ml Erlenmeyer flasks. Appropriate quantities of gluconeogenic substrates (L-alanine, β-D-fructose, L(+)-lactic acid, and sodium pyruvate) were added to produce specified molar concentrations. All substrates were obtained from the Sigma Chemical Co., St. Louis, MO. The flasks were gassed with 95% O_2–5% CO_2 and incubated at 37° and 60 oscillations/min in a Dubnoff metabolic shaker bath. At the end of the incubation period, viability was again estimated by the trypan blue exclusion test. The incubations were terminated by rapid freezing to −15°. Upon thawing the samples were deproteinized with 1 ml each of 1.8% barium hydroxide and 2.0% zinc sulfate and centrifuged at 1000*g*; then 1-ml duplicate samples of the supernatant were analyzed for glucose using the glucose oxidase method in Tris buffer (Worthington Biochemicals Corp., Freehold, NJ). Cell aliquots were analyzed for protein using the Oyama and Eagle modification (17) of the Lowry method. Gluconeogenesis was calculated as the micromoles of glucose produced per g of cell protein per min.

Liver enzyme measurements. For the determination of cell integrity, isolated hepatocytes from 24-hr-fasted rats were either incubated at 37° for 120 min or kept on ice at 4° and then centrifuged at 500*g* for 10 min at 10°. The resuspended cell pellets were assayed for glucose-6-phosphatase, EC 3.1.3.9, (18); lactate dehydrogenase, EC 1.1.1.27, (19); malate dehydrogenase, EC 1.1.1.37, (20); and acid phosphatase, EC 3.1.3.2, (21) by the specified standard techniques. Hepatic glycogen was also determined (22).

Data analysis. All data were compared for statistical significance at the 95% confidence level using Student's paired or unpaired *t* test as indicated. Tabulated data are expressed as the mean ± standard error.

Results. Hepatocyte integrity during incubation. The effect of incubation for 120 min at 37° on enzyme activities of the resuspended cell pellets of 24-hr-fasted hepatocytes from four rats was minimal. No significant

changes in cellular protein and inorganic phosphate (P_i) levels were observed in the incubated hepatocytes as compared to control aliquots kept at 4°. Respective activities after 4 vs 37° incubation of the microsomal marker enzyme, glucose-6-phosphatase (66.0 ± 2.2 vs 59.4 ± 1.9 mg P_i/g protein/30 min), and the cytoplasmic marker enzyme, lactate dehydrogenase (7.13 ± 0.84 vs 6.37 ± 0.97 μmoles pyruvate/g protein/min), demonstrated small nonsignificant decreases. The 10.8% decrease in lactate dehydrogenase, a readily soluble enzyme, is similar to the change in the percentage of hepatocytes able to exclude trypan blue which decreased from approximately 90% prior to the incubation to 80% after incubation. Activities of malate dehydrogenase (10.1 ± 0.85 vs 14.1 ± 0.60 μmoles NADH/g protein/min) and acid phosphatase (9.57 ± 0.17 vs 14.4 ± 0.49 mg P_i/g protein/30 min) which are contained in the mitochondrial and lysosomal fractions of the cell, respectively, increased during incubation at 37°.

Factors influencing isolated hepatocyte gluconeogenesis. The effects of substrate concentration and incubation duration on *in vitro* gluconeogenesis by 24-hr-fasted rat hepatocytes were examined. As shown in Table I, 10 m*M* alanine, lactate, and pyruvate yielded maximal gluconeogenic rates for those substrates in the isolated parenchymal cells. The substrate concentrations for half-maximal gluconeogenesis were 2 m*M* for pyruvate and 1 m*M* for alanine and lactate. Increasing the lactate concentration above 10 m*M* produced significant decreases in the gluconeogenic rate from 1.37 μmoles/g protein/min for 10 m*M* lactate to 1.06 for 20 m*M* and 0.50 for 40 m*M*. Although a maximal rate for fructose was not attained in the present study, the glucogenic rate tended to plateau at fructose concentrations of 10–20 m*M*.

Evaluation of the time course of gluconeogenesis from 10 m*M* alanine and lactate revealed a substantial lag in glucose production by fasted rat parenchymal cells (Table II). The rate of gluconeogenesis by isolated hepatocytes increased up to 120 min of incubation for the two substrates and then declined slightly by 240 min. Therefore, an incubation duration of 120 min was selected as optimal for the *in vitro* hepatocyte system. In contrast to substrate-linked gluconeogenesis, endogenous glucose production in buffer was stable throughout the 240 min of hepatocyte incubation.

