

# THE EFFECTS OF 5-BROMODEOXYURIDINE ON YOLK SAC ERYTHROPOIESIS IN THE CHICK EMBRYO

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## ABSTRACT

The effects of the thymidine analog, 5-bromodeoxyuridine (BUdR), on the formation of red cells in the yolk sac of the chick embryo were examined. The prospective area opaca vasculosa from a definitive primitive streak embryo was excised, disaggregated, and deposited into a cell clump, and the cell clump was placed in organ culture. Hemoglobin synthesis is detectable after about 16 hr in culture. The formation of erythropoietic foci and incorporation of  $^{55}\text{Fe}$  into heme were used to measure the extent of erythropoiesis. Exposure to 40  $\mu\text{g}/\text{ml}$  of BUdR within 6 hr after explantation almost completely eliminated red cell formation; subsequent transfer to thymidine medium showed that the inhibition was reversible, and there was no histological evidence of analog toxicity. Between 6 and 12 hr after initiation of organ culture, the tissue became completely refractory to BUdR. DNA synthesis, as monitored by thymidine- $^3\text{H}$  and BUdR- $^3\text{H}$  pulses, was extensive both during and after the period of BUdR sensitivity. Hence, during both BUdR sensitive and insensitive periods the analog was incorporated into DNA of cells which had not yet synthesized hemoglobin. It is proposed that between 6 and 12 hr a crucial regulatory event for terminal differentiation is perturbed by the presence of BUdR in the chromosomes.

## INTRODUCTION

The developing area opaca vasculosa (AOV)<sup>1</sup> of the early chick embryo contains precursor cells which shortly will become identifiable vascular elements, red cells and endothelium. If this tissue is cultured in the presence of 5-bromodeoxyuridine (BUdR) when the explants issue from embryos of the definitive primitive streak (DPS) stage, a time prior to the grouping of precursor cells into blood islands, little or no erythropoiesis occurs, even though the tissues remain apparently quite healthy. In a matter of a few hours, the tissue can now complete its destiny in the presence of BUdR

and forms normal red cells (Wilt, 1965). We wish to report experiments designed to analyze the sensitivity to BUdR in greater detail. A dissociated-reaggregated cell culture system has been employed (Miura and Wilt, 1970); we have confirmed the sensitivity of cells from a DPS embryo, and quantitatively described the time course of the development of insensitivity. DNA synthesis and cell division occur during both sensitive and insensitive periods, and BUdR enters the cells and is incorporated into DNA at both times. The BUdR inhibition is reversible.

Recently, other systems have been analyzed for the effect of BUdR on specialized synthesis. For example, BUdR prevents myoblasts from fusing and forming myotubes (Stockdale, Okazaki,

<sup>1</sup> *The following abbreviations are used in this paper:* AOV, area opaca vasculosa; BUdR, 5-bromodeoxyuridine; DPS, definitive primitive streak; Hb, hemoglobin.

Nameroff, and Holtzer, 1964), prevents matrix accumulation around chondrocytes in culture (Abbott and Holtzer, 1968; Lasher and Cahn, 1969), and inhibits terminal differentiation in pancreatic epithelial rudiments (Wessells, 1964). Some of these examples, e.g. pancreas and myotube, involve interference with terminal differentiation in postmitotic cells; others, e.g. chondrocytes and retinal pigment cells (Coleman and Coleman, 1970), involve interference with maintenance or re-expression of already differentiated cell functions. The present system represents the first example of the effects of BUdR on a system which does not cease extensive DNA synthesis as it overtly differentiates and continues DNA synthesis after overt differentiation.

