Thus recent researches on the chick embryo are directed towards a deeper analysis of the differentiation processes. Biochemical, immunochemical and electron microscopic techniques are called on in an attempt to explain induction or invagination processes, positioning of migrating cells, and developmental abnormalities. The studies presented in this issue, apart from that concerning the origin of blood cells, lead to explanations which are yet only hypotheses and which call for further experiments. Science progresses by small steps and major problems are still to be solved.

The differential susceptibility of early chick embryos to 5-bromodeoxyuridine*

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The thymidine analogue, 5-bromodeoxyuridine (BrdU), has been shown to inhibit the synthesis of cell specific macromolecules without interfering markedly with cell growth and multiplication[1–10]. Since BrdU is readily incorporated into DNA synthesized in vitro and into DNA of both prokaryotic and eukaryotic cells, it has been assumed that its action is DNA-linked[10,11]. Teratogenicity of BrdU has been reported in sea urchin[12,13], amphibian[13], mouse[14,15], rat[16], hamster[17], and chick embryos[18–20]. This paper is concerned primarily with the effects of BrdU in explanted early chick embryos at different stages of development.

Effects of BrdU on early chick embryos

Explanted chick embryos at stages 3–7[21] were cultured[22] on nutrient medium (thin albumen) containing different concentrations of BrdU until the majority of corresponding controls had reached stages 9–11. 1 μg/ml caused a growth retardation of stage 4 or younger embryos, but had no apparent effect on older embryos. Irrespective of stage at explantation progressively larger doses of BrdU resulted in higher persentages of disturbed development. Of all the concentrations of BrdU tested, 8–10 μg/ml at stages 3–5 and 10–14 μg/ml at stages 6–7 appeared appropriate for examination of teratologic effects because of elevated percentages of surviving embryos with one or more discernable abnormalities (figures 1–3).

BrdU, at teratologic doses, inhibited segmentation of axial mesoderm regardless of stage at treatment. The blastodermal expansion and erythropoiesis were usually unaffected. Heart development was significantly affected only in those treated at stage 3. The magnitude of inhibitory action of BrdU in brain development was clearly stage-dependent: at stage 4 or earlier, it resulted in an open brain region (figure 1); if applied at stage 5, it inhibited the closure of the midbrain and, to some extent, the hindbrain, but the forebrain closure was unaffected (figure 2). However, BrdU had no apparent effect on brain roof closure of embryos treated at stages 6–7 (figure 3). BrdU-insensitive processes are not uncommon in developing systems, e.g., echinochrome synthesis by sea urchin embryos[12], haemoglobin synthesis by erythrocytes[2], chondroitin sulphate synthesis by chondrocytes[4], etc. Weintraub et al.[5] 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. The observed brain defects were not due to impairment of inductive interactions between chordamesoderm (Hensen's node) and competent epiblast[19]. Microscopic studies revealed that neural tissue was most susceptible to BrdU treatment. Neuroepithelial cells often had an enlarged nucleus, a characteristic of undifferentiated cells[19]. Pycnosis and chromosomal abnormalities were also observed. Mitotic figures were

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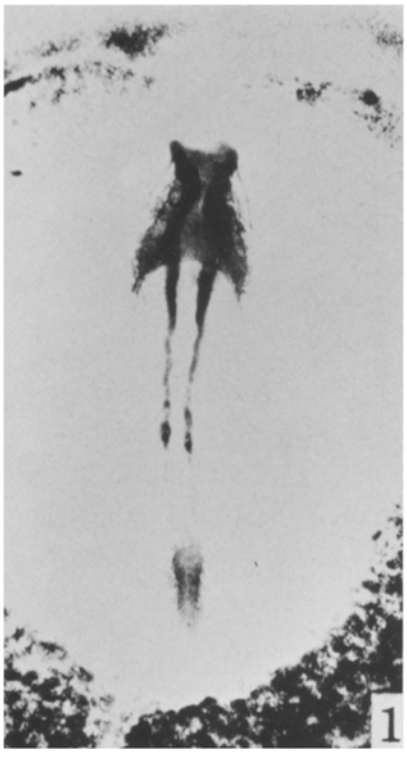

