Metabolism of $^{[14]C}$glucose by postimplantation mouse embryos in vitro

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SUMMARY

Carbon dioxide and lactate production from $^{[14]C}$glucose were measured for postimplantation mouse embryos aged 6 to 9½ days post coitum in static cultures with a defined medium. The rate of metabolism increased rapidly and paralleled the increase in protein content indicating a fairly uniform rate of metabolism throughout the period. At all stages studied more than 90% of the glucose utilized was converted to lactate. Over a quarter of carbon dioxide produced was derived from the C-1 position resulting in high C-1: C-6 ratios, indicating that the Pentose Phosphate Shunt is a major oxidative pathway.

The influence of various culture condition on CO$_2$ production showed that high concentrations of glucose did not affect glucose utilization whilst high lactate concentrations had a significant inhibitory effect. Pyruvate had no discernible effect.

INTRODUCTION

Despite much recent progress in the culture of postimplantation rodent embryos (Hsu, 1979; New, 1978; Sadler, 1979; Tam & Snow, 1980) little is known of the biochemistry underlying the complex requirements of these embryos. Certainly glucose is the major energy source for rat embryos cultured for 2 days from the early head-fold to the 25-somite stage (Cockroft, 1979). A variety of experiments (Cox & Gunberg, 1972a,b; Shepard, Tanimura & Rabkin, 1970; Spielmann, Meyer-Wendecker & Spielmann, 1973; Tanimura & Shepard, 1970) suggest that this utilization of glucose occurs principally via the glycolytic pathway with some contribution from the pentose phosphate route.

No comparable information exists for mammalian embryos between implantation and early somitogenesis. In this paper we describe the metabolism of glucose by early postimplantation mouse embryos cultured in a defined medium in vitro. These embryos, in contrast to preimplantation embryos, produce large amounts of lactate even under aerobic conditions.

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MATERIALS AND METHODS

Preparation of media

The media were based on modified Krebs-Ringer solutions used for the collection and culture of preimplantation mouse embryos. To maintain a constant pH in air (7.2 to 7.4) during the isolation of postimplantation embryos the mouse embryo culture medium M16 (Whittingham, 1971) was buffered with HEPES. This medium designated M2 consisted of 94.59 mM-NaCl, 4.78 mM-KCl, 1.19 mM-KH2PO4, 1.19 mM-MgSO4, 1.71 mM-CaCl2, 4.0 mM-NaHCO3, 21 mM-HEPES (Ultrol grade, Calbiochem), 23.28 mM-Na lactate, (0.33 mM-Na pyruvate and 5.56 mM-glucose supplemented with 60 µg/ml penicillin G (sodium salt), 50 µg streptomycin sulphate and 10 mg/ml crystalline bovine serum albumin (Miles Serovac Ltd.). M16 is identical to M2 except for replacement of HEPES with 21 mM-NaHCO3. The higher levels of BSA for postimplantation culture necessitated final adjustment of the pH of both media to pH 7.2–7.4 with 1 M-NaOH. All media were prepared from concentrated stock solutions immediately prior to use. When substrates such as lactate were omitted from the medium, additional NaCl was added to maintain the osmolarity of the medium at about 290 mosmolar.

For the preparation of media containing radioactively-labelled glucose ([U-14C], [1-14C] or [6-14C] glucose, (Amersham Corp., Amersham, UK)), labelled and unlabelled glucose dissolved in 2× glass distilled water were added to 1.25× concentrated M16 to yield a final concentration of 5.56 mM-glucose with a specific activity of 1 mCi/mmol.

All experimental values were at least twice the background level of radioactivity (100 c.p.m.), to achieve this higher specific activities were used in some experiments (3 or 4 mCi/mmol).

