Studies on effects of 5-bromodeoxyuridine on the development of explanted early chick embryos

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SUMMARY

Chick embryos were explanted at stages ranging from the definitive streak to one-somite and cultured for 19–21 h on thin albumen with or without 5-bromodeoxyuridine (BrdU) (2–12 µg/ml). BrdU primarily inhibited neurulation which was found to be stage dependent. If exposed to BrdU at the definitive streak stage, it resulted in an open brain region; if treatment was delayed until the head-process stage, it inhibited the closure of the midbrain and, to some extent, the hindbrain, but the forebrain closure was mostly unaffected. BrdU had no appreciable effect on later stages. It was also found that BrdU inevitably inhibited somite formation when exposed to 8 µg/ml or higher concentrations. The blastodermal expansion, heart development, and blood island formation were usually not affected.

Cytological changes were observed in adversely affected tissues, particularly the neural tissue. BrdU produced nuclear enlargement and clumped metaphase, but did not cause extensive necrosis.

The grafting experiments showed that an increase in the concentration of BrdU from 4 to 12 µg/ml progressively inhibited the self-differentiation of Hensen's node grafts. However, BrdU (8 µg/ml) had no significant effect on the inducing capacity of node grafts nor the competence of the epiblast.

Autoradiographic studies showed that the application of BrdU, at 6 µg/ml or lower, caused no obvious variations in the incorporation of [3H]uridine into chick embryonic cells. BrdU, at 10–12 µg/ml, selectively inhibited the uptake of [3H]uridine in the neural tissue.

INTRODUCTION

The thymidine analogue, 5-bromodeoxyuridine (BrdU = BUdR), has proved to be a useful tool in the study of cell differentiation (Wilt & Anderson, 1972). The precise mode of the inhibitory action of BrdU remains unclear, although the evidence suggests that it is incorporated into DNA (Bischoff & Holtzer, 1970; Stellwagen & Tomkins, 1971; Ostertag et al. 1973; O'Neil & Stockdale, 1974) and/or interferes with the synthesis of carbohydrates necessary for the formation of some cell membrane constituents (Schubert & Jacob, 1970).

So far BrdU has been shown to inhibit the synthesis of proteins characteristic of the differentiated state in several developing systems (Abbott & Holtzer, 1968; Miura & Wilt, 1971; Stellwagen & Tomkins, 1971; Weintraub, Campbell 1

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& Holtzer, 1972, etc.) and to interfere with the early development of sea urchin eggs (Gontcharoff & Mazia, 1967; Tencer & Brachet, 1973), tunicate and amphibian embryos (Tencer & Brachet, 1973). Our previous study (Lee, Deshpande & Kalmus, 1974) showed that BrdU (3 × 10⁻⁴ M) selectively inhibited the morphogenesis of the brain and somites in explanted definitive streak stage chick embryos. It was also noted that the inhibitory action of BrdU could be alleviated by subsequent treatment with an equimolar concentration of thymidine, suggesting its incorporation into DNA. However, very little is known about its effects on other important aspects of early chick development. In view of this, the present study was undertaken (1) to extend our previous investigation by including older embryos and cytological observations, (2) to study effects of BrdU on the self-differentiation and inducing capacity of Hensen’s node and the competence of the epiblast, and (3) to investigate autoradiographically whether BrdU affects RNA synthesis in chick embryonic cells.

MATERIALS AND METHODS

Cultivation and treatment of chick embryos

Fertile White Leghorn eggs were incubated at 37.5 °C for varying periods of time to obtain embryos at stages ranging from the definitive streak to 1-somite (stages 4–7; Hamburger & Hamilton, 1951). The embryos were cultured by New’s (1955) technique. A stock solution of BrdU (Sigma Chemical) was prepared in Pannett-Compton (PC) saline at a concentration of 1 mg/ml (3.3 × 10⁻³ M) and stored in the dark at 4 °C. The desired amounts of stock solution were added to thin albumen immediately before use. Similarly, equal volume of PC saline was added to thin albumen for the control series. Explanted embryos were exposed to BrdU before incubation according to the procedure described by Billett, Bowman & Pugh (1971). Unless otherwise stated, embryos were grown for 19–21 h on thin albumen with or without BrdU.