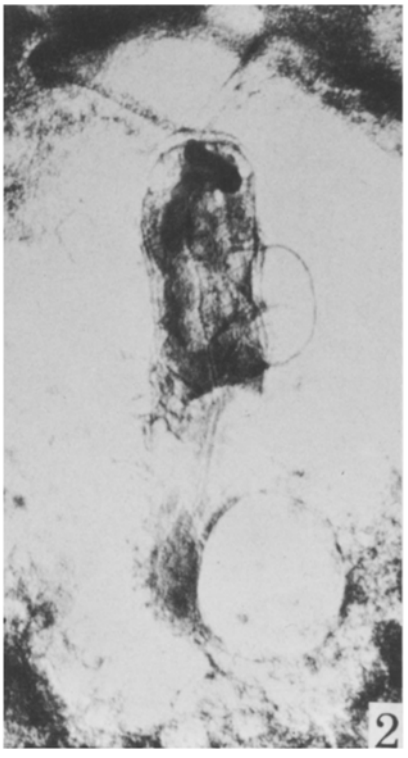

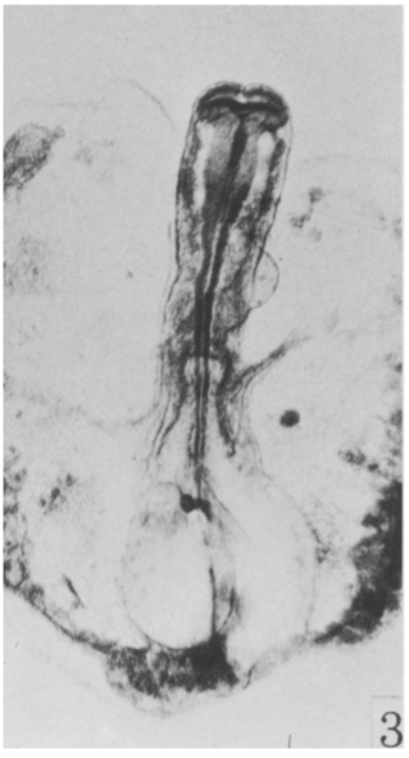

Fig. 1. Embryo explanted at stage 4 and cultured for 21 h on thin albumen with 8 μg/ml BrdU[19]. Fig. 2. Embryo explanted at stage 5 and cultured for 19 h on thin albumen with 8 μg/ml BrdU[19]. Fig. 3. Embryo explanted at stage 6 and cultured for 19 h on thin albumen with 10 μg/ml BrdU[19]. ×28.

Table 1. Morphological analysis of stages 3–7 embryos pretreated with BrdU (10–12 μg/ml) for 4 h, followed by subculturing for 24–26 h on plain nutrient medium. Control embryos were treated the same except that plain nutrient medium was used throughout cultivation

H and H stage(s) at treatment	Group	No. of embryos at subculturing	Structures showing abnormalities (% of embryos) Forebrain	Midbrain	Hindbrain	Heart	Somites
3	Control	28	14.3	10.7	10.7	14.3	14.3
	Treated	68	57.4*	41.2*	72.1*	60.3*	27.9
4	Control	52	11.5	7.7	7.7	7.7	9.6
	Treated	74	81.1*	52.7*	60.8*	14.9	33.9*
5	Control	42	7.1	7.1	4.8	2.4	7.1
	Treated	58	24.1	60.3*	29.3*	13.8	58.6*
6–7	Control	36	2.8	2.8	2.8	0.0	5.6
	Treated	42	16.7	23.8*	11.9	9.5	66.7*

* For significance of difference between control and treated groups, $p < 0.01$.

Table 2. The development of stage 4 chick embryos, subcultured for 20 h on different media following pretreatment with BrdU (3×10^{-4} M)[a] for 4–5 h (groups I–V). The embryos of group VI were treated the same except that plain nutrient medium was used throughout cultivation

Group No.	No. of embryos at subculturing	Subculture medium	Embryos (%) showing abnormalities in Brain	Neural tube	Heart	Somites
I	32[c]	BrdU (3×10^{-4} M)	100.0	84.4	21.9	100.0
II	34[b]	Thymidine (6×10^{-4} M)	20.6[d]	14.7[d]	11.8	17.6[d]
III	42[c]	Methionine (3×10^{-4} M)	33.3[d]	16.7[d]	14.3	19.1[d]
IV	36[c]	Homocysteine (3×10^{-4} M)	86.1	77.8	27.8	91.7
V	48	Uridine (3×10^{-4} M)	77.1[d]	72.9	22.9	79.2[d]
VI	34[c]	Thin albumen	14.7[d]	11.8[d]	11.8	14.7[d]

[a] Approximately 10 μg/ml. [b] Lee et al.[18]. [c] Lee and Redmond[20]. [d] Statistically significant at the 0.01 level with the same structure in group I.

distributed throughout the neuroepithelium, suggesting that interkinetic nuclear migration had been inhibited[23–26].