Embryo collection

Decidua containing embryos aged 6 to 9½ days p.c. were dissected from naturally mated random-bred MF1 females (Olac, Bicester, Oxon) into M2. The decidua were cut to reveal the conceptus which was gently removed with watchmakers forceps and then transferred to fresh medium with a mouth-operated 2 or 10 µl capillary tube (Drummond). Reichert’s membrane and the ectoplacental cone were removed with electrolytically-sharpened tungsten needles for stages up to 8 days. All the extraembryonic membranes except the amnion were removed from later stages with watchmakers forceps.

Carbon dioxide production

The method used for determining carbon dioxide production was modified from that described by Brinster (1967). Embryos were washed once in M2 followed by a final wash in M16 containing radiolabelled glucose. Single embryos
were transferred to 100 or 200 µl drops of the same medium under paraffin oil
equilibrated with 5 % CO₂ in air. Each culture was carried out in a 5 ml plastic
culture tube (Falcon Plastics) cut to fit a scintillation vial with the tube cap acting
as a support (Fig. 1). Vials were gassed with 5 % CO₂ in air for 30 s and sealed
with a Suba-Seal cap which allowed the desired pH of 7·2–7·4 to be maintained
during the 3 h culture period at 37 °C. Control culture drops without embryos
were run in parallel for each group of embryos.

Incubations were terminated by injecting 1 ml of citrate-phosphate buffer
pH 4·0 directly through the Suba-Seal into the culture tube and 1 ml of hyamine
hydroxide (BDH, Poole, Dorset) on to the floor of the vial. After leaving over-
night at room temperature, to allow the hyamine to absorb the CO₂, the culture
tubes were removed and 10 ml of a xylene-based scintillation cocktail (Scintillant
299, Packard) was added to each vial. Samples were counted in a Packard Tricarb
3771 scintillation counter on the [¹⁴C] setting at an efficiency of about 75 %.

**Lactate production**

After collection and a final wash in radioactive medium, embryos were
cultured individually in small volumes of M16 (10–50 µl) in microtest well plates
(Falcon) usually for 1 h. At the end of the culture period a 5 or 10 µl aliquot of
medium was taken and chromatographed on Whatman 4 paper using butanol:
water: acetic acid (12:5:3). Dried chromatograms were cut into strips and
counted at an efficiency of 45 % in Scintillant 299. In some experiments samples
were further chromatographed in ethanol: ammonia: water (16:1:3) and

![Fig. 1. Scintillation vial method for CO₂ release. (A) shows culture tube with
medium containing radiolabel overlaid with oil, and (B) shows vial after termination
of the culture with a CO₂-releasing buffer. See text for details.](image)

**Mouse embryo glucose metabolism**
autoradiographed using Kodak X-ray film (RP-Royal X-Omat). Lactate was identified by its $R_F$ value in the two solvents and by its comigration with cold lactate identified by the silver nitrate–bromphenol blue test of Smith (1958).

**Protein measurement**

Protein content of embryos was measured with the technique of Bramhall, Noack, Wu and Loewenberg (1969). Embryos were spotted on to discs of Whatman No. 1 paper, washed extensively in 7.5% trichloroacetic acid, dried and stained in 10 mg/ml xylene brilliant cyanin G (Hopkins & Williams) in 10% acetic acid. Filters were thoroughly destained in several washes of 10% acetic acid at 56°C and then blotted. Dye bound to protein was eluted with 1 ml of methanol: water: ammonia (66:33:1) and the absorbance measured spectrophotometrically at 610 nm.

**Analysis of data**

Each value represents the mean of three or more replicates with at least three embryos per replicate. Most experiments were tested statistically using single- and two-factor analysis of variance usually on logarithmically-transformed data.

