THE INCORPORATION OF CARBON DIOXIDE INTO THE MAJOR CLASSES OF RNA DURING CULTURE OF THE PREIMPLANTATION MOUSE EMBRYO

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(Received 30th December 1974)

Summary. The fixation of CO₂ into major classes of RNA in the mouse embryo was studied in culture. Total fixation of CO₂ was low at the two-cell stage and no label was found in RNA. Between the eight-cell and morula/early blastocyst stages of development, total fixation increased markedly but decreased again at the late blastocyst stage. On a per cell basis, the level of incorporation of CO₂ decreased steadily throughout the preimplantation period. A significant acceleration in the accumulation of ¹⁴CO₂ into all classes of RNA occurred between eight-celled embryos and morulae/early blastocysts, and this effect was more evident when results were calculated in relation to cell number. At the late blastocyst stage, incorporation of label into RNA decreased on a per embryo and a per cell basis.

Most of the label from CO₂ was incorporated into the r-RNA fraction at all stages of development and incorporation into s-RNA was always less. The pattern of labelling of RNA with ¹⁴CO₂ was similar to that previously obtained for the incorporation of [³H]uridine into embryonic RNA, suggesting that most of the CO₂ entering the RNA pool may be incorporated into nucleotide bases. The s-RNA and r-RNA fractions were susceptible to digestion with both pancreatic ribonuclease and 0·3 M alkali. Approximately 31% of the label in the TD-RNA fraction remained after hydrolysis with ribonuclease and a similar proportion of the TD-RNA was resistant to alkali treatment.

Incorporation of CO₂ by morulae/early blastocysts was substantial during culture in substrate-free medium but was increased significantly in medium containing lactate plus pyruvate. Carbon dioxide fixation into RNA was decreased by preculture for 48 hr before incubation in radioactive medium. When compared with freshly collected morulae/early blastocysts, the proportion of the total label in the s-RNA fraction of precultured embryos was low, and a correspondingly greater proportion of the total label was found in the TD-RNA fraction.

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INTRODUCTION

Activation of the genome and the nature of early genetic expression has been examined in the preimplantation mouse embryo by descriptive studies of RNA synthesis. Synthesis of transfer and ribosomal RNA (t-RNA and r-RNA) has been demonstrated at the four-cell stage of development (Mintz, 1964; Woodland & Graham, 1969). Furthermore, Knowland & Graham (1972), in their studies of [3H]uridine incorporation into RNA, have suggested that limited synthesis of these RNA classes has commenced as early as the two-cell stage. After the initial cleavage division, mouse embryos display marked sensitivity to actinomycin D (Thomson & Biggers, 1966; Monesi et al., 1970), indicating the presence of DNA-dependent RNA synthesis. Synthesis of all RNA classes increases until blastocyst formation (Ellem & Gawatin, 1968; Pikó, 1970), and most of the uridine label is incorporated into r-RNA.

Nucleic acid precursors such as uridine and thymidine are not essential requirements of media for the culture of mammalian embryos (Thomson TenBroeck, 1968). Thus the cultured embryo relies on the utilization of endogenous reserves or exogenous carbon sources such as essential energy substrates and CO₂ for the synthesis of RNA. There is a significant decrease in the total RNA complement of the mouse embryo between the one- and two-cell stages of development (Olds et al., 1973) and degradation of RNA may supply intracellular precursor for new RNA synthesis.

Considerable amounts of glucose carbon are incorporated into mouse embryonic RNA after the third cleavage division (Murdoch & Wales, 1973). Fractionation of the RNA by chromatography on methylated bovine serum albumin-coated kieselguhr (MAK; Mandell & Hershey, 1960) indicated that glucose carbon was utilized for the synthesis of s-RNA, r-RNA and RNA with a similar base composition to DNA and having high affinity for the MAK (TD-RNA). An abrupt acceleration in the incorporation of label into all classes of RNA was observed between the eight-cell and morula stages.