## MATERIALS AND METHODS

### *Culture*

Portions of dissociated-reaggregated chick embryos (white Leghorn, DPS stage) were prepared and cultured by a technique described in detail previously (Miura and Wilt, 1970). In brief, small fragments of the prospective AOV posterior to the primitive streak are dissociated in trypsin, filtered, washed, and deposited into a clump by centrifugation. The cell mass is placed on an egg-agar medium whereupon the precursor cells of erythroblasts "sort out" and form recognizable erythropoietic foci within 6-18 hr; hemoglobin (Hb) is present by 18 hr after initiation of the culture. By 36 hr, *in vitro*, (the standard time in the present experiments), the erythropoietic foci form isolated blood islands. Counting of numbers of foci was done after staining explants by O'Brien's (1961) benzidine technique. The number of blood island foci form a reliable index of the number and degree of development of erythroblast precursor cells.

### *5-Bromodeoxyuridine (BUdR)*

BUdR (Calbiochem, Los Angeles, Calif.) was dissolved in chick embryo saline (DeHaan, 1967) and included in the agar medium at 10-100  $\mu\text{g}/\text{ml}$  ( $3.25 \times 10^{-5}$ - $3.25 \times 10^{-4}$  M). In experiments designed to determine the time of sensitivity to BUdR, explants on control medium were transferred to BUdR medium at 0, 3, 6, 9, 12, 15, 18, and 24 hr of incubation.

When recovery from BUdR was being investigated, explants were transferred from BUdR medium (40  $\mu\text{g}/\text{ml}$ ,  $1.3 \times 10^{-4}$  M) after 6 or 12 hr of culture to normal medium containing  $2.6 \times 10^{-4}$  M thymidine. These cultures were continued for an additional 36

hr and compared to control cultures (with thymidine) cultured for 36 hr.

### *Transfer of Explants*

A number of experiments required transfer of intact explants from one medium to another. This was accomplished by explanting the tissue onto medium in which a millipore filter (type HA, 20  $\mu$  thick) was embedded 0.1-0.2 mm below the agar surface. This was accomplished by pouring the medium in two steps, allowing gelation of the first to occur before covering the filter. When tissue was ready for transfer, the filter, agar overlay, and tissue were picked up, rinsed by floating on saline, and transferred as a unit to the surface of new medium, filter surface down. Several controls showed that this constituted a complete and rapid "chase" of isotope.

Entrance of small molecules from the new medium into the explant across the millipore filter and its agar overlay was complete and rapid. A model experiment was carried out in which a 3.5 mm diameter glass filter disc (H. Reeve Angel and Co., Inc., Clifton, N. J., type 934 AH) was placed on a millipore strip coated with nonradioactive agar medium. This was placed on medium containing nucleoside- $^3\text{H}$ . Fig. 1 shows rapid penetration of nucleoside through the filter, and apparent equilibration was reached within 30 min. Placing the glass disc directly on radioactive medium gave identical results.

### *Incorporation of $^{55}\text{Fe}$ into Hemoglobin (Hb)*

A method for determining Hb accumulation was devised by using as a standard criterion the incorporation of  $^{55}\text{Fe}$  into protein-bound heme. Lack of specificity observed in some previous studies, caused by the trapping of radioiron in yolk (cf. Wilt, 1967), was successfully overcome by using iron bound to putative transferrin. The radioiron ( $^{55}\text{Fe}$ , as ferrous citrate, New England Nuclear Corp., Boston, Mass. 8.06 mCi/mg or 36 mCi/mg) was diluted with chick saline, neutralized, and hen serum was added to a final concentration of 10% (v/v). The serum-iron mixture was incubated for 1 hr at 37°C to allow interaction of iron and transferrin, then diluted into agar medium to attain a final concentration of 10  $\mu\text{Ci}/\text{ml}$ .

