SHIFTS IN ATP SYNTHESIS DURING PREIMPLANTATION STAGES OF MOUSE EMBRYOS

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(Received 6th August 1974)

Summary. The actual and potential activities of the cytochrome system were studied in cleavage-stage mouse embryos. Activities were determined by assaying embryos for total ATP and the rates of \([^{32}P]ATP\) synthesis both before and after their incubation in medium supplemented either with an energy coupling site inhibitor (antimycin, amytal or cyanide) or with the FADH-linked substrate, succinate. The data indicate that there are three major shifts in the mode of ATP production during preimplantation stages: the first, between the two-cell and late four-cell stages; the second, between the eight-cell and late morula stages; and the third, between the late morula and late blastocyst stages. These data are discussed in relation to studies on the energy metabolism of cleavage and blastocyst stage mouse embryos.

INTRODUCTION

Mitochondrial configurational changes have been described during the cleavage stages of mouse embryos (Hillman & Tasca, 1969; Stern, Biggers & Anderson, 1971; Pikó & Chase, 1973). These changes occur between the two-cell and late four- to early eight-cell stages, the late eight-cell and late morula stages, and the late morula and late blastocyst stages of development (Hillman & Tasca, 1969; Nadijcka & Hillman, 1975). Significant differences in the levels of ATP synthesis are also found between these same preimplantation stages (Ginsberg & Hillman, 1973). The data suggest that an altered concentration or availability of energy coupling sites at these specific times might account for the observed differences in the levels of ATP synthesis.

This study was undertaken to determine the comparative activity of the three mitochondrial energy-coupling sites during cleavage and blastocyst stages. Comparative activity can be established by using site-specific inhibitors and by assaying the effect of these inhibitors on the levels of total ATP and ATP synthesis both within and between specific developmental stages. The inhibitors used in the present study were amytal (Site I), antimycin (Site II) and cyanide (Site III). In addition, a series of studies were undertaken to compare the actual and potential aerobic rates of ATP synthesis by preimplantation embryos.

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Obtaining embryos

Random-bred Swiss albino female mice were induced to superovulate by an intraperitoneal injection of 10 i.u. PMSG (Gestyl: Organon, West Orange, New Jersey), followed 48 hr later by an intraperitoneal injection of 10 i.u. HCG (Pregnyl: Organon). After the second injection, single females were placed overnight with single males. The presence of copulation plugs was checked the following morning and, if present, two-cell embryos were flushed from the oviducts 24 hr later. The embryos were either treated and assayed immediately or were cultured in Brinster's medium (BMOC-3, 1970 modification; Grand Island Biological Co., Grand Island, New York) until they reached the desired cleavage stage (four-cell, 10 to 14 hr; eight-cell, 24 hr; early morula, 36 hr; late morula, 45 hr; late blastocyst, 60 hr). The culture medium contained 20 mM-lactate and 0.56 mM-pyruvate. The blastocysts used in these experiments were sub-stage 3 blastocysts (Nadijcka & Hillman, 1975).

Inhibitor studies

The culture medium was supplemented with amytal (10\(^{-3}\) M, 10\(^{-4}\) M) (Sigma, St Louis, Missouri), antimycin (10\(^{-6}\) M, 5 \times 10\(^{-7}\) M, 10\(^{-7}\) M) (Sigma), or cyanide (10\(^{-4}\) M) (J. T. Baker, Phillipsburg, New Jersey). The criteria used for deciding the optimum concentration of amytal (10\(^{-3}\) M), antimycin (5 \times 10\(^{-7}\) M, 10\(^{-7}\) M), or cyanide (10\(^{-4}\) M) were (1) that the inhibitor gave a significant decrease in total ATP after a short treatment for 10 min, and (2) that the embryos continued to develop to the blastocyst stage after treatment for 1 hr followed by reincubation in standard medium. The second criterion was not used for antimycin since its inhibitory effect is pseudoreversible (Slater, 1973).