The suggestion that CO₂ may be an important carbon source during early development of the mouse embryo (Biggers et al., 1967) has been confirmed in studies of CO₂ incorporation into a variety of intracellular pools, including protein, nucleic acid and lipid (Wales et al., 1969; Graves & Biggers, 1970; Quinn & Wales, 1971; Murdoch & Wales, 1971). After determination of the activities of enzymes involved in the process of CO₂ fixation in the embryo, Quinn & Wales (1971) deduced that the main route of entry of CO₂ is through condensation with pyruvate. In view of the probable importance of CO₂ fixation as a carbon source for the developing embryo, the incorporation of CO₂ in vitro into the major classes of embryonic RNA before implantation, and the effects of energy substrate and preculture on CO₂ accumulation have been investigated.

MATERIALS AND METHODS

General

Embryos were obtained at specific times after ovulation from random-bred, albino mice that had been stimulated to superovulate by the intraperitoneal injection of PMSG followed 48 hr later by HCG (Brinster, 1963). The embryos
Carbon dioxide fixation into mouse embryo RNA

at the two-cell, eight-cell, morula/early blastocyst and late blastocyst stages were flushed from the reproductive tracts approximately 36, 60, 84 and 108 hr after ovulation respectively. The flushing medium was basic culture medium consisting of a modified Krebs–Ringer bicarbonate solution supplemented with 25 mM-sodium pyruvate, 1 mg bovine serum albumin/ml, 60 µg penicillin/ml and 50 µg streptomycin sulphate/ml (Brinster, 1965). After collection the embryos were washed through two changes of culture medium which lacked energy substrate (2 ml/wash) and then cultured for 5 hr at 37°C. Culture conditions were identical to those described by Wales et al. (1969); 20 µl droplets of medium (50 to 100 embryos/droplet) containing 25 mM-NaH$_{14}$CO$_3$ were used under an atmosphere of 5% $^{14}$CO$_2$ in air (sp. act. 12 µCi/µmol; The Radiochemical Centre, Amersham, England).

To estimate total fixation of CO$_2$, five to ten embryos were separated from the medium by centrifugation through isotonic sucrose or non-radioactive basic medium depending upon the stage of development reached (Wales & Whittingham, 1970). These embryos were acidified with 0·1 ml of 2 N-H$_2$SO$_4$ and placed in a closed container for 24 hr together with a strip of filter paper impregnated with 0·2 ml of 1 N-NaOH to absorb liberated CO$_2$ (Wales et al., 1969). Assay of the acid resistant radioisotope in the embryos afforded an estimate of total incorporation. The remaining embryos were washed twice in 2 ml substrate-free medium and stored at −20°C in 1 ml of 0·01 M-acetate buffer (pH 5·0) containing 4 µg polyvinylsulphate before RNA extraction. The number of cells within the embryos at the later stages of development has been determined previously (Murdoch & Wales, 1973).

Extraction and chromatography of RNA

Before RNA extraction the embryos were thawed and mixed with 2 ml mouse liver carrier. The carrier was prepared by homogenizing one lobe of fresh mouse liver (approximately 0·5 g) in 10 ml of 0·01 M-acetate buffer (pH 5·0). Following mixing, the embryo/liver homogenate was diluted to 10 ml in the acetate buffer and the RNA extracted at room temperature by the phenol/sodium lauryl sulphate (SLS) method (Brown & Littna, 1964). Nucleic acids were precipitated from the aqueous phase at −20°C by the addition of 1·0 M-NaCl to a final concentration of 0·1 M and 2 vols of ethanol. The precipitate was treated with DNase and the RNA reprecipitated in NaCl/ethanol. After collection by centrifugation, the RNA pellet was dissolved in 2 ml of 0·05 M-NaCl in 0·05 M-tris-HCl buffer (pH 6·7) and applied to a MAK column (1 x 12 cm) constructed by the method of Monier et al. (1962). The MAK column was washed with 60 ml buffer to remove unbound label and the RNA eluted with a linear gradient of 0·1 to 2·0 M-NaCl in 0·05 M-tris-HCl buffer (pH 6·7). The full procedure of RNA extraction and MAK chromatography has been described previously (Murdoch & Wales, 1973). The eluate from the MAK columns was continuously monitored for u.v. absorption and 2 ml fractions were collected for radioassay.

Experiments

Experiment 1. The incorporation of fixed CO$_2$ into the RNA of various classes

B
of mouse embryos at successive developmental stages was examined. Two-cell, eight-cell, morula/early blastocyst and late blastocyst stage embryos were cultured for 5 hr in basic culture medium buffered with isotopically labelled sodium bicarbonate under a 14CO2 atmosphere.