Explants were usually cultured on medium containing  $^{55}\text{Fe}$  for the duration of the culture period. After cessation of incubation, explants were washed three times with chick saline and frozen until use. The labeled explants were thawed in 1 ml of ice-cold distilled water and allowed to stand for 15-30 min with occasional agitation on a Vortex mixer. The extract was then centrifuged at 27,000 *g* for 20 min. The supernatant from this centrifugation was ex-

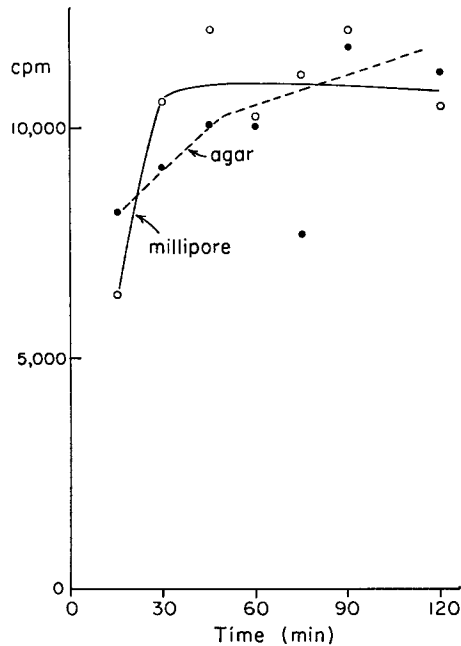


FIGURE 1 Glass fiber discs of 3.5 mm diameter were placed on millipore filters coated with a thin layer of agar (○—○) and placed on organ culture medium, or placed directly (●—●) without the millipore support on the medium, containing 16  $\mu\text{Ci/ml}$  of  $\text{BUdR-}^3\text{H}$ . The glass filter discs were removed at the indicated times, placed in scintillation vials, and counted.

tracted with 3 ml of methyl ethyl ketone adjusted to pH 2 with 0.1 N HCl (Teale, 1959, Krantz et al., 1963). This procedure quantitatively extracts heme. A sample of the ketone layer was dried in a counting vial, and radioactivity was determined by liquid scintillation counting by use of a toluene-based fluor. All samples were corrected for quenching by the channels ratio method with methyl ethyl ketone as a quencher to construct standard curves.

Radioactivity in the methyl ethyl ketone layer is almost exclusively in heme. An extract was prepared as described above, together with carrier unlabeled hemoglobin, and a sample was subjected to thin-layer chromatography. Silica gel plates (Brinkmann Instruments, Westbury, N.Y.) were used as supplied by the manufacturer. After the plate was spotted with 20  $\mu\text{l}$  of sample, it was developed with a mixture of 3 parts 2,6-lutidine: 2 parts water in an atmosphere saturated with ammonia vapor. The carrier heme migrates with an  $R_f$  of 0.63, and all the radioactivity migrates in identical fashion.

Furthermore, most of the radioactivity in the homogenates of cells cultured on medium containing  $^{56}\text{Fe}$  sedimented in a sucrose gradient the same as carrier Hb (Fig. 2). It is evident that the bulk of the

radioactivity is associated with the 4S peak, although some of the radioactivity and optical density does not enter the gradient, probably because of binding to lipid-rich yolk.

Other controls were consistent with the view that this technique measures heme iron associated with the formation of new Hb. The incorporation into cultures of AOV increased with time only after the equivalent of the 6-somite stage had been attained. Fragments of anterior area opaca, which cannot synthesize Hb, did not show incorporation above background levels. We conclude that this is a reliable and quantitative measure of the extent of Hb formation in this system.

### Radioautography

Explants were incubated for 2 hr on the agar containing 10  $\mu\text{Ci/ml}$  of thymidine- $^3\text{H}$  (methyl-thymidine- $^3\text{H}$ , New England Nuclear Corp., specific activity 2,050 Ci/mole); or  $\text{BUdR-}^3\text{H}$  (5-bromo-deoxyuridine-6- $^3\text{H}$ , New England Nuclear Corp., specific activity 5.86 Ci/mole). Explants were then transferred to the chase media containing  $10^{-4}\text{M}$  cold thymidine or 40  $\mu\text{g/ml}$  ( $1.3 \times 10^{-4}\text{M}$ )  $\text{BUdR}$ , a 10,000-fold excess of unlabeled nucleoside. After 36 hr of incubation, the explants were fixed with Carnoy's solution. In some experiments the explants were fixed just after labeling without chasing with cold nucleosides.

