Preparation of Extended In Vitro Cultures of Bovine Hepatocytes That Are Hormonally Responsive

S. S. Donkin and L. E. Armentano

Department of Dairy Science, University of Wisconsin, Madison 53706

ABSTRACT: Hepatocytes isolated from male dairy calves were used in monolayer culture or in suspension culture to determine their suitability for the study of hormonal regulation of hepatic gluconeogenesis. The rate of gluconeogenesis (nanomoles of 2.5 mM \(^{14}C\)propionate incorporated into glucose microgram of DNA\(^{-1}\)·hour\(^{-1}\)) was higher for monolayers than for suspension cultures. Gluconeogenesis and ureagenesis (nanomoles of urea N formed microgram of DNA\(^{-1}\)·3 hours\(^{-1}\)) were similar in monolayers cultured for 24 and 48 h but declined by 120 h. Ureagenesis was barely detectable in suspension cultures. Glucagon (10 nM) increased gluconeogenesis from propionate in monolayers but was without effect on suspension cultures. Actinomycin D (800 nM) and cycloheximide (200 \(\mu M\)) abolished glucagon stimulation of gluconeogenesis, suggesting that glucagon acts to mediate gene expression. Prolonged exposure (45 h) of monolayers to insulin (1,000 nM) decreased basal gluconeogenic rates but did not affect glucagon-stimulated gluconeogenesis. Prior incubation with glucose or valerate did not affect gluconeogenesis. Cells can be successfully maintained in serum-free media for 41 h at the expense of diminished basal gluconeogenic activity. Culture of bovine hepatocytes as monolayers provides a useful tool for the study of chronic and acute hormonal regulation of specific liver functions in the bovine.

Key Words: Bovine, Liver, Insulin, Glucagon, Propionate

Introduction

The liver occupies a complex central role in mammalian homeostasis. For this reason, several methods have been devised that permit the study of hepatic metabolism in vitro. Previous in vitro studies of liver functions in ruminants have used perfused lobes, tissue homogenates, liver slices, or hepatocyte suspension cultures. These techniques have increased our understanding of regulation of ruminant hepatic metabolism; however, each system has limitations. Some of these limitations include nonuniformity of oxygen and(or) nutrient diffusion across liver slices; nonhomogeneity of cell types in liver slices, lobes, and homogenates; and the large number of preparations required to account for animal effects on random error in experiments that use perfused lobes.

Functional hepatocytes from small ruminants, isolated by collagenase dispersion, have been used as freshly isolated hepatocyte suspension cultures but may not be suited to the study of hormonal regulation of metabolism. Suspensions of rat hepatocytes show diminished hormonal responsiveness as a result of plasma membrane receptor damage, which occurs during the isolation process (Johnson et al., 1972). Likewise, the relatively short lifespan of hepatocyte suspensions precludes their utility in the study of hormonal effects that take several hours to be fully manifested.

Culturing hepatocytes as monolayer primary cultures may provide a system that permits repair of membrane damage and provides culture periods required to test long-term hormonal and(or) nutrient effects on cell function. Several abstracts (Donkin et al., 1990, 1991, 1992; Cadorniga et al., 1992) and one paper (Emmison et al., 1991) have reported the use of ruminant parenchymal cell monolayers to study hormonal regulation of hepatic metabolism. Xenobiotic metabolism has also been investigated using ruminant hepatocyte monolayers (Shull et al., 1986; Van Klooster et al., 1992). The purpose of this report is to provide a complete description of the technique for establishing bovine hepatocyte monolayers and to characterize changes in cellular metabolism during their extended culture.
Materials and Methods

Reagents

Sodium thiamylal was obtained from Parke-Davis (Elk Grove, IL) and [2-14C]propionate was from Amersham (Arlington Heights, IL). All chemicals were cell-culture grade or the highest purity available from Sigma Chemical (St. Louis, MO). Perfusion media (pH 7.4) consisted of calcium-free Krebs-Ringer bicarbonate (KRB) buffer containing .45 mM pyruvate and 5 mg/L phenol red, fortified with .04 mM L-asparaginase, L-aspartic acid, L-glutamic acid, glycine, L-methionine, L-proline, and L-serine; .16 mM L-isoleucine, L-leucine, L-lysine, L-threonine, and L-valine; .03 mM L-alanine, .29 mM L-arginine, .08 mM L-cystine, .09 mM L-histidine, .08 mM L-phenylalanine, and .02 mM L-tryptophan and .08 mM L-tyrosine. Wash media (pH 7.4) was Ca-free KRB containing 1% BSA (bovine albumin Cohn fraction V and essentially fatty acid-free). The BSA was prepared as a 20% solution in Ca-free KRB and dialyzed against 60 volumes of the same, then filtered through a .22-μm nylon filter (Corning Glass Works, Corning, NY). Basal culture medium (BCM) was arginine-free Dulbecco's Modified Eagle's Medium containing .5 mM ornithine, 24 mM sodium bicarbonate, 10 mM HEPES, 4.0 mM glutamine, 1.0 mM pyruvate, 5.5 mM glucose, 100 UI/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of amphotericin. The absence of arginine was designed to retard the proliferation of nonparenchymal cells, which, unlike hepatocytes, cannot synthesize arginine from ornithine. Fetal bovine serum (FBS) was heated to 55°C for 30 min to inactivate complement.