Twenty-five to thirty embryos at various cleavage stages were cultured (Brinster, 1963) for 10 min in medium containing the specific inhibitor. Similar numbers of control embryos at corresponding stages were incubated for the same length of time in non-supplemented medium. At the end of the treatment, both the experimental and control embryos were immediately collected and the total ATP of each sample was assayed by a modified luciferin–luciferase method (Ginsberg & Hillman, 1973).

Gross ATP synthesis (Ginsberg & Hillman, 1973) was determined in control and experimental embryos after incubation of embryos at known cleavage stages for 1 hr in medium containing either \(^{32}\)PO\(_4\) alone or in combination with a specific inhibitor. These experiments involved two-cell, eight-cell, and early morula stage embryos. The \(^{32}\)P-labelled \(\text{H}_2\text{PO}_4\) (HCl-free) was obtained from International Chemical and Nuclear Corp., Waltham, Massachusetts (sp. act., 285 Ci/mg) and diluted to 150,000 ct/min/10 \(\mu\)l medium before use (Ginsberg & Hillman, 1973). Each inhibitor experiment was performed at least four times at each specified developmental stage.

Metabolism of ATP with supplemented succinate

Brinster's medium received additions of 10 mM-succinate; the pH of the medium was adjusted to 6.8 and the osmolarity was corrected by decreasing the concentration of sodium chloride (Biggers & Brinster, 1965; Brinster, 1965).
Thirty to fifty embryos were incubated for 1 hr at the two-cell, eight-cell, late morula and late blastocyst stages in medium supplemented with succinate alone or with succinate and $^{32}$PO$_4$. Total ATP and rates of $[^{32}P]$ATP synthesis were then measured.

Similar numbers of control embryos were cultured in unsupplemented medium (or in medium containing only $^{32}$PO$_4$) for 1 hr, and their total ATP or gross synthetic rates were compared with those of the experimental embryos. The ATP/ADP ratios of the embryos developing in supplemented medium were also compared with those of control embryos (Ginsberg & Hillman, 1973). The succinate experiments were performed at least four times at each stage tested.

**Statistical studies**

Standard errors of the mean are included in the tables. Significant differences between the means ($P$<0.05) were determined by Student’s $t$ test.

**RESULTS**

**Amytal; Site I**

After treatment for 10 min, amythal (optimal concentration $10^{-3}$ M) reduced the total ATP by 13% at the two-cell stage, by 46% at the late four-cell stage, by 70% at the eight-cell stage and by 70% at the early morula stage. The same concentration caused a reduction of only 38% of the total ATP at the late morula/early blastocyst stage and of 44% at the late blastocyst stage (Table 1).

**Table 1.** The effect of three energy-coupling site-specific inhibitors on the total ATP metabolized by mouse embryos at different preimplantation stages

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Control</th>
<th>Amytal (Site I) $10^{-3}$ M</th>
<th>Antimycin (Site II) $5 \times 10^{-7}$ M</th>
<th>Antimycin (Site II) $10^{-7}$ M</th>
<th>Antimycin (Site II) $10^{-8}$ M</th>
<th>Cyanide (Site III) $10^{-4}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-cell</td>
<td>1.47 ± 0.07</td>
<td>1.28 ± 0.01</td>
<td>0.36 ± 0.01</td>
<td>1.19 ± 0.03</td>
<td>1.37 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Late four-cell</td>
<td>0.99 ± 0.01</td>
<td>0.53 ± 0.04</td>
<td>0.39 ± 0.03</td>
<td>1.03 ± 0.03</td>
<td>0.59 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Late eight-cell</td>
<td>0.91 ± 0.01</td>
<td>0.65 ± 0.01</td>
<td>0.57 ± 0.01</td>
<td>—</td>
<td>0.42 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Early morula</td>
<td>0.65 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>0.27 ± 0.01</td>
<td>0.67 ± 0.04</td>
<td>0.32 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Late morula</td>
<td>0.43 ± 0.01</td>
<td>0.24 ± 0.03</td>
<td>0.27 ± 0.01</td>
<td>—</td>
<td>0.32 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Late blastocyst</td>
<td>0.43 ± 0.01</td>
<td>0.24 ± 0.03</td>
<td>0.27 ± 0.01</td>
<td>—</td>
<td>0.32 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as Means ± S.E. in pmol ATP remaining/embryo after a 10-min treatment.