Microtubules have been implicated as essential in nuclear migration[25,26]. This was found not to be the case in BrdU-treated cells[27]. Microfilaments and cell flanges were less numerous and conspicuous than in controls. Whether these findings are directly associated with the observed neural tube defects remain to be answered, although microfilaments are thought to be responsible for changes in the cell shape during neurulation[28].

Among inhibitors of nuclei acid synthesis, actinomycin D has been most extensively used for causal analysis of morphogenesis in early chick embryos[29–33]. Therefore, it is of special interest to compare the effects of BrdU and actinomycin D. Morphological defects produced by BrdU (8–10 μg/ml) and actinomycin D (0.02–0.04 μg/ml) are similar, but differ in detail. Both agents strongly inhibit neural tube closure and somite formation. In contrast to BrdU, actinomycin D limits blastodermal expansion. At the cellular level, actinomycin D differs from BrdU in that it produces smaller nuclei and causes extensive necrosis. The inhibitory action of both agents can be alleviated by subsequent treatment with excess thymidine[18,20,32]. These findings suggest that both agents act on DNA complexes.

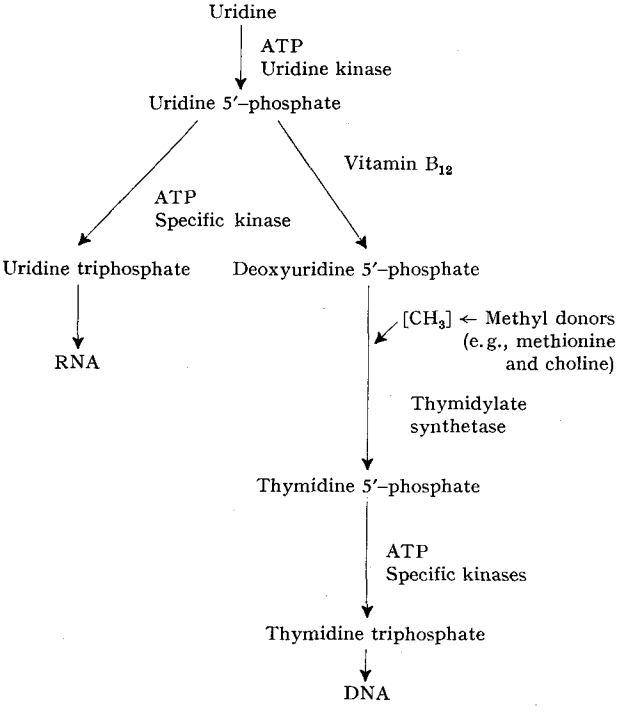

Fig. 4. Some steps involved in the interconversion of pyrimidine nucleotides (modified from Levine et al.[39]). Because DNA contains thymine instead of uracil present in RNA and ribonucleotides, a pathway for thymidine 5′-phosphate synthesis is required. It is furnished by thymidylate synthetase, which catalyzes the methylation of deoxyuridine 5′-phosphate to thymidine 5′-phosphate. Methionine and choline serve as the major methyl donors in vertebrate cells[38].

Removal of BrdU during incubation

This experimental series was carried out to further test the stage-dependent sensibility of chick embryos to BrdU. Stages 3–7[21] embryos were cultured[22] on nutrient medium with or without BrdU (10–12 μg/ml) for 4 h. Embryos were then carefully washed several times in warm PC saline, followed by subculturing for 24–26 h on plain nutrient medium. After the incubation, all embryos were examined on a warm stage (37–38 °C) under a dissecting microscope to determine morphological defects. Those with no apparent malformations were serially sectioned and reexamined. Embryos with the following abnormalities were recorded systematically: brain – open along length or irregularly folded; somites – unsegmented axial mesoderm or less numerous and poorly defined somite pairs; heart – primordia not formed or fused, primordia fused but not flexed, or small and/or nonpulsatile. Results are summarized in table 1.

It can be seen that the highest frequency of abnormalities in the forebrain, midbrain, hindbrain, somites, and heart occurred when embryos were pretreated with BrdU at stage 4, stage 5, stage 3, stages 6–7, and stage 3, respectively. In general, if treatment was begun at stage 3, it yielded acardiac embryos with an open neural tube and nearly normal somites; if treatment was delayed until stage 6 or later, it inhibited somite formation without interfering with brain roof closure and heart development. Actinomycin D is known to produce similar effects[33].