Isolation of Liver Parenchymal Cells

Male dairy calves aged 7 to 14 d (n = 16) or 3 mo (n = 3) and castrated yearling Saanen goats (n = 9) were given heparin (1,000 USP) intravenously and anesthetized with sodium thiamylal (2 g) via intrajugular injection. The caudate process was excised with 150 mL of perfusion media, and the outflow was discarded. The lobe was then perfused with 300 mL of perfusion media in a recirculating system at a flow rate of approximately 50 mL/min. After 5 min of recirculating perfusion, collagenase (Sigma Type IV) was added to the media (.5 mg/mL). The perfusion was continued for another 5 min, at which time CaCl2 was added to a final concentration of .5 mM (.75 mL of a 200 mM solution) to activate collagenase (Seglen, 1976). Throughout the course of the perfusion the lobe was gently massaged to facilitate even perfusion. The perfusion continued until tissue definition was lost and the lobe became mushy, which usually occurred within 20 min. At the end of the perfusion, the lobe was removed from the apparatus and immersed in 100 mL of the previously recirculated perfusion media and deoxyribonuclease I (Sigma DNase; type IV) was added (15 mg/100mL). In a sterile environment, Gilman's capsule was removed and cells gently teased away from blood vessels into the media using blunt scissors. The tissue suspension was then placed in 250-mL flasks, gassed with 95% O2:5% CO2, and capped and shaken in an oscillating water bath (90 strokes/min) at 37°C for 4 min. In the interest of time and improved cell viability, the prior step was successfully omitted in well-digested preparations. The suspension was filtered through a single layer of cheesecloth into a flask at 5°C that contained 1 mL of a 20% BSA solution and 45.6 mg of ethylene glycol tetraacetic acid (EGTA; .6 mL of a 100 mM solution). Cheesecloth was rinsed with wash media (1% BSA KRB) and the effluent added to the first filtrate. Cells were separated from debris in the resulting 200 to 220 mL of wash media and tissue suspension mixture by centrifugation (40 × g for 3 min at 5°C). The supernatant was discarded and cells were resuspended and washed three times in wash media at 5°C. A liver cell suspension was prepared in the same media at a packed cell volume of 10 to 15% (25 to 30 mg of DM/mL of cell suspension), gassed with 95% O2:5% CO2 and stored for < 30 min on ice before use. Throughout the procedure buffers and media were continuously gassed with 95% O2:5% CO2 to prevent cell hypoxia. The cell suspension obtained was virtually free of cellular debris as judged by light microscopy. Viability of cells was estimated by trypan blue (.2% solution) and only preparations in which ≥ 90% of the hepatocytes excluded dye were used in subsequent experiments. The entire procedure from excision of the caudate process to procurement and evaluation of the cell suspension took < 60 min.

Suspension Cultures

Suspension cultures were established by the addition of 12.5 to 15 mg of cell DM to 25-mL side-arm stoppered, Erlenmeyer flasks containing incubation media. The flasks were gassed briefly with 95% O2:5% CO2 and placed in an oscillating water bath.
Monolayer Cultures

Liver cells were maintained in monolayer culture for up to 5 d. Cells (approximately 5.3 x 10^4 cells/cm²) were seeded onto Falcon Primaria culture dishes (Becton Dickinson, Lincoln Park, NJ) containing plating media that consisted of BCM supplemented with 20% FBS, 1,000 nM insulin, and 100 nM dexamethasone. In excess of 150, 50-mm x 15-mm plates or 300, 35-mm x 15-mm plates can be prepared from a suspension of cells obtained from a single animal. Special efforts were taken to ensure that minimal time elapsed between isolation of hepatocytes and introduction onto plastic cultureware; 1 h was sufficient time to seed 300 plates.

Cells were incubated at 37°C in 95% air:5% CO₂. After an attachment period of 4 h, media and unattached cells were removed, discarded, and replaced with fresh media. The FBS content was reduced to 10% or eliminated, dexamethasone was deleted, insulin was reduced or deleted, and other supplements were added as outlined in the text and figure legends. Cells were given fresh media 24 h after initial plating and every 24 h thereafter. Monolayers were routinely monitored for morphological changes using a bright field inverted microscope.