Experiment 2. The effect of energy substrate within the culture medium was investigated by culturing morula/early blastocyst-stage embryos for 5 hr in media containing NaH14CO3 as above and the following energy substrates: (i) none (control); (ii) 0·25 mm-sodium pyruvate; (iii) 25 mm-sodium dl-lactate; (iv) 25 mm-sodium dl-lactate + 0·25 mm-sodium pyruvate; (v) 5·56 mm-glucose.

Experiment 3. The effect of preculture upon the incorporation of fixed CO2 was examined by culturing two-celled embryos for 48 hr in unlabelled basic medium. Embryos displaying normal development were washed in two changes of energy-free medium and transferred to basic culture medium containing NaH14CO3 for 5 hr. Uptake and incorporation of label was compared with that accumulated by embryos freshly collected approximately 84 hr after ovulation.

Experiment 4. The biochemical nature of the extracted RNA was assessed after mouse morulae/early blastocysts were cultured for 24 hr in basic medium containing NaH14CO3 under an atmosphere of 14CO2. After culture, the RNA was extracted from the embryos, dissolved in 2 ml of 0·05 m-NaCl in 0·05 m-tris–HCl buffer (pH 6·7) and divided into two equal aliquots. One aliquot served as a control for the MAK chromatography while the other aliquot received further treatment as follows. Predigested pronase (25 mg) was added and the resultant solution incubated for 1 hr at room temperature. Alternatively, an aliquot received treatment for 18 hr at room temperature with 100 µg bovine pancreatic ribonuclease which had been preboiled in NaCl:tris–HCl buffer (pH 6·7). A further aliquot was alkali hydrolysed by adjusting the solution to 0·3 m with respect to NaOH. The sample was neutralized with HCl after incubation at 37°C for 18 hr. The RNA in all treated samples was re-extracted by the addition of fresh mouse liver homogenate and phenol/SLS, re-precipitated with ethanol and subjected to MAK chromatography as described above.

Determination of radioactivity

Samples were assayed by liquid scintillation techniques using Triton X100-toluene (1:2 v/v) containing 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis (5-phenyloxazol-2-yl) benzene. The scintillation mixture was added in the ratio of 5 ml scintillator for each 0·4 ml aqueous sample, and a Nuclear-Chicago liquid scintillation spectrometer was used for counting. The incorporation of CO2 was calculated (in pg-atoms or fg-atoms) from the radioactivity detected in the samples and the specific activity of the NaH14CO3.

Statistical analyses

The data for the incorporation of CO2 were transformed to logarithms and
examined for significance by analysis of variance. Data expressed as percentages were converted to angles for analysis. All values given in the text and tables are means ± S.E.M. of unconverted data.

RESULTS

Experiment 1

At least 90% of the embryos at all stages showed normal morphology after culture for 5 hr. Carbon dioxide fixation was demonstrated at all developmental stages and the data obtained for total $^{14}$C incorporation are shown in Table 1. Calculated on a per embryo basis, incorporation doubled between the two-cell and eight-cell stages and doubled again with formation of morulae and early blastocysts. A significant decrease was observed at the late blastocyst stage when embryos had started to implant before being washed from the uterus. Calculated on a per cell basis the level of incorporation steadily decreased, however, as the embryos aged.

Total incorporation of carbon dioxide into RNA was determined by summation of the label in the three peaks eluted from the MAK column. The results (see Table 1) reveal that on a per embryo basis the pattern of incorporation into RNA was superficially similar to that obtained for total $^{14}$CO$_2$ fixation with a rapid increase in accumulation of label between the eight-cell and morula/early blastocyst stages of development followed by a decrease at the late blastocyst stage. When calculated per cell, however, the label detected in embryonic RNA rose to a maximum at the morula/early blastocyst stage, a time when total fixed CO$_2$ was decreasing rapidly. Fixed carbon could not be detected in the RNA of the two-celled embryos (a mean of 806 embryos extracted per replicate) although approximately 2% of the total fixed label was found in the washes of the MAK column before gradient elution. The difference between the percentage incorporation at the eight-cell and late blastocyst stages was significant ($P<0.05$) and the greatest proportion of the incorporated label was found at the morula/early blastocyst stage ($P<0.05$). A further 3.9%, 4.0%, and 3.5% respectively of the total fixed carbon was found in the washes of the MAK column.