Grafting procedure

The grafts used in this study were obtained from the node area (size = 0.3 × 0.3 mm) of stage 3+ embryos (average length of streak = 1.32 mm). Each graft was inserted between the epiblast and hypoblast in the area pellucida of a host embryo at stage 4 following the technique described by Waddington (1932). The host embryos were grown for 24 h on thin albumen with or without BrdU.

Histological preparations

At the end of the incubation period, some embryos were fixed in Bouin’s fluid, stained with Delafield’s hematoxylin, and kept as whole mounts. Others were fixed in Bouin’s fluid or 10% neutral formalin, embedded in paraffin, sectioned at 6 μm, and stained with Delafield’s hematoxylin and eosin or methyl green-pyronin.
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 Autoradiographic techniques

Stage 4 embryos were grown for different lengths of time on thin albumen containing 5 μCi/ml [3H]uridine (New England Nuclear). This labelling process did not interfere with the subsequent development of control embryos. After the incubation period, the blastoderms were washed several times in PC saline, fixed in absolute ethanol-glacial acetic acid (3:1), embedded in paraffin, sectioned at 4 μm, and mounted on slides. The slides were deparaffinized, divided into 4 groups, and were treated for 1 h at 37 °C with one of the following: (1) distilled water, (2) 0.8 mg/ml RNase (Worthington Biochem.) boiled at 100 °C for 30 min to inactivate DNase, (3) 0.1 mg/ml DNase-RNase free (Worthington Biochem.), and (4) 0.8 mg/ml RNase plus 0.1 mg/ml DNase. All the slides were covered with Kodak NTB-2 liquid photographic emulsion using the dipping technique of Kopriwa & Leblond (1962) and exposed for 10 days at 4 °C. The emulsion was developed for 6 min in Kodak D-19 at 20 °C and fixed for 10 min in Kodak acid-fixer. The sections were stained with hematoxylin-eosin, dehydrated through a graded ethanol series, and mounted in Permont. For quantitative evaluation of autoradiographs, the following procedure was used. For example, a section through the tip of the notochord of each embryo was initially located. Grain counts were then made over nuclei and cytoplasm of the cells in the neural fold region (unit area = 40 x 40 μm). Similar procedure was followed to study the distribution of labelled materials in other tissues such as notochord, epidermis, and somite mesoderm. Background counts were made near the section to which the reading would apply.

RESULTS

Effects of BrdU on the development of early chick embryos

In this series of experiments, chick embryos at stages ranging from 4 to 7 were exposed to different concentrations of BrdU for 19–21 h. It was found that BrdU, at a concentration of 1 μg/ml, caused a growth retardation of stage 4 embryos, but had no obvious effect on older embryos. Concentrations of 16 μg/ml or higher caused extensive disintegration of all embryos. Four categories were used to record the degree of development: (1) little or no development – embryos at the same stage of development as when explanted; (2) very abnormal – severely malformed embryos (Fig. 1A); (3) abnormal – embryos with one or more discernible abnormalities (Fig. 1B, C, D), and (4) normal (Fig. 1E). The results are summarized in Table 1.

BrdU (8 μg/ml or higher) inhibited somite formation, regardless of the developmental stage of embryos at treatment. The blastodermal expansion, heart development, and blood island formation, except in severely malformed embryos, were not significantly affected. In contrast, the magnitude of the inhibitory action of BrdU (2–12 μg/ml) on the brain development was stage
Table 1. Effects of BrdU on the development of chick embryos explanted at stages 4–7 and cultured for 19–21 h