Measurement of Cell Functions

Gluconeogenesis. Suspension cultures containing 2.5 mM [2-¹⁴C]propionate in BCM were incubated for 3 h at 37°C, as previously described (Aiello and Armentano, 1987a,b), except that BCM containing 1% BSA replaced KRB as the incubation medium. Glucose was isolated by ion-exchange chromatography (Mills et al., 1981) using [U-³H]glucose as an internal standard. Radioactivity in glucose was determined by liquid scintillation counting (Packard Tri-carb 4000 Series). Radioactivity appearing in glucose was corrected for incomplete trapping of [2-¹⁴C]propionate by ion-exchange chromatography, which was < 1% of total disintegrations per minute of [2-¹⁴C]propionate added to the columns. Gluconeogenic activity was expressed as nanomoles of ¹⁴C-precursor incorporated into glucose microgram of DNA^{-1}·hour^{-1} calculated as disintegrations per minute recovered in glucose (corrected for [U-³H]glucose loss) divided by the specific activity of the precursor. Recovery of [³H]glucose was 87.2% (SD = 2.3; n = 234 samples).

Gluconeogenesis was determined in a similar manner for monolayer cultures. Cells were incubated with BCM containing 1% BSA, 2.5 mM [2-¹⁴C]propionate, and hormones as detailed in the text and figure legends. Incubations were terminated by the removal of media. Cells were then washed with 1 mL of KRB, which was combined with the incubation media. Newly synthesized glucose was determined in the media as detailed above.

Survival Efficiency. Media and KRB rinse were aspirated from the plates as described above and cells were layered with 1 mL of ice-cold dissociation buffer (pH 7.4) (2.68 mM KCl, 1.57 mM KH₂PO₄, 137 mM NaCl, 8.06 mM Na₂HPO₄, 1.0 mM EDTA) and cells were then stored frozen on culture plates for up to 1 wk. The EDTA was added to chelate Mg and thereby inhibit constitutive DNase activity. Upon thawing, cells were scraped into 3-mL vials, and plates were rinsed with an additional 1 mL of dissociation buffer. Aliquots were removed for determination of protein (Pierce BCA assay using BSA as a standard, Pierce Chemical, Rockford, IL) and DNA (Labarca and Paigen, 1980). Survival efficiency was determined by dividing the amount of protein and DNA contained on the plates at the end of the incubation period by the amounts contained in six aliquots of the cell suspension identical to those used to establish the monolayers.

Ureagenesis. Cell suspensions or monolayers were incubated in KRB containing 1% BSA, 5.5 mM glucose, 1.0 mM pyruvate, and 5 mM NH₄Cl. After 3 h of incubation, cells and media were separated and urea formation was determined in media by a colorimetric procedure (Sigma kit 535). The rate of urea synthesis was expressed as nanomoles of urea formed microgram of DNA^{-1}·3 hours^{-1}. Measurements of ureagenesis in suspension cultures were corrected for urea present in cells at time 0 and released during separation of cells and media. This value represented < 3% of total ureagenesis.

Other Measures. Effects of incubation media were assessed on cell survival (micrograms of DNA/plate), cell protein (micrograms/plate), cell glycogen content, and gluconeogenic activity. Four hours after plating, the effects of BSA, FBS, glucose, and valerate were tested using the following combinations: a) 10% FBS, b) 10% FBS plus insulin (1,000 nM), c) 1% BSA, d) 1% BSA plus insulin (1,000 nM), e) 10% FBS plus insulin (1,000 nM) plus 1.25 mM valerate, f) 10% FBS plus insulin (1,000 nM) plus 1.25 mM valerate and the deletion of glucose (contained at 5.5 mM unless noted otherwise), and g) 10% FBS plus insulin (1,000 nM) and the deletion of glucose but without added valerate. Media were refreshed 24 h after plating and treatments were terminated after an additional 24 h, corresponding to a culture time of 48 h and total exposure to treatments of 44 h. The DNA and protein were determined as described above and glycogen was measured as glucose equivalents by the glucose oxidase method after amyloglucosidase digestion (Hoffer and Lowenstein, 1986). Effects of previous media on subsequent gluconeogenic capacity was...
Table 1. Effect of culture time on cell characteristics and functions