A previous study from our laboratory[19] showed that the application of BrdU, at 6 μg/ml or lower, caused no obvious variations in RNA metabolism, whereas 10–12 μg/ml inhibited the incorporation of uridine-H^3 into chick embryonic tissues, particularly neuroepithelium. If we assume that BrdU is incorporated into DNA[9–11,34] and consequently inhibits the transcription of certain genes into messenger RNA[6,9], then the observed stage-dependent sensibility would indicate that the synthesis of tissue specific nuclear RNA takes place at a particular time in the development of early chick embryos. This synthesis occurs at or about stage 3 for heart and hindbrain, at stage 4 for the forebrain,

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at or about stage 5 for the midbrain, and at stage 5 and later stages for somites. We are aware that these suggestions are speculative, but may lead to further meaningful studies.

Methyl groups vs. BrdU action

Experiments conducted in our laboratory have shown that in early chick embryos the BrdU effect could be greatly alleviated by subsequent treatment with excess thymidine or methionine, but not homocysteine (table 2). The precise mode of action of BrdU remains unclear, but the evidence indicates that the specific effects of BrdU stem from non-random and differential substitutions by BrdU in those DNA sequences responsible for the control of specialized cell functions[34–37]. However, BrdU inhibition is not the result of mutagenic action, because it is reversible[8,9,18,20] and no altered proteins can be detected[9]. Since BrdU has a bromine atom substituting for the methyl group of thymidine, and methionine is a major methyl donor for vertebrate cells[38], we have been led to interpret the BrdU effect in terms of the role of methyl groups. Methyl groups are required for the conversion of deoxyuridine 5′-phosphate to thymidine 5′-phosphate in DNA synthesis (figure 4). This conversion requires, in addition to thymidylate synthetase, adequate methylating agents[39]. Methionine, by virtue of its labile methyl group, is known to serve in this capacity[38], thus stimulating the synthesis of thymidine 5′-phosphate (and hence its dephosphorylated form, thymidine). This in turn may be related to the observations that methionine, like thymidine[8–10,18], can reverse the BrdU effect (table 2). The alleviation of BrdU effect by thymidine is correlated with a decreased incorporation of BrdU into DNA[9].

A number of studies have shown that the methyl group of methionine plays an important role in cell differentiation. For example, Parsa et al.[38] reported that 1. acinar cells of the rat pancreatic anlage cultured in vitro requires a certain level of methionine to differentiate; 2. homocysteine, a demethylated derivative of methionine, in equimolar concentrations cannot substitute for methionine to initiate morphologic and enzymatic differentiation in acinar cells. There is also evidence that the distribution of methyl groups in DNA is not random, and transmethylation is indispensable to cell differentiation[40]. Thus the failure of homocysteine to alleviate the BrdU effect appears to be directly attributable to the methyl group. Furthermore, about 30% of exogenous uridine can be incorporated into DNA of chick embryonic cells[19,31]. This incorporation is enhanced as much as 18% by the presence of excess methionine in a developing system[27]. Since BrdU is a competitive inhibitor of thymidine, the observations that uridine cannot alleviate the BrdU action as effectively as thymidine is not unexpected. Tencer and Brachet[13] reported that BrdU-treated amphibian cells were larger in diameter than their untreated counterparts. This finding raises the possibility that the effects of BrdU on cell surface[42] may be mediated by methyl groups (protons)[43].

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Chondrogenesis in chick limb buds and somites*

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The literature dealing with chondrogenesis in embryonic somite cells and limb bud cells is extensive and polarized[1–5]. The central issues are whether the sulfated proteoglycans synthesized and deposited by the definitive chondroblasts are identical to those synthesized by 1. the presumptive chondroblasts, and 2. by cells outside the chondrogenic lineage. Many investigators[6–13] have claimed that presumptive chondroblasts and a variety of nonchondrogenic cells synthesize the same sulfated proteoglycans as are synthesized by definitive chondroblasts. Somite or limb bud cells are claimed to possess an active 'chondrogenic genotype'[14], and the primary differences between presumptive and definitive chondroblasts are visualized as quantitative and incremental rather than qualitative. According to this view, the presumptive chondroblast itself transforms into the definitive chondroblast, the transition requiring merely the enchancement of synthesis of the sulfated proteoglycans which the cell already was producing. This view, that presumptive and definitive chondroblasts are one and the same cell, renders untenable the contention that pre-