Hepatic tissue regression during fasting. As indicated in Table III, fasting rats from 24 to 168 hr produced 10.7–30.8% losses in body weight. Correspondingly, the protein content of isolated hepatocytes declined such that by 168 hr of fasting 53.9% of the cellular protein was lost. Values previously determined in this laboratory (23) correlating liver weight to percentage of body weight for specified fasting periods were then used to calculate the percentage decreases in liver weight. Even though the hepatocyte protein content decreased 34.1% by 24 hr of fast-

TABLE I. Effect of Substrate Concentration on Isolated Hepatocyte Gluconeogenesis.[a]

Substrate concentration (m*M*)	Gluconeogenesis (μmoles glucose/g protein/min)			
	Alanine	Lactate	Pyruvate	Fructose
1	0.54 ± 0.05[b]	0.74 ± 0.04[b]	0.57 ± 0.05[b]	1.31 ± 0.06[b]
2	0.63 ± 0.04[b]	0.91 ± 0.06[b]	0.93 ± 0.05[b]	2.06 ± 0.11[b]
5	0.88 ± 0.09	1.33 ± 0.14	1.50 ± 0.09[b]	5.72 ± 0.07[b]
10	1.03 ± 0.08	1.37 ± 0.16	1.84 ± 0.13	8.11 ± 0.40
20	1.04 ± 0.06	1.06 ± 0.20[b]	1.84 ± 0.16	9.33 ± 0.20[b]

[a]Data are expressed as means ± standard error. Each group consists of cells isolated from four rats fasted for 24 hr. Hepatocytes were incubated for 120 min at 37° with specified substrate. The gluconeogenic rate with substrates is less the endogenous rate of 0.22 ± 0.02 μmoles/g protein/min in Krebs–Ringer bicarbonate buffer.

[b]$P < 0.05$ as compared to respective value in 10 m*M* substrate concentration group by Student's paired *t* test.

TABLE II. Effect of Incubation Duration on Isolated Hepatocyte Gluconeogenesis.[a]

Incubation time	Krebs–Ringer bicarbonate (KRB)	Gluconeogenesis (μmoles glucose/g protein/min) 10 m*M* Alanine	10 m*M* Lactate
15	0.25 ± 0.03	0.69 ± 0.16[b]	0.48 ± 0.15[b]
30	0.22 ± 0.03	0.86 ± 0.14[b]	0.61 ± 0.12[b]
60	0.22 ± 0.02	1.03 ± 0.11	1.20 ± 0.12[b]
120	0.22 ± 0.02	1.08 ± 0.05	1.51 ± 0.09
180	0.22 ± 0.02	1.11 ± 0.06	1.48 ± 0.07
240	0.20 ± 0.02	0.95 ± 0.04	1.28 ± 0.06[b]

[a]Data are expressed as means ± standard error. Each group consists of cells isolated from four rats fasted for 24 hr. Hepatocytes incubated at 37° with specified substrate. The gluconeogenic rate with substrates is less the endogenous rate in KRB buffer.

[b]$P < 0.05$ compared to respective value in 120 min incubation group by Student's paired *t* test.

ing, the liver protein concentration, as previously reported (23, 24) actually increased since liver weight decreased proportionately more—36.5%. On this basis, even greater increases in hepatocyte protein concentration were noted for the other fasting periods.

Hepatocyte gluconeogenesis during fasting. The rates of *in vitro* rat hepatocyte gluconeogenesis as a function of fasting duration are presented in Table IV. Endogenous glucose production in Krebs–Ringer bicarbonate buffer without added substrate for parenchymal cells of rats fasted from 24–168 hr was uniformly low. Correspondingly, the hepatic glycogen level in eight 24-hr-fasted rats in the present study was 0.43 ± 0.09 mg of glucose/100 mg of liver wet weight as compared to a value of 6.35 ± 0.14 for eight fed rats. The addition of 10 m*M* alanine, lactate, and pyruvate produced 5- to 10-fold increments in glucose synthesis over the endogenous rate in fasted rat hepatocyte. Fructose at a concentration of 1 m*M* uniformly augmented the amount of glucose formed in fasting hepatocytes. Interestingly, the rates of gluconeogenesis expressed per gram of cellular protein for all four substrates demonstrated no striking changes from 24–168 hr of fasting. However, based on the hepatocyte protein content presented in Table III,

TABLE III. Effect of Fasting[a] on Hepatic Tissue Parameters.