<table>
<thead>
<tr>
<th>Culture time, h</th>
<th>DNA (^{bc})</th>
<th>Protein (^{bd})</th>
<th>Protein:DNA (^{be})</th>
<th>Ureagenesis (^{fg})</th>
<th>Gluconeogenesis (^{fi})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>53 ± 4</td>
<td>2,019 ± 268</td>
<td>35 ± 3</td>
<td>16.2 ± 13.8</td>
<td>4.99 ± 1.36</td>
</tr>
<tr>
<td>24</td>
<td>30 ± 4 (^*)</td>
<td>1,334 ± 266</td>
<td>40 ± 3</td>
<td>77.7 ± 13.8(^*)</td>
<td>9.46 ± 1.28(^*)</td>
</tr>
<tr>
<td>48</td>
<td>32 ± 5 (^*)</td>
<td>1,534 ± 324</td>
<td>50 ± 3(^*)</td>
<td>55.8 ± 13.8</td>
<td>6.83 ± 1.32</td>
</tr>
<tr>
<td>120</td>
<td>43 ± 4</td>
<td>1,725 ± 240</td>
<td>42 ± 2</td>
<td>47.1 ± 13.8</td>
<td>2.08 ± 1.36</td>
</tr>
</tbody>
</table>

\(^a\)Least squares means and associated standard errors using animal \(\times\) culture time as the error term and Type I sums of squares.
\(^b\)The DNA and protein seeded onto culture plates (60-mm diameter) and amount remaining was determined at time points indicated; \(n = 3\) animals.
\(^c\)DNA (micrograms/60-mm plate), culture time effect \((P < .05)\).
\(^d\)Cellular protein (micrograms/60-mm plate).
\(^e\)Culture time effect \((P < .05)\).
\(^f\)Gluconeogenesis and ureagenesis was measured in freshly isolated suspensions (time 0) and monolayers at times indicated; \(n = 3\) animals.
\(^g\)Urea formed during 3 h (nanomoles urea \(\mathrm{N/}\mu\mathrm{g}\) of DNA\(^{-1}\)), culture time effect \((P < .10)\).
\(^h\)Incorporation of 2.5 mM \([2-\text{14C}]\)propionate into glucose (nanomoles/micrograms of DNA\(^{-1}\)hour\(^{-1}\)), culture time effect \((P = .04)\).
\(^i\)Root mean square error for animal \(\times\) culture time.
\(^j\)Degrees of freedom associated with the root mean square error.
\(^*\)Differs from culture time 0 \((P < .10)\).

determined during the last 3 h (culture time 45 to 48 h) of culture, during which time all cells were incubated in BCM containing 1% BSA and 2.5 mM \([2-\text{14C}]\)propionate. In those instances cells received long-term insulin and media treatments for 41 h and in some cases glucagon during the final 3 h of incubation coincidental with measurement of gluconeogenesis.

**Statistical Analysis**

A minimum of three separate cell preparations constituted an experiment, and treatments within each preparation were replicated in three to six culture flasks or plates. All data from individual plates or flasks were used in the analysis of variance. Culture time effects were compared using Type I sums of squares in a split block design with culture time \(\times\) animal as the error term. All other analyses used Type III sums of squares. Glucagon effects on suspensions and monolayers were compared in a randomized block design that tested for the effects of culture type, glucagon treatment, and their interactions using animal \(\times\) culture type \(\times\) glucagon treatment as the error term. Effects of additions to or deletions from media on cell survival and function were tested using orthogonal contrasts with animal \(\times\) media alteration as the test statistic denominator. Where applicable, subsequent hormonal treatment effects were tested using animal \(\times\) alteration \(\times\) hormonal treatment as the error term. Data are reported as least squares means, except the data in Tables 2 and 3, which are unadjusted means. The mean value of gluconeogenesis for all monolayers was determined using control incubations of cells previously cultured in FBS only and cells cultured in FBS plus insulin (1,000 nM). For the latter, the effects of long-term insulin exposure (45 h) were removed by analysis of variance using the GLM procedure of SAS (1985).

**Results and Discussion**

Freshly isolated hepatocytes tended to cluster in groups of two to four cells, were rounded, and had visible nuclei and granular contents. Attachment of cells in groups of two to four to plastic substratum was apparent within 30 min of seeding. Monolayer confluence was achieved within 24 h (Figure 1) and was maintained during the 120-h culture period. Survival efficiency, determined at 24 h, was approximately 62% of the DNA seeded onto the plates (Table 1). Cells covered approximately 85 to 95% of the available surface of the culture plates. The DNA and protein contained on the plates remained relatively constant during the culture period with slight increase by 120 h. This late increase may indicate the proliferation of co-isolating nonparenchymal cells, because hepatocytes generally do not replicate in culture. Unsuccessful attempts to culture hepatocytes did result in the growth of cells that could be mistaken for hepatocytes if evaluated by light microscopy only. In contrast to bovine hepatocytes, these cells proliferated in culture and contained several nucleoli per nucleus. The precise origin of these cells has not been determined; however, preliminary experiments indicated that they did not synthesize glucose and could only be grown in arginine-supplemented media. Epithelial-like cells displaying similar morphology have been cultured from rat liver (Williams et al., 1971).