<table>
<thead>
<tr>
<th>Conc. of BrdU (μg/ml)</th>
<th>Stage(s) treated</th>
<th>No. of embryos</th>
<th>Little or no development</th>
<th>Very abnormal</th>
<th>Abnormal</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>4</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>6–7</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>2–4</td>
<td>4</td>
<td>33</td>
<td>0</td>
<td>6</td>
<td>64</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>28</td>
<td>0</td>
<td>4</td>
<td>50</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>6–7</td>
<td>30</td>
<td>0</td>
<td>3</td>
<td>27</td>
<td>70</td>
</tr>
<tr>
<td>6–8</td>
<td>4</td>
<td>38</td>
<td>0</td>
<td>11</td>
<td>89</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20</td>
<td>0</td>
<td>15</td>
<td>65</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>6–7</td>
<td>21</td>
<td>0</td>
<td>10</td>
<td>47</td>
<td>43</td>
</tr>
<tr>
<td>10–12</td>
<td>4</td>
<td>25</td>
<td>8</td>
<td>20</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>24</td>
<td>0</td>
<td>25</td>
<td>67</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>6–7</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>62</td>
<td>38</td>
</tr>
</tbody>
</table>

dependent: if treatment was begun at stage 4, it resulted in an open brain region (Fig. 1B); if treatment was delayed until stage 5, it inhibited the closure of the midbrain and, to some extent, the hindbrain, but the forebrain closure was usually unaffected (Fig. 1C, 2A). However, BrdU had no apparent effect on the brain development of embryos treated at stages 6–7 (Fig. 1D). Furthermore, in many malformed embryos, particularly those treated at stages 5–7, large vesicles were found along the posterior axis (Fig. 1C, D). These vesicles appeared to be formed from the disintegration of somite mesoderm and accumulation of fluid between ectoderm and endoderm.

One of the most conspicuous changes seen in transverse sections of BrdU-treated embryos was a marked enlargement of the dorsal aortae (compare Fig. 2A with Fig. 2B).

**Figure 1**

(A) Embryo explanted at stage 4 and cultured for 21 h on thin albumen with 12 μg/ml BrdU. Scale line = 0.63 mm.
(B) Embryo explanted at stage 4 and cultured for 21 h on thin albumen with 8 μg/ml BrdU. Scale line = 0.63 mm.
(C) Embryo explanted at stage 5 and cultured for 19 h on thin albumen with 8 μg/ml BrdU. Scale line = 0.63 mm.
(D) Embryo explanted at stage 6 and cultured for 19 h on thin albumen with 10 μg/ml BrdU. Scale line = 0.63 mm.
(E) Embryo explanted at stage 4 and cultured for 21 h on thin albumen. Scale line = 0.63 mm.
Cytological effects of BrdU

The neural tube of malformed embryos showed various types of abnormal cells depending largely on the concentration of BrdU used. Treatment with 2–4 μg/ml BrdU increased the number of cells with nucleus larger than normal. Nuclear enlargement, whether slight or pronounced, appeared to be the first sign of the nucleotoxic effect of BrdU. The enlarged nuclei stained diffusely with methyl green, showing a barely recognizable network of chromatin. At 6–12 μg/ml, some neural tube cells showed, besides the nuclear enlargement, pycnosis and chromosomal abnormalities (compare Fig. 2C with Fig. 2D). Most cells in division were at metaphase, although examples of other mitotic phases were scattered throughout the adversely affected neural tissue. Chromosomal abnormalities such as clumped metaphase (‘star’ metaphase), C-metaphase, and chromosomal bridge were frequently observed (Fig. 2C).

In some severely malformed embryos the head mesenchyme was represented
Effects of 5-bromodeoxyuridine on chick embryos

Table 2. Effects of different concentrations of BrdU on the self-differentiation and inducing capacity of Hensen’s node grafts

<table>
<thead>
<tr>
<th>Conc. of BrdU (µg/ml)</th>
<th>Total no. of grafts</th>
<th>Total % of grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Differentiated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induction No induction</td>
</tr>
<tr>
<td>0 (control)</td>
<td>18</td>
<td>72.2 11.1</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>33.3 8.3</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>33.3 0.0</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>25.0 0.0</td>
</tr>
</tbody>
</table>

by fewer cells than usual. These cells were large compared to the controls, often rounded, and lost the usual spindle-shaped character.