The gross rate of $[^{32}P]$ATP synthesis was reduced by 23% at the two-cell stage, by 56% at the eight-cell stage, and by 78% at the early morula stage (Table 2).

**Antimycin; Site II**

At the two-cell stage, treatment for 10 min with $5 \times 10^{-7}$ m-antimycin produced a 76% loss of total ATP. The same concentration produced a 61% loss at the late four-cell stage, a 69% loss at the eight-cell stage, a 63% reduction at the early morula stage, a 12% loss at the late morula stage and a 37% loss at the late blastocyst stage (Table 1). Concentrations of $5 \times 10^{-7}$ m-antimycin caused a 64% decrease in gross $[^{32}P]$ATP synthesis at the two-cell stage, a 63%
reduction at the eight-cell stage, and a 59% decrease at the early morula stage (Table 2).

Although a concentration of $10^{-7}$ M-antimycin caused a 19% reduction in total ATP at the two-cell stage, it produced a 22% gain in total ATP at the eight-cell stage and had no significant effect at either the late four-cell or early morula stages (Table 1).

**Table 2.** The effect of three energy-coupling site-specific inhibitors on gross $[^{32}\text{P}]$ATP synthesis by mouse embryos at different preimplantation stages

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Control</th>
<th>Site I Amytal (10$^{-3}$ M)</th>
<th>Site II Antimycin (5 x 10$^{-7}$ M)</th>
<th>Site III Cyanide (10$^{-4}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-cell</td>
<td>0.22 ± 0.01</td>
<td>0.17 ± 0.01 (23%)</td>
<td>0.08 ± 0.01 (64%)</td>
<td>0.17 ± 0.01 (23%)</td>
</tr>
<tr>
<td>Eight-cell</td>
<td>0.63 ± 0.06</td>
<td>0.28 ± 0.05 (56%)</td>
<td>0.23 ± 0.03 (63%)</td>
<td>0.18 ± 0.01 (71%)</td>
</tr>
<tr>
<td>Early morula</td>
<td>0.27 ± 0.01</td>
<td>0.06 ± 0.02 (78%)</td>
<td>0.11 ± 0.02 (59%)</td>
<td>0.08 ± 0.02 (70%)</td>
</tr>
</tbody>
</table>

Values are expressed as Means ± S.E. in pmol $[^{32}\text{P}]$ATP/embryo/hr.

**Table 3.** Metabolism of ATP by mouse embryos in Brinster's medium supplemented with 10 mM-succinate

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Control</th>
<th>Succinate</th>
<th>ATP/ADP ratio</th>
<th>Gross $[^{32}\text{P}]$ATP synthesis†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>Succinate</td>
</tr>
<tr>
<td>Two-cell</td>
<td>1.47 ± 0.07</td>
<td>1.75 ± 0.05</td>
<td>10.2 ± 0.43</td>
<td>2.20 ± 0.15</td>
</tr>
<tr>
<td>Early eight-cell</td>
<td>0.91 ± 0.01</td>
<td>0.87 ± 0.01</td>
<td>2.48 ± 0.14</td>
<td>2.40 ± 0.06</td>
</tr>
<tr>
<td>Late morula</td>
<td>0.65 ± 0.01</td>
<td>0.35 ± 0.05</td>
<td>1.80 ± 0.10</td>
<td>0.56 ± 0.01</td>
</tr>
<tr>
<td>Late blastocyst</td>
<td>0.43 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>1.33 ± 0.06</td>
<td>1.30 ± 0.03</td>
</tr>
</tbody>
</table>

Values expressed as Means ± S.E.

* pmol ATP/embryo.
† pmol $[^{32}\text{P}]$ATP/embryo/hr.