Relative to freshly isolated hepatocytes, monolayer cultures 24 h after isolation showed higher rates of gluconeogenesis and had similar rates by 48 h in
culture, but gluconeogenic activity decreased by 120 h in culture (Table 1). Accumulation of labeled glucose was directly proportional to incubation time in the presence of labeled propionate for either 2 or 4 h (Figure 2). As a result, gluconeogenic activity (nano-
moles of $^{14}$C-precursor incorporated into glucose/microgram of DNA·hour$^{-1}$) did not differ between plates incubated for either 2 or 4 h with labeled substrate ($P = .63\ n = 2$). These relationships were evident in monolayers cultured for 24, 48, or 120 h despite declining gluconeogenic capacity by 120 h in culture (Figure 2).

Leakage across the plasma membrane can be a problem in freshly isolated cell suspension models (Pogson et al., 1984) but can be circumvented with monolayer culture (Ichihara et al., 1980). Higher metabolic activity observed for monolayers after 24 h in culture may result from plasma membrane repair and decreased cellular energy expenditure in ion pumping and other processes. Additionally, only viable cells are capable of producing the extracellular matrix proteins necessary for adherence to stationary surfaces. The attachment phase of the monolayer culture system, therefore, selects against the culture of damaged cells. Loss in capacity to synthesize glucose by 120 h, expressed per unit of DNA, may result from detachment of expired parenchymal cells and the concomitant growth of other cell types, a loss of differentiated functions within parenchymal cells, or both. Similar results were seen for ureagenesis (Table 1), except monolayers sustained higher rates of urea synthesis than freshly isolated cells during the 120-h experimental period.

Cells isolated from goats have been used in both hepatocyte suspension (Aiello and Armentano, 1987a,b; Aiello et al., 1989) and monolayer culture systems (Van Klooster et al., 1992). However, in the present study, satisfactory monolayers were obtained with much lower repeatability from goat liver. Nine attempts to establish caprine hepatocyte monolayers resulted in only one culture containing adequately populated culture plates; however, satisfactory monolayers were obtained from 16 of 19 preruminant calves. Monolayers have been obtained from liver of ruminant calves (aged 3 mo) using the procedures detailed in this report. These findings suggest that species differences may be more important than age differences in the application of these techniques to the study of hepatic metabolism in older ruminants.

Rates of in vitro propionate incorporation into glucose in preruminant calves (present study) are similar to rates obtained using hepatocytes from ruminating animals (Table 2), with the exception of those presented by Ash and Pogson (1977). Higher rates obtained in those experiments may reflect the contribution of glycogenolysis to glucose output. Other researchers (Clark et al., 1976; Looney et al., 1987) have minimized this contribution by withholding feed from donor animals for 24 h before liver removal. Rates obtained by Forsell et al. (1985) are likely to be maximal for both processes due to inclusion of dibutyryl cAMP in the basal incubation media. Hepatocyte suspensions had higher gluconeogenic
Table 2. Comparison of in vivo and in vitro rates of propionate incorporation into glucose

<table>
<thead>
<tr>
<th>Animal</th>
<th>Experimental model</th>
<th>Propionate, mM</th>
<th>Propionate to glucose(^a)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep (6 mo to 2 yr)</td>
<td>Suspensions</td>
<td>10</td>
<td>13.2</td>
<td>Ash and Pogson, 1977</td>
</tr>
<tr>
<td>Lamb (10 d)(^b)</td>
<td>Suspensions</td>
<td>10</td>
<td>4.6</td>
<td>Clark et al., 1976</td>
</tr>
<tr>
<td>Lamb (4 to 8 mo)</td>
<td>Suspensions</td>
<td>5</td>
<td>7.3</td>
<td>Looney et al., 1987</td>
</tr>
<tr>
<td>Cows, steers, calves(^b)</td>
<td>Suspensions</td>
<td>10</td>
<td>3.0</td>
<td>Forsell et al., 1985</td>
</tr>
<tr>
<td>Lactating cows(^cd)</td>
<td>In vivo(^e)</td>
<td>.36</td>
<td>4.9</td>
<td>Baird et al., 1980</td>
</tr>
<tr>
<td>Nonlactating cows(^cd)</td>
<td>In vivo</td>
<td>.35</td>
<td>4.1</td>
<td>Baird et al., 1980</td>
</tr>
<tr>
<td>Lactating cows(^e)</td>
<td>In vivo</td>
<td>.50</td>
<td>6.1</td>
<td>Reynolds et al., 1988(^a,b)</td>
</tr>
<tr>
<td>Calf (ruminating)(^b)</td>
<td>Suspensions</td>
<td>2.5</td>
<td>4.0</td>
<td>Aiello et al., 1989</td>
</tr>
<tr>
<td>Calf (7 to 14 d)(^f)</td>
<td>Suspensions</td>
<td>2.5</td>
<td>4.9</td>
<td>Present experiments</td>
</tr>
<tr>
<td>Calf (7 to 14 d)(^g)</td>
<td>Monolayers</td>
<td>2.5</td>
<td>5.0</td>
<td>Present experiments</td>
</tr>
<tr>
<td>Nonlactating sheep(^h)</td>
<td>Monolayers</td>
<td>2.0</td>
<td>2.3</td>
<td>Emmison et al., 1991</td>
</tr>
<tr>
<td>Lactating sheep(^h)</td>
<td>Monolayers</td>
<td>2.0</td>
<td>4.7</td>
<td>Emmison et al., 1991</td>
</tr>
</tbody>
</table>