Consistently reproducible elution profiles were obtained from MAK chromatography of the embryonic RNA. Labelled RNA from the mouse embryos and optical density peaks from the mouse liver cells always corresponded and s-RNA, r-RNA and TD-RNA were eluted in turn as shown in Text-fig. 1. From the eight-cell stage onwards, label was found in all three classes of RNA studied. Accumulation of CO$_2$ into these classes increased markedly between the eight-cell and morula/early blastocyst stages, as shown in Text-fig. 2. Incorporation of label into all RNA classes decreased ($P<0.01$) following differentiation and formation of the late blastocyst. Of the radioactivity present in the RNA, 50% was found in the r-RNA, 14% in the s-RNA and the remaining 36% in the TD-RNA. There were no significant differences between these percentages for the developmental stages studied. A highly significant peak of incorporation into s-RNA, r-RNA and TD-RNA was recorded on a per cell basis during the transition from morula to early blastocyst.
Table 1. Total fixation of CO₂ and incorporation of fixed CO₂ into major classes of RNA in the mouse embryo

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>No. of cells/embryo</th>
<th>No. of replicates</th>
<th>Mean no. of embryos/replicate</th>
<th>Total fixation of CO₂ (pg-atoms incorporated/5 hr)</th>
<th>Fixation of CO₂ in RNA (fg-atoms incorporated/5 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Per embryo</td>
<td>Per cell</td>
</tr>
<tr>
<td>Two-cell</td>
<td>2</td>
<td>2</td>
<td>806</td>
<td>0.31 ± 0</td>
<td>0.155</td>
</tr>
<tr>
<td>Eight-cell</td>
<td>8</td>
<td>3</td>
<td>528</td>
<td>0.65 ± 0.18</td>
<td>0.081 ± 0.02</td>
</tr>
<tr>
<td>Morula/early blastocyst</td>
<td>34</td>
<td>3</td>
<td>408</td>
<td>1.44 ± 0.19</td>
<td>0.042 ± 0.006</td>
</tr>
<tr>
<td>Late blastocyst</td>
<td>94</td>
<td>3</td>
<td>215</td>
<td>0.94 ± 0.22</td>
<td>0.010 ± 0.002</td>
</tr>
</tbody>
</table>

Values represent the Means ± S.E.M. The % of the total incorporated label present in the RNA is shown in parentheses. N.D. = not detectable.
**Text-fig. 1.** Chromatography of RNA from mouse embryos on a MAK column. Embryos of the (a) eight-cell (○), (b) morula/early blastocyst (●) and late blastocyst (■) stages were cultured for 5 hr in 25 mM-NaH\(^{14}\)CO\(_3\) and under 5% \(^{14}\)CO\(_2\) in air before chromatography. Each point represents the mean of three replicates. The thin line in (a) is the u.v. absorption curve.

**Text-fig. 2.** Total incorporation of CO\(_2\) into the s-RNA (●), r-RNA (x) and TD-RNA (○) of mouse embryos at the two-cell, eight-cell, morula/early blastocyst (M) and late blastocyst (LB) stages of development. Values are expressed in terms of (a) whole embryos or (b) in relation to the number of cells present at each stage. Each point represents the mean for two replicates at the two-cell stage and three replicates at the later stages of development.
Table 2. Effect of energy substrate on the fixation of CO₂ by the mouse embryo and the incorporation of fixed CO₂ into embryonic RNA