**Cyanide; Site III**

The effects of incubation in $10^{-4}$ M-cyanide, which were reported earlier (Ginsberg & Hillman, 1973), were extended so that a comparison could be made between this inhibitor and the others used in the present study. Our present data showed a 56% decrease in total ATP at the early morula stage and a 34% loss at the late morula stage following a 10-min treatment. The other stages were as reported previously (two-cell, 7%; four-cell, 40%; eight-cell, 42%; late blastocyst, 26%). The levelling off of these decreases occurred at the late morula stage (Table 1). The effect of a 60-min incubation in $^{32}\text{PO}_4$ medium containing $10^{-4}$ M-cyanide on gross ATP synthesis was also repeated. There was a 23% loss in gross $[^{32}\text{P}]$ATP synthesis at the two-cell stage, 71% at the eight-cell and 70% at the early morula stage (Table 2). The effects of cyanide on both total ATP and on the gross synthesis of $[^{32}\text{P}]$ATP closely paralleled those of amyntal.
The effect of succinate on ATP metabolism

After incubation for 1 hr in medium supplemented with succinate, there was a 20% increase in total ATP at the two-cell stage, which was greater than a fourfold decrease in the ATP/ADP ratio and a twofold increase in the rate of gross [³²P]ATP synthesis (Table 3). At the eight-cell stage, this same concentration of succinate had no significant effect on the total ATP level, ATP/ADP ratio, or the gross rate of [³²P]ATP synthesis. At the late morula stage, succinate had an inhibitory effect on [³²P]ATP synthesis, and caused a reduction in the ATP/ADP ratio and the total ATP level. At the late blastocyst stage, the addition of succinate had no significant effect on total ATP, the ATP/ADP ratio or the rate of [³²P]ATP synthesis (Table 3).

DISCUSSION

The data indicate that there are three major shifts in ATP metabolism during the cleavage and blastocyst stages of development in the mouse. The first shift, between the two-cell and late four- to early eight-cell stages, is accompanied by a sharp increase in ATP metabolism (Ginsberg & Hillman, 1973). This increase could be caused by changes in the concentration or availability of cytochromes, by changes in the activities of glycolytic or TCA cycle enzymes, or a combination of the two. There is evidence to support both of these possibilities.

None of the three coupling site-specific inhibitors (amytal, cyanide or 10⁻⁷ m-antimycin) produces more than a 20% inhibition of total ATP at the two-cell stage. Beginning at the late four-cell stage and continuing into the eight-cell stage, the inhibitory effect of amytal and cyanide on total ATP increases two- to threefold. However, 10⁻⁷ m-antimycin results in a stimulation of ATP production by eight-cell embryos, indicating that this concentration is too low (Ahmad, Schneider & Strong, 1950). At the eight-cell stage, a fivefold greater concentration of antimycin is required to give results comparable to those of the other two inhibitors.

The inhibitory concentration of antimycin, unlike that of cyanide or amytal, is proportional to the availability or concentration of the cytochromes (Slater, 1973). The higher inhibitory concentration of antimycin required at the eight-cell stage indicates, therefore, that either the concentration or availability of the cytochromes increases between the two-cell and eight-cell stages, since such an increase could effect the increased rate of oxidative phosphorylation which begins at the late four-cell/early eight-cell stages (Ginsberg & Hillman, 1973).

Alternatively, the higher rate of ATP metabolism observed at the late four-cell stage could result from increased rates of TCA cycle activity. The high ATP/ADP ratio of embryos at the two-cell stage (Ginsberg & Hillman, 1973) would reduce the activity of TCA cycle enzymes, notably isocitrate dehydrogenase (IDH), thereby decreasing the rate of oxidative phosphorylation (Atkinson, 1965). Although there are data which indicate that IDH is functional at the two-cell stage (Wales & Whittingham, 1973), the levels of aerobic ATP synthesis at this stage suggest that the activity of this enzyme is low. It is possible, however, to determine both the relative activity and the levels of control by this NADH-linked enzyme by adding an FADH-linked substrate (succinate) to the
medium, thus by-passing the control point enzyme. Any increase in embryonic ATP synthesis in the presence of the added substrate would indicate a low level of IDH activity. The present studies show that incubation in this medium for 1 hr causes a twofold increase in gross ATP synthesis and a 78.4\% decrease in the ATP/ADP ratio at the two-cell stage. The accumulative data suggest, therefore, that the activity of the TCA cycle is rate limiting in the production of ATP at the two-cell stage.