\(^a\)Nanomoles of propionate converted to glucose microgram of DNA\(^{-1}\)hour\(^{-1}\).
\(^b\)Assumes 20 \(\mu\)g of DNA/mg of cell dry weight (present experiments SD = 3, n = 15).
\(^c\)Assumes lactating cow liver weight = BW \times .0165, nonlactating cow liver weight = BW \times .013 (Baldwin et al., 1980), 28.5 mg of liver dry weight/100 mg liver (Smith and Baldwin, 1975), and 20 \(\mu\)g of DNA/mg of liver dry weight.
\(^d\)Estimated BW = 450 kg.
\(^e\)Trans-organ balance.
\(^f\)Mean of suspension cultures in 1% bovine serum albumin and Dulbecco's Modified Eagle's Medium; SD = 3.7, n = 10.
\(^g\)Mean of 48-h monolayers cultured in 10% fetal bovine serum; SD = 2.6, n = 16.
\(^h\)Assumes 33.5 \(\mu\)g of protein/\(\mu\)g of DNA (Present experiments, Table 3).

activity than liver slices (Forsell et al., 1985); therefore, gluconeogenic rates obtained using monolayers are also likely to be greater than those obtained using liver slices.

Gluconeogenic rates obtained in the present experiments were similar to in vivo gluconeogenic rates (Baird et al., 1980; Reynolds et al., 1988\(^a,b\)) listed in Table 2. These calculations assume total conversion of hepatically extracted propionate into glucose and do not account for propionate loss via oxidation or other routes. In vivo rates calculated by those methods seem highest in lactating animals. One would expect a greater gluconeogenic capacity in lactating animals because whole-body glucose turnover is augmented (Baird et al., 1983).

Variation among animals for gluconeogenesis under identical in vitro conditions is large mean = 5.0, SD = 2.5, n = 16, making it difficult to detect main effects of treatments applied in vivo. However, the effects of treatments applied in vitro, as well as any possible interactions between in vivo treatments and subsequently applied in vitro treatments, can be tested with much greater precision. This precision is increased most with a large number of in vitro treatments and a correspondingly large number of total plates per animal preparation. Even in relatively small experiments involving < 100 plates, we are able to detect differences of 25% using only three animals (Table 3, Figure 4 and 5).

Gluconeogenesis from propionate in vitro is a substrate-dependent and saturable process (Looney et al., 1987). Experimentally elevating portal propionate concentrations increases hepatic glucose output and maximal gluconeogenesis from propionate (Baird et al., 1980; Peters et al., 1983). Determination of gluconeogenesis in vitro, using hepatocyte suspensions, monolayers, or other batch-type cultures necessitates the presence of substrates at concentrations 5- to 10-fold higher than physiological portal levels. In vitro rates thus obtained are likely to be artificially elevated in comparison to in vivo gluconeogenic rates.

Culture of bovine hepatocytes in monolayer for 24 h increased their rate of urea formation. Rates of urea synthesis determined using conversions given in Table 2 indicate that ureagenesis is 11.7 nmol of urea N formed/\(\mu\)g of DNA\(^{-1}\)h\(^{-1}\) for lactating cows (Reynolds et al., 1988\(^a,b\)) and is 9.9 mol of urea N

![Figure 2. Effect of duration of exposure of bovine hepatocyte monolayers to 2-[\(^{14}\)C]propionate on incorporation into [\(^{14}\)C]glucose. Incorporation of 2.5 mM [2-\(^{14}\)C]propionate into glucose (nanomoles microgram of DNA\(^{-1}\)) was determined in parallel for bovine hepatocyte monolayers subsequent to culture of cells for 24, 48, or 120 h. Values represent least squares means and standard errors.](image)
Table 3. Effect of previous media conditions on cellular components