**RESULTS**

The medium chosen for these experiments (M16) is used for the culture of preimplantation stages in the mouse. It contains three energy substrates, glucose, lactate and pyruvate. Initial experiments tested the effects of varying these components on CO$_2$ production from [U-$^{14}$C] glucose by 7½-day embryos. Two series of experiments were performed. The first using complete M16 with varying concentrations of glucose showed that increasing the glucose fourfold to 22.24 mM did not alter the rate of CO$_2$ production (Table 1). The effect of the other two components was also tested (Table 2). This revealed that the high lactate concentration caused a significant reduction in the metabolism of radio-labeled glucose to carbon dioxide but pyruvate had no discernible effect. There was no significant interaction between the pyruvate and lactate ($F_{6,24} = 0.8$).

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>pmol CO$_2$/embryo/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.56</td>
<td>864 ± 66* †</td>
</tr>
<tr>
<td>11.12</td>
<td>894 ± 56-7†</td>
</tr>
<tr>
<td>22.4</td>
<td>996 ± 33-9†</td>
</tr>
</tbody>
</table>

* All mean values given ± s.e.m.
† Not significantly different $F_{2,18} = 0.45$
Table 2. The effect of lactate (24 μM) and pyruvate (0.3 μM) on CO₂ production from [U-14C] glucose by 7½-day embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pmol CO₂/embryo/h</th>
<th>pmol CO₂/embryo/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>M16 + lactate</td>
<td>1362 ± 80*</td>
<td>1104 ± 49†</td>
</tr>
<tr>
<td>+ pyruvate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>− pyruvate</td>
<td>2118 ± 109‡</td>
<td>2004 ± 201‡</td>
</tr>
<tr>
<td>M16 − lactate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ pyruvate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>− pyruvate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*All mean values given ± S.E.M.
†, ‡ Treatments with the same superscript are not significantly different. Effect of lactate is significant (F₂,₂₄ = 20.3; P < 0.001).

Table 3. Carbon dioxide and lactate production from [U-14C] glucose by post-implantation embryos in M16 without lactate

<table>
<thead>
<tr>
<th>Age (p.c.)</th>
<th>pmol CO₂/embryo/h</th>
<th>nmol lactate/embryo/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>egg cylinder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 days</td>
<td>61.2 ± 17.5*</td>
<td>n.d.†</td>
</tr>
<tr>
<td>6½ days</td>
<td>143.1 ± 5.9</td>
<td>2.18 ± 0.17</td>
</tr>
<tr>
<td>7 days</td>
<td>388.0 ± 50.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>7½ days</td>
<td>2075.0 ± 89.9</td>
<td>28.86 ± 1.22</td>
</tr>
<tr>
<td>8 days</td>
<td>2772.0 ± 189</td>
<td>n.d.</td>
</tr>
<tr>
<td>embryo + amnion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8½ days</td>
<td>2786.0 ± 201.9</td>
<td>69.33 ± 0.99</td>
</tr>
<tr>
<td>9½ days</td>
<td>9378.0 ± 536</td>
<td>96.60 ± 4.37</td>
</tr>
</tbody>
</table>

* All values given ± S.E.M.
† Not determined.

For subsequent measurements M16 without lactate was used on the assumption that this gave more favourable conditions to the embryo.

Quantitative changes in carbon dioxide production for stages from the pro-amniotic egg cylinder (6 days) through to 22 somites (9½ days) are shown together with lactate production in Table 3. These same data are shown in Table 5 normalized to protein content. Figure 2 shows the normalized data for CO₂ together with similar data on preimplantation embryos (Brinster, 1967) for comparison. Unlike preimplantation embryos, where conversion of glucose to CO₂ increases exponentially from fertilization to the blastocyst without any increase in protein content, the postimplantation stages produce similar amounts of CO₂ when output is normalized to protein content (Table 5 and Fig. 2).

Lactate production shows the same type of pattern as CO₂ output over the stages studied but represents a much greater utilization of glucose (between 20
Fig. 2. CO$_2$ production (pmol/µg protein/h) from [U-$^{14}$C] glucose by mouse embryos. ○ Data for preimplantation embryos from Brinster (1967). ● Measurements on whole egg cylinder, or ▲ embryo with amnion only.