<table>
<thead>
<tr>
<th>Substrate in medium</th>
<th>Total CO₂ fixed (fg-atoms/embryo)</th>
<th>Total CO₂ incorporated into RNA (fg-atoms/embryo)</th>
<th>Incorporation of CO₂ into RNA classes (fg-atoms/embryo)</th>
<th>% of RNA radioactivity in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>s-RNA</td>
<td>r-RNA</td>
</tr>
<tr>
<td>Substrate-free</td>
<td>0.99 ± 0.08</td>
<td>120.3</td>
<td>19.5 ± 4.0</td>
<td>66.6 ± 8.7</td>
</tr>
<tr>
<td>0.25 mm-pyruvate</td>
<td>0.96 ± 0.16</td>
<td>139.1</td>
<td>22.0 ± 2.3</td>
<td>73.5 ± 2.3</td>
</tr>
<tr>
<td>25 mm-lactate</td>
<td>1.46 ± 0.16</td>
<td>123.8</td>
<td>15.8 ± 4.9</td>
<td>69.1 ± 9.2</td>
</tr>
<tr>
<td>0.25 mm-lactate +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.56 mm-glucose</td>
<td>1.50 ± 0.10</td>
<td>170.9</td>
<td>35.0 ± 5.0</td>
<td>86.3 ± 12.4</td>
</tr>
<tr>
<td></td>
<td>1.06 ± 0.22</td>
<td>163.6</td>
<td>27.3 ± 1.4</td>
<td>96.4 ± 7.0</td>
</tr>
</tbody>
</table>

Values represent the Means ± S.E.M. for the three replicates. In each replicate the average number of embryos used per substrate was 311. Embryos were cultured in labelled medium for 5 hr.

Summary of analysis of variance (data transformed to logarithms)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Variance ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between substrates</td>
<td>4</td>
<td>2.91*</td>
</tr>
<tr>
<td>Between replicates within substrates</td>
<td>10</td>
<td>1.14</td>
</tr>
<tr>
<td>Between RNA classes</td>
<td>(i) s-RNA versus TD-RNA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(ii) r-RNA versus other classes</td>
<td>1</td>
</tr>
<tr>
<td>RNA x substrates interaction</td>
<td>8</td>
<td>0.67</td>
</tr>
<tr>
<td>Within substrates (error variance)</td>
<td>20</td>
<td>0.0229</td>
</tr>
</tbody>
</table>

* P<0.05; ** P<0.01.
Experiment 2

Data for the fixation of CO₂ into the RNA of mouse morulae/early blastocysts during incubation in a variety of culture media containing NaH¹⁴CO₃ are shown in Table 2. Total incorporation by the embryos was no greater in medium containing pyruvate or glucose than in substrate-free medium. The inclusion of lactate in the medium, with or without the addition of pyruvate, significantly increased total label accumulated in the embryos during the 5 hr incubation (P<0·01). Data for the fixation of carbon dioxide into the RNA of the embryos are also shown in Table 2. There was a significant effect of substrate on the incorporation of carbon dioxide into RNA and comparison of each of the substrate-containing media with substrate-free medium showed a significant increase in incorporation in the presence of lactate + pyruvate (P<0·05). Incubation in the presence of lactate, pyruvate, or glucose alone did not increase the level of incorporation above that in substrate-free medium. Most of the radioactive carbon was again found in the r-RNA and incorporation of label into s-RNA was least. Furthermore, the proportion of the label in these three classes, relative to total isotope extracted in the RNA fraction, was unchanged by addition of the substrates.

Experiment 3

The fixation of CO₂ by two-cell embryos precultured for 48 hr was compared with that of fresh embryos collected approximately 84 hr after ovulation (Table 3). Of the two-celled embryos cultured in non-radioactive medium, 76% developed into morulae but rarely displayed cavitation, whereas approximately 50% of the freshly collected embryos had reached the stage of early blastocyst formation. Thus normal development was slightly retarded during the 48 hr preculture.

Although there was a 35% reduction in the total uptake of CO₂ by embryos which had been precultured, there was considerable variability between replicates and the decrease was not significant. However, preculture caused a depression of 61% (P<0·01) in the incorporation of carbon dioxide into RNA (Table 3). Incorporation of label was decreased in all three RNA classes. The decrease in the proportion of the total radioisotope accumulated in the RNA did not reach statistical significance, although the pattern of labelling of individual RNA classes differed in the cultured embryos compared with that in freshly collected embryos (P<0·05). The label in the s-RNA fraction, expressed as a percentage of the total label in embryonic RNA, was lowered after preculture for 48 hr while there was a corresponding increase in the percentage of the total label in the TD-RNA fraction.