The somite mesoderm did not show such conspicuous cytological changes as the neural tissue, although its differentiation into dermatome, myotome, and sclerotome was inhibited where the neural tube was very degenerate. Nuclear enlargement and metaphase clumping were occasionally found in severely affected somite mesoderm.

The notochordal, cardiac, and endodermal cells were not noticeably affected by BrdU, at concentrations of 12 µg/ml or lower. The blood island formation was also unaffected.

Effects of different concentrations of BrdU on self-differentiation and inducing capacity of Hensen’s node

Stage 3 embryos (average length of streak = 0.76 mm) were grown on thin albumen with or without BrdU. The node area was excised from the embryos which had advanced to stage 3+ (average length of streak = 1.32 mm), washed several times in PC saline to ensure removal of unbound BrdU, and grafted on to untreated host embryos at stage 4 (average length of streak = 1.86 mm). The hosts were grown for 24 h on thin albumen. Four categories were used to record the results: (1) the graft had differentiated and had caused induction (Fig. 3A); (2) the graft had differentiated but had not caused induction (Fig. 3B); (3) the graft had not differentiated but had caused induction (Fig. 3C); (4) the graft had neither differentiated nor caused induction. The results are summarized in Table 2. Fig. 4 clearly shows that the inhibitory effect of BrdU on the graft differentiation is concentration dependent. In contrast, BrdU had no appreciable effect on the capacity of Hensen’s node to induce a secondary neural tissue in the epiblast of host embryos.
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Differentiated grafts

Induced neural tissue

Fig. 4. Effects of different concentrations of BrdU on the self-differentiation and inducing capacity of Hensen's node grafts.

Effects of BrdU on the competence of epiblast

Embryos serving as the hosts were explanted at stage 3, grown for 3–5 h on thin albumen with or without 8 μg/ml BrdU (a concentration found to inhibit strongly the morphogenesis of stage 3 embryos), and then washed several times in PC saline. The node areas of untreated stage 3+ embryos were grafted on to the hosts which had advanced to the stage 3+. The hosts were grown for 24 h on thin albumen. Three categories were used to record the results: (1) strong induction – in which the graft had induced in the host epiblast a neural tube of

Figure 3

(A) Transverse section through embryo with 4 μg/ml BrdU-treated node graft. Note neural tissue and differentiated node graft. G, graft; HNT, host neural tissue; INT, induced neural tissue. Scale line = 0.07 mm.

(B) Transverse section through embryo with 4 μg/ml BrdU-treated node graft. Note differentiated node graft. G, graft; HNT, host neural tissue. Scale line = 0.07 mm.

(C) Transverse section through embryo with 8 μg/ml BrdU-treated node graft. Note induced neural tissue and undifferentiated node graft. G, graft; HNT, host neural tissue; INT, induced neural tissue. Scale line = 0.07 mm.
considerable dimensions; (2) weak induction – in which the graft had caused in the host epiblast the formation of thickened neural ectoderm; (3) no induction. The results are summarized in Table 3. It can be seen that BrdU (8 μg/ml) had no appreciable inhibitory effect on the competence of the epiblast.

**Table 3. Effect of 8 μg/ml BrdU on the competence of the epiblast**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of host embryos</th>
<th>% of host embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Strong induction</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>62.5</td>
</tr>
<tr>
<td>Treated</td>
<td>24</td>
<td>58.3</td>
</tr>
</tbody>
</table>

**Effects of BrdU on the uptake of [3H]uridine**

Autoradiography was employed to study whether BrdU had any effect on RNA metabolism of chick embryonic tissues.