Fig. 3. Diagram of autoradiograph of two dimension chromatogram of [U-$^{14}$C] glucose-labelled M16 after 3 h culture of an 8½-day embryo. First dimension run with butanol: water: acetic acid (BuAc), second with ethanol: ammonia (EtAm). G, remaining glucose; L, lactate; P, pyruvate.
Table 4. Carbon dioxide production (pmol/embryo/h) from specifically labelled glucose by postimplantation embryos.

<table>
<thead>
<tr>
<th>Age (p.c.)</th>
<th>[1(^{-14})C] glucose</th>
<th>[6(^{-14})C] glucose</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>M16 - lactate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6(\frac{1}{2}) days</td>
<td>79.8 ± 12.7*</td>
<td>21.1 ± 4.4</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>7(\frac{1}{2}) days</td>
<td>536.5 ± 25.0</td>
<td>28.4 ± 5.7</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>8(\frac{1}{2}) days</td>
<td>1283 ± 95.0</td>
<td>51.2 ± 9.0</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>M16 - lactate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7(\frac{1}{2}) days</td>
<td>566.0 ± 72.0</td>
<td>12.3 ± 4.3</td>
<td>45 ± 9</td>
</tr>
</tbody>
</table>

* All mean values given ± S.E.M.

Table 5. Percentage of glucose catabolised to lactate and CO\(_2\), and lactate and CO\(_2\) production normalized to embryo protein content.

<table>
<thead>
<tr>
<th>Age (p.c.)</th>
<th>% glucose as:</th>
<th>Catabolite output (pmol/(\mu)g protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO(_2)</td>
<td>Lactate</td>
</tr>
<tr>
<td>6(\frac{1}{2}) days*</td>
<td>2.0</td>
<td>97.8</td>
</tr>
<tr>
<td>7(\frac{1}{2}) days*</td>
<td>6.7</td>
<td>93.3</td>
</tr>
<tr>
<td>8(\frac{1}{2}) days†</td>
<td>3.9</td>
<td>96.1</td>
</tr>
<tr>
<td>9(\frac{1}{2}) days†</td>
<td>8.85</td>
<td>91.15</td>
</tr>
</tbody>
</table>

* egg cylinder
† embryo and amnion

and 30 times the amount metabolized to CO\(_2\). The metabolites excreted into the culture medium by 8\(\frac{1}{2}\)-day embryos were analysed by two-dimensional chromatography. Only two spots apart from unused glucose were observed on autoradiographs (Fig. 3). The larger corresponds to lactate as judged by RF values whilst the minor spot is most likely to be pyruvate by the same criteria. This latter spot contains a hundredfold-less label than the lactate, so pyruvate appears to be a very minor excretory product. The data suggest that the main catabolic utilization of glucose is via glycolysis which can occur under aerobic conditions.

Further analysis of the pattern of metabolism was performed using specifically labelled glucose as energy substrates. C-1: C-6 ratios are high for the three stages studied (Table 4) especially so for 7\(\frac{1}{2}\)- and 8\(\frac{1}{2}\)-day embryos. For these latter stages the oxidation of the first carbon accounts for about 30% of total CO\(_2\) production, whereas the C-6 position only accounts for 1–2% of the total. In the presence of a high concentration of lactate, which reduces total CO\(_2\) output from uniformly labelled glucose by 40% (Table 2), oxidation of the C-1 position is not significantly altered but C-6 oxidation is halved.
DISCUSSION

Whilst the improvement of culture techniques for postimplantation embryos over the past 10 years has been considerable, further advances are needed before in vitro techniques can be widely applied in such areas as teratology, developmental biochemistry and manipulative embryology. Present methods have been essentially derived using empirical methodology and thus little understanding exists of the roles that various media components have in growth and differentiation of the embryo. Two approaches are available for the elucidation of this problem. One, already initiated (Hsu, 1980), is to fractionate the serum components of the culture medium thus attempting to isolate factors essential for the embryo's well being. The alternative is to study explanted embryos in simple culture systems, which can subsequently be made more elaborate. Although such media will not support normal development for long periods, they do not have pathological effects as embryos can be kept in them for several hours without significantly altering their ability to develop normally in complex media. For short periods, then, simple media can be used to acquire useful information, related to the embryo's in vivo status and its potential needs in vitro. We have adopted this latter modus operandi.