Experiment 4

During culture for 24 hr in basic medium buffered with radioactive bicarbonate and CO₂, all the morulae/early blastocysts developed into blastocysts, some of which had hatched from the zona pellucida, and fixed 4·35±0·28 pg-atoms CO₂/embryo. The level of label incorporated into the individual RNA classes was slightly decreased by treatment with pronase, probably because of losses during re-extraction and recovery of the RNA. More than 95% of the
Table 3. Effect of culture on the incorporation of CO$_2$ by mouse embryos

<table>
<thead>
<tr>
<th>Pretreatment of embryos</th>
<th>Mean no. of embryos/replicate</th>
<th>Total CO$_2$ incorporation (fg-atoms/embryo)</th>
<th>Incorporation of CO$_2$ into RNA (fg-atoms/embryo)</th>
<th>Incorporation of CO$_2$ into RNA classes</th>
<th>% of RNA radioactivity in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>s-RNA</td>
<td>r-RNA</td>
</tr>
<tr>
<td>Collected 84 hr after ovulation</td>
<td>335</td>
<td>1.40 ± 0.18</td>
<td>25.9 ± 3.3</td>
<td>201.8 (14.4)</td>
<td>57.4 ± 0.4</td>
</tr>
<tr>
<td>Collected 36 hr after ovulation and cultured for 48 hr</td>
<td>492</td>
<td>0.91 ± 0.07</td>
<td>6.4 ± 1.6</td>
<td>78.7 (8.7)</td>
<td>8</td>
</tr>
</tbody>
</table>
s-RNA and r-RNA fractions was susceptible to ribonuclease hydrolysis but 31% of the labelled TD-RNA fraction remained after treatment with this enzyme. In addition, the s-RNA and r-RNA fractions were extremely vulnerable to alkali digestion and quantities of label slightly greater than background remained after treatment. Again, 31% of the radioactivity originally present in the TD-RNA fraction was retained in this peak following digestion in 0.3 M alkali.

DISCUSSION

The secretions of the oviduct and uterus contain a considerable concentration of bicarbonate (Restall & Wales, 1966; David et al., 1969) and a bicarbonate-buffered system is essential for successful culture of the preimplantation mouse embryo (Biggers et al., 1967). In addition to its role in pH regulation, CO₂ is an important carbon source for the embryo during early cleavage. The fixation of CO₂ is not restricted to mammalian embryos but has also been demonstrated in amphibian embryos (Cohen, 1954). Radioactivity from CO₂ has been detected in protein, nucleic acid and acid-soluble fractions of mouse embryos cultured in the presence of [¹⁴C]bicarbonate (Graves & Biggers, 1970; Quinn & Wales, 1971) and incorporation is maximal at the time of blastocyst formation.

Total incorporation of CO₂, as determined in the present study, is in close agreement with the earlier findings at all developmental stages. Furthermore, the experiments demonstrate that incorporation into all major RNA classes commences between the two-cell and eight-cell stages of development and increases during the subsequent 24 hr. At the morula/early blastocyst stage, accumulation of CO₂ into embryonic RNA was considerable and represented 11.7% of the total label in the embryos.

When considering parameters of gene activity such as protein and RNA synthesis, calculation of data on the basis of cell number probably relates changes more closely to the actual genetic events (Epstein & Smith, 1973). On this basis, a highly significant peak of incorporation into all RNA classes was found at the morula/early blastocyst stage of development, a time when total CO₂ incorporation/cell is decreasing rapidly. As this peak of incorporation occurs with the formation of the early blastocyst it may be correlated with the initial subcellular events leading to differentiation into the two distinct cell types, trophoblast and inner cell mass. Although uridine incorporation into r-RNA increases abruptly, when calculated per cell, between the eight-cell and morula stages (Ellem & Gwatkin, 1968), the pattern of incorporation of uridine/cell into the various classes of RNA as found by Ellem & Gwatkin (1968) differed considerably from the pattern obtained with CO₂, especially at the later stages of development. The morulae and blastocysts used by Ellem & Gwatkin (1968) had been cultured from the two-cell stage and the differences in incorporation of uridine and CO₂ could be related to developmental changes during culture. Even under optimal conditions of culture the rate of cleavage of embryos is reduced when compared with the situation in vivo (Bowman & McLaren, 1970), and blastocysts which are cultured from the eight-cell stage have a lower metabolic rate than fresh uterine blastocysts.
(Menke & McLaren, 1970). In the present experiments the accumulation of CO₂ in embryonic RNA, after preculture for 48 hr, was significantly decreased. The most significant decrease was in the s-RNA fraction indicating that translation in particular may be hindered by a decreased synthesis of t-RNA. Structural alterations and subcellular localization of t-RNA molecules may be involved in regulation of cell differentiation (de Vellis, 1970) and any change in t-RNA metabolism could have a significant effect on the rate of maturation of embryos.