Experimental group	Body weight (g) Prior	Post	% Decrease	Liver weight (g) % Body wt at sacrifice	Total organ	% Decrease	Hepatocyte protein g/10^9 cells	% Decrease
Fed	298 ± 3 (12)			4.40	13.10		1.72 ± 0.16 (4)	
24-hr Fast	300 ± 3 (12)	268 ± 3[b] (12)	10.7	3.12	8.35	36.6	1.13 ± 0.04[b] (12)	34.1
48-hr Fast	298 ± 2 (8)	244 ± 4[b] (8)	18.1	2.83	6.90	47.2	1.04 ± 0.07[b] (4)	39.4
96-hr Fast	293 ± 2 (8)	231 ± 2[b] (8)	21.2	2.74	6.32	50.8	1.01 ± 0.06[b] (4)	41.1
168-hr Fast	299 ± 3 (8)	207 ± 3[b] (8)	30.8	2.57	5.32	59.4	0.79 ± 0.05[b] (4)	53.9

[a]No food, but water *ad libitum*. Data are expressed as means ± standard error. Parentheses enclose number of rats per group.

[b]$P < 0.05$ as compared to respective value in the fed group by Student's unpaired *t* test.

TABLE IV. Effect of Fasting on Isolated Hepatocyte Gluconeogenesis.[a]

Experimental group	Krebs-Ringer bicarbonate (KRB)	Gluconeogenesis[b] (μmoles glucose/g protein/min) 10 m*M* Alanine	10 m*M* Lactate	10 m*M* Pyruvate	1 m*M* Fructose
24-hr Fast (12)	0.21 ± 0.02	1.04 ± 0.05	1.50 ± 0.10	1.98 ± 0.12	1.47 ± 0.08
48-hr Fast (4)	0.17 ± 0.02	0.98 ± 0.06	1.37 ± 0.11	1.86 ± 0.06	1.48 ± 0.10
96-hr Fast (4)	0.21 ± 0.03	1.13 ± 0.07	1.59 ± 0.14	2.04 ± 0.12	1.64 ± 0.05
168-hr Fast (4)	0.21 ± 0.04	1.06 ± 0.18	1.25 ± 0.22	2.01 ± 0.19	1.79 ± 0.09

[a]Data are expressed as means ± standard error. Parentheses enclose number of cell preparations per group. Hepatocytes were incubated for 120 min at 37° with specified substrate.

[b]All values with added substrate are $P < 0.05$ compared to buffer alone for each experimental group by Student's paired *t* test.

the gluconeogenic rates per 10^9 cells were calculated to fall significantly during prolonged fasting, *e.g.*, glucose production from alanine decreased from 1.17 μmoles/10^9 cells/min after a 24-hr fast to 0.84 after 168 hr. Corresponding gluconeogenic rates from alanine for 24- and 168-hr-fasted cells expressed as μmoles/g protein/min were not different, *i.e.*, 1.04 and 1.06, respectively (Table IV).

Discussion. The structural and metabolic integrity of parenchymal cells isolated from rat liver by an enzymatic technique was verified in the present study by a high percentage of trypan blue exclusion, a low degree of soluble enzyme release into the incubation media, and a constant rate of gluconeogenesis from lactate and other precursors. The small loss (10.8%) of lactate dehydrogenase from isolated hepatocytes during incubation for 120 min could be accounted for by cells with damaged plasma membranes as indicated by their uptake of trypan blue. Correspondingly, Berry and Friend (6) demonstrated that the specific activity of lactate dehydrogenase was as high in isolated cells as in the initial tissue dispersate. In contrast to the observed ability of enzymatically isolated hepatocytes to exclude vital dyes (12, 14, 15) and retain soluble enzymes (14, 15), mechanically isolated cells have invariably been shown by these same techniques to possess severely damaged plasma membranes (5, 9, 11). Further verification of the integrity of hepatocytes isolated using collagenase and hyaluronidase was the findings that the fasting-induced physiological pathways of fatty acid metabolism were operative (15) and that there was no change in the antigen profile of the cell surface membrane (13).