Under our experimental conditions the early stages of postimplantation development in the mouse show a considerable aerobic glycolysis. At the stages examined (i.e. 6½–9½ days p.c.) more than 90% of glucose catabolized is converted to lactate (Table 5). The embryos were cultured in 20% oxygen, which favours oxidative pathways, yet only a small amount of glucose was oxidised. Embryos cultured at lower oxygen tensions produce even less CO$_2$ from glucose: i.e. a Pasteur effect is operating (unpublished observations). These characteristics suggest that the embryos are committed to glycolysis even when conditions favour the more efficient pathways of energy metabolism.

Lactate reduces $^{14}$CO$_2$ output (Table 2), an effect which could be attributed either to lactate reducing the specific activity of the pyruvate pool by dilution, or to an effect on mass action ratios such that the actual rate of metabolism is reduced. If the latter case holds, the large amounts of lactate produced by the embryo would accumulate and inhibit metabolism. This could be a limiting factor in present culture systems; with the rate of lactate production measured, 8½-day embryos cultured as described by Tam & Snow (1980) would make their medium more than 5 mM with respect to lactate within 24 h. Higher concentrations of glucose do not overcome this effect (Table 1) or increase metabolic rate in the absence of lactate (data not shown).

Minor alterations in the pattern of energy metabolism also occur; for instance 7½- and 9½-day embryos produce more CO$_2$ relative to lactate than do other stages (Table 5). Indeed for the latter stage about half of the ATP equivalents generated from glucose catabolism could be derived from oxidative pathways. It is however impossible to tell whether these properties are indicative of any real
biological change. More informative are the data obtained from metabolism of specifically-labelled glucose (Table 4). The first and sixth carbons of glucose (designated C-1 and C-6) are metabolized identically by the Krebs' cycle but C-1 is preferentially oxidized by the Pentose Phosphate Shunt. Thus the ratio of labelled CO$_2$ produced from these carbon atoms is indicative of the relative activities of these two pathways. The very high C-1: C-6 ratios observed suggest that the Pentose Phosphate Shunt is very active compared with the Krebs' cycle at these stages, when both cell number (Snow, 1977) and protein content are increasing rapidly. Interestingly a correlation between rapid cell proliferation in embryonic tissues and elevated Pentose Phosphate Shunt activity has long been known (Papaconstantinou, 1967).

Although once considered by Warburg to be an irreversible response to adverse conditions (Krebs, 1981) aerobic glycolysis occurs in several tissues in vivo (e.g. retina, gut mucosa). Whether the postimplantation embryo can be added to this group is open to question as availability of oxygen to the embryo and surrounding decidual tissue is unknown. Certainly the inability of these embryos to oxidize glucose fully under aerobic conditions implies that glycolysis is the predominant pathway in vivo. If the embryo's environment is low in oxygen or anoxic then the embryo's adoption of an energetically inefficient metabolism during a period of very rapid growth and cell division is less perplexing. However a decidual vasculature inadequate for oxygenation would also be unable to supply sufficient nutrients to the embryo although decidual cells may be capable of providing such needs during the early postimplantation period. Alternatively, if oxygen is available another, more intriguing, possibility is that a fully functional Krebs' cycle might be incompatible with developmental processes during this period of embryogenesis. Morriss & New (1979), for instance, have shown that failure of neural tube closure in rat embryos cultured at high oxygen concentrations is associated with a mitochondrial morphology typical of active oxidative phosphorylation.

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REFERENCES


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