Graves & Biggers (1970) have reported that 10% of the total fixed carbon is extracted with RNA at all stages of development subsequent to the second cleavage division. In the present study, proportions of the same order were found at the morula and late blastocyst stages but were considerably lower at the eight-cell stage (approximately 2%). The less specific sequential separation employed in the earlier study could account for these differences and presumably labelled RNA precursors would have been included in the estimates of Graves & Biggers (1970). With the phenol extraction used here, some of the label extracted as RNA fails to bind to the MAK column, suggesting the presence of components other than the major RNA classes.

Synthesis of RNA by early developing embryos has been used widely as a model for the study of control of eukaryote differentiation. Echinoderm and amphibian embryos synthesize little new RNA prior to gastrulation (Wilt, 1963, 1964; Gross & Cousineau, 1964), and protein synthesis during early stages is apparently directed by m-RNA preformed in the unfertilized egg (Tyler & Tyler, 1970). Fertilization is thought to involve activation of this 'masked' messenger-RNA (mm-RNA). Although net synthesis of RNA and associated rapid protein synthesis have not been found until blastulation in the rabbit (Manes, 1969), RNA turnover and concomitant salvage synthesis may play a significant role and net synthesis of RNA may not be necessary for continued development. Newly synthesized m-RNA associated with polyribosomes has been detected as early as the sixteen-cell stage in the rabbit embryo (Schultz, 1972). Furthermore, cleavage of the rabbit embryo, which is accomplished without synthesis of r-RNA (Manes, 1971), may be arrested immediately by actinomycin D and more slowly by α-amanitin, an inhibitor of RNA polymerase II (Manes, 1973), suggesting that transcription is an important early event in this species. Very limited RNA synthesis occurs before the eight-cell stage in the mouse embryo (Knowland & Graham, 1972), but actinomycin D sensitivity has again been demonstrated at certain stages of the cell cycle (Molinaro et al., 1972). The sensitivity of sea urchin and amphibian embryos to actinomycin D contrasts with that of rabbit and mouse embryos (Thomson & Biggers, 1966; Monesi et al., 1970) and high levels of this antibiotic do not significantly block cleavage divisions in the non-mammalian species. Although actinomycin D may have non-specific effects which are responsible for the immediate arrest of cleavage of the mammalian embryo (Manes, 1973), the above evidence suggests that some DNA-dependent RNA synthesis is essential for continued cleavage of the mouse embryo. The observation that CO₂ is not incorporated into mouse embryonic RNA at the two-cell stage of development provides further evidence of lack of significant RNA
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synthesis at this early stage. Total RNA content of the mouse embryo decreases during the first 2 days of pregnancy (Olds et al., 1973). Thus a large proportion of early protein synthesis in both the rabbit and mouse embryo may be directed by mm-RNA. Furthermore, Bernstein & Mukherjee (1972) suggest that synthesis of nuclear RNA is suppressed during the first two cleavage divisions of the preimplantation mouse embryo, possibly by cytoplasmic factors, and sequential reactivation of transcription results in formation of major RNA classes after this time.

Without prior knowledge of important criteria such as precursor pool sizes and their turnover characteristics, increased incorporation of radioisotope from the environment into RNA cannot be interpreted as increased RNA synthesis per se (Epstein & Daentl, 1971; Murdoch & Wales, 1973). On the other hand, the detection of label from precursor within the RNA molecule does imply some new synthesis of RNA. Assuming that RNA precursor pools do not change radically during development, increased incorporation of CO₂ into RNA suggests increased synthesis, which follows a similar overall pattern to the incorporation of [³H]uridine as reported by others (Ellem & Gwatkin, 1968; Pikó, 1970; Murdoch & Wales, 1973). From the onset of genome activation, synthesis of r-RNA from carbon dioxide and uridine predominates over incorporation of label into both s-RNA and TD-RNA. By contrast, more exogenous [¹⁴C]glucose label is accumulated by the embryo in the TD-RNA than in either s-RNA or r-RNA. Although these conflicting results may be due in part to the complexity of possible metabolic pathways by which glucose carbon could enter the RNA molecule and its precursor pools, labelled polysaccharide closely associated with the RNA may be responsible for some of the high level of incorporation of [¹⁴C]glucose into TD-RNA (I. L. Pike and R. G. Wales, unpublished).