The primary advantage of the *in vitro* parenchymal cell system over the perfused liver are the homogeneity of the isolated hepatocytes and the notable increase in the number of variables together with internal controls which can be determined for cells obtained from a single rat. In this regard, the cell yield for this study was invariably at least 50 × 10^6 cells/g of liver weight. Ingebretsen and Wagle (12) reported a similar cell yield and calculated that it was 10 times that for the enzymatic technique of Howard and Pesch (11) utilizing liver slices instead of liver perfusion. In comparing isolated hepatocytes to liver slices *per se* as an *in vitro* test system, Berry and Friend (6) emphasized that the problem of oxygen diffusion in liver slices was overcome in the isolated cell preparation.

Recently, other investigators (8, 12, 14, 15) have reported that enzymatically isolated rat parenchymal cells are capable of gluconeogenesis from a variety of substrates at rates similar to or greater than those observed in the present study. In the absence of a common unit or expression, conversion factors such as those suggested by Scrutton and Utter (25) for liver (activity per g wet wt = activity per g dry wt × 0.28 = activity per g protein × 0.20) were necessary for comparisons to other studies. We chose to express

our data per gram of cell protein since this determination is convenient, precise, and reproducible. The importance of the unit of expression is emphasized by the finding in the present study that the gluconeogenic rate as a function of fasting duration was unchanged on a cell protein basis but decreased per 10^9 cells. The effects of substrate concentration and incubation duration on gluconeogenesis by isolated hepatocytes in the present study are basically in accord with findings in other *in vitro* systems, particularly the perfused rat liver. Exton and Park (1), utilizing the perfused liver, found the highest gluconeogenic rate from frustose and half-maximal gluconeogenic rates from lactate and pyruvate at 1 and 2 m*M* concentrations. They also observed no change in the rate of glucose production from lactate above 10 m*M* (1) while for enzymatically isolated hepatocytes Ingebretsen and Wagle (12) found a marked increase and we found a marked decrease. A lag in the rate of gluconeogenesis from lactate as seen in the present study was also described by Ontko (15) even though Exton and Park (1) reported a time of only 75 sec for conversion of lactate to glucose in the perfused liver.

Fasting rats for 24, 48, 96, or 168 hr produced a marked stimulation of gluconeogenesis from all substrates tested over the endogenous rate. The endogenous rate of glucose synthesis in fasted cells probably reflects that from amino acids and other precursors in the liver rather than glycogenolysis since glycogen was found to be depleted markedly in rats by 24 hr of fasting in this and other studies (4, 14). As described by Filkins (23) and Harrison (24), during prolonged starvation the liver undergoes a drastic loss in cytoplasm contributing essential nutrients to extrahepatic tissues. However, Schimmel and Knobil (4) found that hepatic gluconeogenesis in rats was stimulated even during moderate extension of interprandial periods, *i.e.*, a 3- to 9-hr fast. The absence of a marked change in the rates of gluconeogenesis with fasting from 24–196 hr in the present study is in agreement with the concept of Exton and Park (1) that the primary fine control of gluconeogenesis is accomplished by variations in substrate presentation. With reference to alanine, Felig, Owen, Wahren, and Cahill (16) have reported evidence supporting a similar substrate-limited control model of gluconeogenesis. They believed that during prolonged fasting alanine ceases to be released from muscle, the body's principal protein store, while the gluconeogenic apparatus of the liver as well as hepatic extraction of alanine remain unchanged (16). The results of the present study support the hypothesis that the ability of the liver to carry out gluconeogenesis is not diminished by a prolonged fast in rats.

Summary. In vitro studies of the structural and metabolic integrity of dispersed parenchymal cells prepared by perfusion of isolated rat livers with collagenase and hyaluronidase were performed. A high percentage of trypan blue exclusion, a low degree of soluble enzyme release into the incubation media, and a constant rate of gluconeogenesis from lactate, as well as other precursors verified the viability and integrity of the isolated hepatocytes. Addition of 10 m*M* alanine, lactate, and pyruvate produced 5- to 10-fold increases in the rate of gluconeogenesis as compared to the endogenous level in cells from fasted rats. Prolongation of the fast from 24 hr up to 168 hr resulted in no significant changes in the gluconeogenic rates when expressed per gram of cell protein. This finding supports the concept that substrate presentation provides control of hepatic gluconeogenesis during prolonged fasts.

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