In the first series, stage 4 embryos were grown on thin albumen containing 5 μCi/ml [3H]uridine (control group) or on thin albumen containing both 5 μCi/ml [3H]uridine and BrdU (experimental group) until the majority of the control embryos had reached the stage 6 or 7. The application of BrdU, at 6 μg/ml or lower, caused no obvious variations in the incorporation of [3H]-uridine into chick embryonic cells. In both control and experimental groups, treatment with RNase resulted in the loss of 60–70% of the labeled materials, the remainder were predominantly found in the nuclei and were removable with DNase. At 8 μg/ml, the incorporation of [3H]uridine into the nuclei, but not into the cytoplasm, of neural tube cells was significantly affected (P < 0.01). BrdU, at 10–12 μg/ml, strongly inhibited the uptake of [3H]uridine in both the nucleus and cytoplasm of neural tube cells. The incorporation of [3H]uridine into DNA of neural tube cells was similarly inhibited by BrdU. However, BrdU, at the concentrations used, had no appreciable effect on the incorporation of [3H]uridine into other tissues.

In the second series, stage 4 embryos were grown for 4 h on thin albumen containing 5 μCi/ml [3H]uridine, washed several times in PC saline, and subcultured on thin albumen containing 8 μg/ml BrdU (experimental group) or on thin albumen (control group) until the majority of the control embryos had reached the stage 6 or 7. In the case of BrdU-treated embryos, cells of various tissues had about 8% less cytoplasmic labeling than the controls. This finding indicates that BrdU has very little inhibitory effect on the transfer of labeled materials from the nucleus to the cytoplasm.

In the third series, stage 4 embryos were grown for 4 h on thin albumen containing 10 μg/ml BrdU (experimental group) or on thin albumen (control group), washed several times in PC saline, and subcultured on thin albumen
containing 5 μCi/ml [³H]uridine until the majority of the control embryos had reached the stage 6 or 7. In the neural tissue, 88–92% of the nuclei in the control series were labeled, whereas only 12–24% of the nuclei in the experimental series were labeled. However, no marked difference was noted in other tissues. This finding indicates that BrdU penetrates neural plate cells and inhibit the uptake of [³H]uridine.

**DISCUSSION**

The present study showed that BrdU (8 μg/ml or higher) inhibited somite formation, regardless of the developmental stage of embryos at treatment. The blastodermal expansion, heart development and blood island formation, except in severely malformed embryos, were usually unaffected. In contrast, the magnitude of the inhibitory action of BrdU (2–12 μg/ml) on the brain development varied from one stage to another, i.e. a critical time of administration is required for BrdU to inhibit different primary divisions of the neural tube. This particular pattern of abnormalities is not restricted to BrdU, because a number of other chemical agents, e.g. actinomycin D (Heilporn-Pohl, 1964; Lee & Poprycz, 1970; Billett *et al.*, 1971; Lee, Auslander & Kalmus, 1973) can produce a similar picture. However, BrdU differs from actinomycin D in that (1) it has no apparent effect on the blastodermal expansion; (2) it does not cause extensive necrosis and cell death in adversely affected tissues.