The sensitivity of the s-RNA and r-RNA classes labelled with ¹⁴CO₂ to pancreatic ribonuclease and alkaline digestion and their resistance to pronase indicates that these fractions are relatively free from contaminants. After 18 hr digestion with alkali at 37°C, approximately 30% of the labelled TD-RNA remained unhydrolysed while another 30% of this material was ribonuclease-resistant. The latter result is not surprising in view of the presence of labile, heterogeneous nuclear RNA and m-RNA in this fraction (Ellem, 1966; Pikó, 1970). RNA with these characteristics displays considerable resistance to ribonuclease digestion (Gorski & Nicolette, 1963) due to its content of long polyadenylic acid sequences (100 to 200 nucleotides) (Darnell et al., 1971; Edmonds et al., 1971).

Although the mouse embryo requires an appropriate energy source for early development in vitro (Whitten, 1957; Brinster, 1965), a proportion of eight-celled embryos will develop in medium totally lacking energy substrate (Brinster & Thomson, 1966; Wales & Whittingham, 1973), and a large proportion of morulae will also develop into blastocysts during culture in substrate-free medium. The present study shows that, at the morula/early blastocyst stage, the fixation of CO₂ was substantial in medium lacking energy substrate and the total incorporation of label was not elevated by the addition of glucose or pyruvate. In the absence of substrate, morulae/early blastocysts are able to mobilize
glycogen stores (Pike & Wales, 1975), and presumably products of glycogenolysis provide the carbon skeleton for condensation with CO₂ and subsequent entry of CO₂ into the general carbon pools of the embryo. The increased level of CO₂ incorporation found when 25 mM-lactate was added to the culture medium may be related to the increased substrate concentration. In a study of CO₂ fixation by ram spermatozoa, O'Shea & Wales (1970) showed that more fixation of carbon dioxide occurred as the concentration of carboxylic acid in the incubation mixture increased.

The effects of substrate on carbon dioxide incorporation into RNA were different from those for total incorporation and possibly indicate different routes of entry of label into the various pools. It is generally assumed in studies of uridine incorporation by tissues that the tracer accumulates in the base moiety of the nucleic acids. The similarity in MAK chromatography patterns for [³H]uridine and ¹⁴CO₂ incorporation into the RNA of the mouse embryo suggests that label from CO₂ may be located in the nucleotide bases. Quinn & Wales (1971) calculated that condensation of carbon dioxide with pyruvate was the major route of CO₂ fixation in these embryos with total activity of the enzymes pyruvate carboxylase (E.C. 6.4.1.1) and malate dehydrogenase (decarboxylating) (E.C. 1.1.1.40) being sufficient to account for the measured fixation of CO₂ in vitro. For RNA synthesis, some CO₂ could be incorporated into the purine bases by conversion of the condensation product of these reactions, oxaloacetate, into aspartate, but the CO₂ may also enter more directly into the biosynthesis of nucleotide precursors by fixation involving carbamoylphosphate synthetase (E.C. 2.7.2a) and phosphoribosylaminoimidazole carboxylase (E.C. 4.1.1.21). Furthermore, it is unlikely that the isotope would be found in the ribose moiety as phosphopyruvate carboxylase (E.C. 4.1.1.32) has not been detected in the mouse embryo (Quinn & Wales, 1971), suggesting gluconeogenesis from pyruvate does not occur.

ACKNOWLEDGMENTS

The authors are indebted to Professor C. W. Emmens for his interest and criticism. The work was aided by grants from the Ford Foundation and the Australian Research Grants Committee. One of us (I.L.P.) was supported by a F. H. Loxton Post-Graduate studentship.

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