<table>
<thead>
<tr>
<th>Cellular measure</th>
<th>BSA or FBS</th>
<th>Insulin</th>
<th>Glucose</th>
<th>Valerate</th>
<th>RMSEb</th>
<th>SEc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNA</strong>d</td>
<td>40.6</td>
<td>38.9</td>
<td>40.2</td>
<td>39.2</td>
<td>42.4</td>
<td>41.6</td>
</tr>
<tr>
<td><strong>Glycogene</strong>e</td>
<td>1,416</td>
<td>2,819*</td>
<td>2,164</td>
<td>2,070</td>
<td>3,076</td>
<td>2,572*</td>
</tr>
<tr>
<td><strong>Protein</strong>d</td>
<td>1,213</td>
<td>1,512*</td>
<td>1,377</td>
<td>1,348</td>
<td>1,527</td>
<td>1,582</td>
</tr>
<tr>
<td><strong>Gluconeogenesis</strong>f</td>
<td>2.56</td>
<td>3.52*</td>
<td>2.42</td>
<td>3.66*</td>
<td>2.85</td>
<td>2.52</td>
</tr>
</tbody>
</table>

a Unadjusted means (n = 3 animals). BSA = bovine serum albumin; FBS = fetal bovine serum.
b Root mean square error (63 df).
c Standard error of the mean associated with animal × incubation conditions.
d Micrograms/60-mm plate.
e Glucose equivalents (nanomoles/60-mm plate).
f Incorporation of 2.5 mM [2-14C]propionate into glucose (nanomoles/μgram of DNA-1-hour-1).
* Contrast differs (P < .05).

formed μg of DNA-1-h-1 in sheep hepatocyte suspensions (Ash and Pogson, 1977). Although in vivo and in vitro rates are similar, the presence of NH₄Cl in vitro may reflect maximal ureagenesis for reasons similar to those stated above.

Glucagon increased propionate incorporation into glucose in hepatocyte monolayers but had no measurable effect on suspension cultures (Figure 3). Plasma membrane receptor damage can result from protease activity found in commercial preparations of collagenase (Johnson et al., 1972). Looney et al., (1987) observed that 1,200 nM glucagon simulated gluconeogenesis (128% of controls) from 5 mM propionate in isolated lamb hepatocytes but had no effect on glucose synthesis from alanine or lactate. Dibutyryl CAMP (10 μM) alternatively stimulated gluconeogenesis from all three substrates, demonstrating the potential for increased gluconeogenic capacity and suggesting an impaired signal transduction system. Regardless of the nature or source of damage to the glucagon cascade, it seems that 45 h in monolayer culture is sufficient for restoration of these functions.

The addition of actinomycin D (800 nM) or cycloheximide (200 μM), inhibitors of transcription and translation, abolished the stimulatory effects of glucagon on gluconeogenesis from propionate (Figure 4). The effects of actinomycin D and cycloheximide during a 3-h incubation period supports a rapid effect of glucagon to mediate gene expression in bovine hepatocytes. In nonruminant species, genes for several gluconeogenic enzymes have been shown to respond to metabolites and endocrine factors (Pilkis and Claus, 1990). The activity of (GTP) phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32), a rate-limiting gluconeogenic enzyme, is transcriptionally regulated by several hormones (O'Brien and Granner, 1990) and CAMP (Lamers et al., 1982). Within 4 h, glucagon maximally induced PEPCK expression and enzyme activity in cultured rat hepatocytes (Christ et al., 1988). The effects of glucagon on gluconeogenesis in the present experiments are a summation of events occurring during the 3-h glucagon exposure period; however, it is apparent that a portion of these effects are at the level of gene expression. Regulation of genes encoding specific gluconeogenic enzymes in ruminants awaits delineation. However, the present experiments prove the utility of bovine hepatocyte monolayer cultures in those determinations.

Sheep hepatocyte monolayers retain characteristics of lactating and nonlactating donor animals after 24 h in culture and display differential responsiveness to prolonged exposure to insulin and growth hormone (Emmison et al., 1991). Culture condition effects may also influence hepatocyte metabolism (Guguen-Guillouzo and Guillouzo, 1983); however, the effects of extracellular metabolites, hormonal factors, and their

![Figure 3. Effect of glucagon on gluconeogenesis in bovine hepatocyte suspensions and monolayers. Incorporation of 2.5 mM [2-14C]propionate into glucose (nanomoles/μgram of DNA-1-hour-1) was determined in isolated hepatocytes cultured as suspensions or monolayers. Values represent least squares means and standard errors. The asterisk denotes differences (P < .05) within type of culture (n = 3). Type of culture × glucagon interaction effect (P < .05).](image-url)
Fig. 4. Effects of glucagon and actinomycin D or cycloheximide on gluconeogenesis in bovine hepatocyte monolayers. Incorporation of 2.5 mM [2-14C]propionate into glucose (nanomoles·microgram of DNA·hour⁻¹) was measured in the presence or absence of glucagon (10 nM) and the transcriptional or translational inhibitors, actinomycin D (800 nM) or cycloheximide (200 μM). Glucagon (10 nM) main effect [P < .01], inhibitor effect [P = .06], and glucagon × inhibitor effect [P = .08] [n = 4; one nonruminating, three ruminating animals]. The asterisk denotes differences [P < .05] between inhibitors and control within glucagon or no addition. The dagger denotes differences [P < .05] between glucagon addition within inhibitor or control. Values represent least squares means and standard errors.