Beginning at the late four-cell stage and continuing through the eight-cell stage, the ATP/ADP ratio is lower than at the two-cell stage. Under these conditions, IDH activity should increase and the full TCA cycle should be functional. This hypothesis is supported by the data showing that ATP synthesis increases significantly and that succinate does not stimulate ATP production at the early eight-cell stage.

The second shift in ATP synthesis occurs between the eight-cell and late morula stage. Although Mills & Brinster (1967) noted a large increase in O₂ uptake starting at the late morula stage, other data have shown no accompanying increase in ATP synthesis (Ginsberg & Hillman, 1973). These conditions (high O₂ uptake, low ATP synthesis) suggest that the late morula mitochondria are uncoupled (Wainio, 1970). The ultrastructural appearance of mitochondria in late morulae mouse embryos corroborate this hypothesis since they are large, multivacuolated and contain a matrix of low density (Hackenbrock, 1968; Hillman & Tasca, 1969).

If the mitochondria of late morulae are uncoupled, ATP synthesis would occur by the glycolytic pathway. The importance of glycolysis in ATP synthesis at this developmental stage is supported by the present data which show that succinate inhibits ATP synthesis by late morulae. In other systems, succinate has been found to inhibit the synthesis of ATP by cells which have a low ATP/ADP ratio and a high rate of glycolysis (Koobs, 1972). The present data also show that amytal, antimycin (5 × 10⁻⁷ M) and cyanide have reduced effectiveness on aerobic respiration at the late morula stage that could be caused by a decrease in aerobic ATP production and an increase in glycolytic ATP synthesis, an hypothesis supported by other evidence (Thomson & Brinster, 1966; Thomson, 1967; Stern & Biggers, 1968).

The third shift in metabolism occurs between the late morula and late blastocyst sub-stages. While late blastocysts have a higher net ATP synthesis than do eight-cell embryos (Ginsberg & Hillman, 1973), the total ATP synthesis in the late blastocyst is significantly lower in the presence of amytal, antimycin (5 × 10⁻⁷ M) and cyanide. On the other hand, succinate has no significant effect on total ATP synthesis in embryos at either of these stages. The similarity in response to the addition of succinate suggests that both stages utilize oxidative phosphorylation as the major pathway for ATP synthesis. The reduced effectiveness of the three inhibitors on late blastocysts may result from several factors, including both a reduced permeability to the inhibitors and an increased potential for glycolytic ATP synthesis to compensate for inhibited oxidative phosphorylation. The paucity of information on late blastocyst energy metabolism makes it impossible to speculate on the possible rôle of these or other factors on the regulation of ATP synthesis at this stage.
ATP synthesis in mouse embryos

It may not be feasible to study ATP metabolism by late blastocysts using in-vitro conditions, since these embryos, unlike the other preimplantation stage embryos, differ in their levels of total ATP when developing in vitro and in utero (Quinn & Wales, 1973). Also, Menke & McLaren (1970) have shown that blastocysts developing in culture have a lower rate of CO₂ production than those obtained directly from the uterus. Recently, Nadijcka & Hillman (1975) have found that blastocyst development can be divided into four ultrastructurally distinct sub-stages. It is possible that the blastocysts of one specific sub-stage would have the same level of oxidative phosphorylation in utero and in vitro and that the reported differences reflect the levels of oxidative aerobic metabolism of different sub-stages. Further work on energy metabolism within and between the four sub-stages of the blastocyst will be necessary to establish the parameters and the control of ATP metabolism during blastocyst development.

ACKNOWLEDGMENTS

The authors would like to thank Dr Ralph Hillman for his help in the preparation of this manuscript. This work was supported by a United States Public Health Service Research Grant (HD-00827).

REFERENCES