Microscopic studies showed that the effects of BrdU on the chick embryo was directed mainly to the developing neural tissue. At lower concentrations (2–4 μg/ml), the affected cells showed either slight or severe nuclear enlargement. The severely enlarged nuclei stained diffusely with methyl green, showing a barely recognizable network of chromatin. These observations imply that the cells were arrested at the early prophase and there might have been an inhibition of DNA synthesis. At higher concentrations (6–12 μg/ml), the affected cells showed, in addition, pycnosis and chromosomal abnormalities. Similar observations have been made in other developing systems, e.g. sea urchin embryos (Mazia & Gontcharoff, 1964) and amphibian embryos (Tencer & Brachet, 1973). Hsu & Somers (1961) reported that BrdU preferentially damaged certain adenine-thymine rich regions in mammalian chromosomes. Whether this is also true in avian chromosomes needs further investigations. Neural tube closure was inhibited in many malformed embryos, particularly those treated at stage 4. This defect did not appear to be caused by mitotic failure in the tissue itself, since cells in various phase of mitosis were often found in the vicinity of degenerating cells. It appears that cell processes associated with the neural tube closure are more sensitive to BrdU than are those essential to growth and cell division. There is little or no evidence that the early chick embryo has differential regional resistance to penetration of BrdU, whereas there is a large body of evidence demonstrating that there are regional metabolic differences in the early chick embryo (Spratt, 1952; Duffey & Ebert, 1957; etc.). Furthermore, it
is at stage 4 that the process of neural differentiation is initiated (England, 1973) and it is precisely at this stage that the presumptive neural tube tissue is highly susceptible to BrdU. These considerations suggest that the interruption of metabolic pathways and/or induction process may account for the observed neural tube defects. Importance of microtubules in neurulation has been pointed out by Waddington & Perry (1966). It is of interest to know whether microtubules of the chick neural plate cell are susceptible to BrdU. This could help us elucidate the effect of BrdU on the morphogenesis of the neural tube. Our autoradiographic studies showed that high concentrations of BrdU (10–12 μg/ml) selectively inhibited the incorporation of [3H]uridine into the neural tissue. Whether or not BrdU may suppress or alter the synthesis of tissue specific nuclear RNA is presently not known. If we assume that in our experiments BrdU is incorporated into DNA and inhibits the transcription of certain genes into mRNA as suggested by Stellwagen & Tomkins (1971), then the results would indicate that a change in the pattern of transcription of genetic information might be involved during the initial phases of brain development. Ingram et al. (1974) reported that BrdU lowered the rate of uptake of adenosine into the ATP pool during the chick erythropoiesis. The differential susceptibility of the neural tissue to BrdU suggests some disturbances in energy supply mechanisms. In this respect the BrdU effects resemble those of cyanide (Spratt, 1952) and 2,4-dinitrophenol (Bowman, 1967). This view is supported by the observation that BrdU, at a concentration of 6 μg/ml, had a selective inhibitory effect on the neural tissue, but had no obvious effect on the incorporation of [3H]-uridine into RNA. Alterations in oxidative phosphorylation, whether immediate consequences of BrdU treatment or secondary results of the interference with nucleic acid synthesis, may have led to the observed neural tube defects.

This study also showed that the brain development often became insensitive to BrdU, if treatment was delayed until the embryo had reached stages 6–7. BrdU-insensitive processes are not uncommon in developing systems, e.g. echinochrome synthesis by sea urchin embryos (Gontcharoff & Mazia, 1967), haemoglobin synthesis by erythrocytes (Miura & Wilt, 1971), chondroitin sulphate synthesis by chondrocytes (Abbott, Mayne & Holtzer, 1972), etc. Weintraub et al. (1972) suggested that normal differentiation involves the institution of a programme that is resistant to BrdU. It does not, however, explain the reason why BrdU selectively inhibits the differentiation of certain tissues and/or cell functions.

Hensen’s node of the stage 4 chick embryo is found to be active in cell proliferation and RNA synthesis (Lee, 1972). It is, therefore, expected that BrdU has a marked inhibitory effect on the differentiation of Hensen’s node grafts. Our grafting experiments did show that an increase in the concentration of BrdU from 4 to 12 μg/ml caused a significant decrease in the degree of graft differentiation which was, however, not accompanied by a proportionally decreased frequency of neural inductions. These findings suggest that the vital
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Activity of the inductor is not prerequisite to elicit typical neural induction in the chick epiblast. Furthermore, BrdU (8 μg/ml) did not impair the competence of the epiblast, although the host embryo proper showed characteristic syndromes of BrdU treatment. The observations that BrdU is incorporated into DNA (Wishnow, Feist & Glowalla, 1974) and inhibits the uptake of [3H]uridine suggest that DNA-mediated RNA synthesis required for initiating the differentiation of the competent epiblast is insensitive to BrdU or it occurs prior to treatment period (stage 3).

This study was supported by a grant from the Rutgers University Research Council No. 07-2189.

REFERENCES


(Received 26 February 1974, revised 30 May 1974)