Metabolism in bovine hepatocyte monolayers depends on the previous extracellular environment of the hepatocytes (Table 3). Only main effects of BSA, FBS, glucose, and long-term insulin exposure are reported because there were no interactions among their combinations. Replacing FBS with BSA decreased (P < .05) glycogen and protein per plate and the rate of propionate incorporation into glucose (Table 3). Removal of FBS from media did not affect cell survival, but cellular anabolic activities were compromised. Inclusion of FBS provides a myriad of potentially interactive nutrients, hormones, and growth factors, which may serve to slow the loss of metabolic capacity observed during extended culture of bovine hepatocyte monolayers. Insulin alone did not replace components of FBS responsible for higher cellular glycogen and protein because interactions between insulin and FBS or BSA were not significant. Insulin did, however, decrease (P < .05) gluconeogenic activity when it was included in the culture media from 4 to 45 h after plating. Increasing glycogen storage through media glucose addition did not result in a change in the rate of propionate incorporation into glucose (P = .34). These data indicate that bovine hepatocytes can be successfully maintained in serum-free media. However, rates of gluconeogenesis are lower than those obtained in serum-supplemented media.

Valerate metabolism results in production of both acetate and propionate; therefore, it may serve as both an energy or anaplerotic carbon source. Exposure of bovine hepatocytes to valerate (1.25 mM) for the 41-h period (Table 3) was without effect on propionate incorporation into glucose and suggests the lack of substrate induction of gluconeogenesis from propionyl coenzyme A.

Hepatocytes exposed to FBS plus 1,000 nM insulin for long periods (41 h) had decreased rates of gluconeogenesis (2.80 for FBS plus insulin vs 4.25 for FBS controls, P < .01). Cells maintained in a serum-free environment containing 1,000 nM insulin showed a similar decrease compared with BSA controls (2.05 vs 3.08; P < .05). The continued exposure of cells to a high concentration of insulin (1,000 nM) may partially explain the loss of gluconeogenic capacity of cells maintained over longer culture times (Table 1). Previous insulin exposure did not impair the ability of glucagon to stimulate gluconeogenesis from propionate (Fig. 5) because no long-term insulin exposure × short-term glucagon treatment interactions were detected (P = .55).

Fig. 5. Effect of previous culture conditions on subsequent response to glucagon exposure in bovine hepatocyte monolayers. Hepatocytes were incubated in basal culture medium supplemented with the additions indicated within the figure (BSA = bovine serum albumin; FBS = fetal bovine serum). Cells were removed from the treatments and incorporation of 2.5 mM [2-14C]propionate into glucose (nanomoles·microgram of DNA·hour⁻¹) was measured during the subsequent 3-h period. Values represent least squares means and standard errors. The asterisk denotes differences within previous incubation media [n = 3].
The bovine hepatocyte monolayer system fills a niche in the study of ruminant hepatic metabolism but does not supplant in vivo or other in vitro techniques, each of which has unique advantages. Hepatocyte monolayer cultures provide a system that permits the relatively inexpensive study of several treatment combinations simultaneously in cells obtained from a single animal. The use of hepatocyte monolayers as presented here, like other in vitro techniques, is not without certain disadvantages. The technique has not been adapted for cells isolated from mature cattle for reasons related to procurement of viable tissue. The culture period of the bovine hepatocytes used in the present experiments is limited to 48 h before hepatocyte-specific functions are diminished. Similarly, cultured rat hepatocytes show phenotypic differentiation and reversion to fetal-like state (Bissell and Guzelian, 1981; Reid and Jefferson, 1984). Despite these drawbacks, hepatocyte monolayers obtained from other species have been effectively used to study long-term hormonal, nutritional, and developmental regulation of gene expression (Guguen-Guillouzo and Guillouzo, 1983).

Implications

Bovine hepatocyte monolayers provide an efficient model for the study of direct hormonal regulation of liver metabolism. The system of hepatocyte culture offers advantages over other systems used to study certain aspects of regulation of bovine hepatic metabolism. The system should be useful for the study of hepatic enzyme induction and(or) liver-specific gene expression in ruminants.

Literature Cited