Radioautographs were made by standard procedure by dipping the slides in Ilford Nuclear Research Emulsion (K-5). Sections were stained with hematoxylin and eosin after they had been exposed for 7–16 days in a refrigerator.

Grain counts were made over the nuclei of the erythroblasts of at least 10 randomly selected different blood islands in the sectioned material. A minimum of five grains per nucleus was regarded as evidence of incorporation.

## RESULTS

### The Effective Dose of $\text{BUdR}$

Widely varying concentrations of  $\text{BUdR}$  have been used to suppress development of specialized cell function. The actual effective level may, of course, be similar in different instances, for tissue structure, cell permeability, and kinase levels would all play a role. We wished to find operationally a level of  $\text{BUdR}$  that would suppress blood island development without excessive toxicity. Explants were incubated in 10, 20, 40, 80, or 100  $\mu\text{g/ml}$  of  $\text{BUdR}$ , and scored, after 36 hr of culture, for number of foci and Hb staining. Faint and diffuse Hb staining and some erythropoietic foci

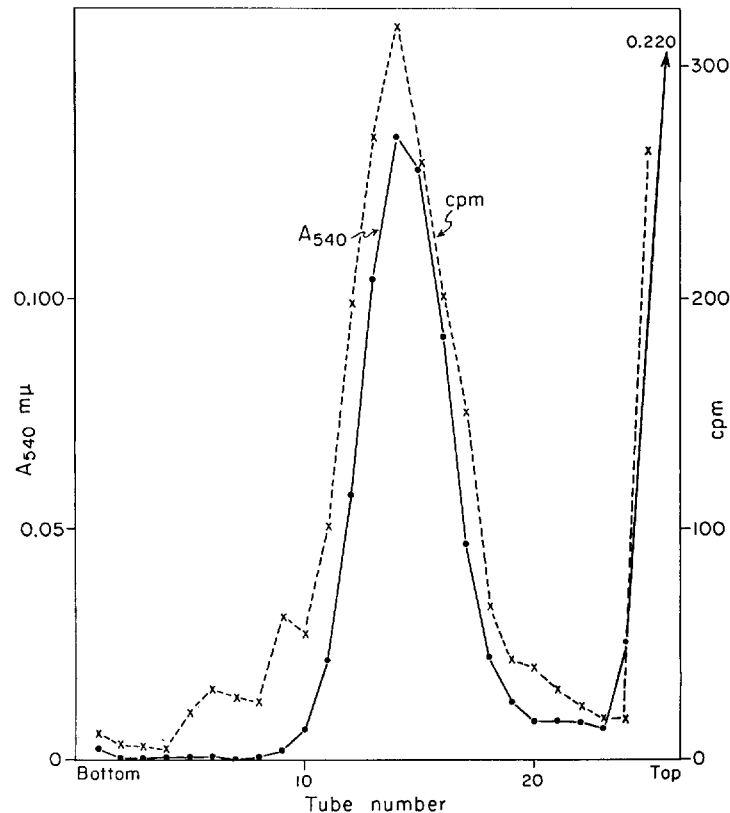


FIGURE 2 Nine fragments of AOV were cultured on standard medium containing  $10 \mu\text{Ci/ml}$  of  $^{55}\text{Fe}$  for 27 hr. The distilled water homogenate was prepared. To  $0.3 \text{ ml}$  of the clarified homogenate was added  $100 \mu\text{l}$  of 1% potassium ferricyanide and  $100 \mu\text{l}$  of cyanomet Hb (1% solution) from 5-day chick embryo red cells (Hashimoto and Wilt, 1966). The mixture was centrifuged through a 5–20% sucrose gradient containing  $5 \times 10^{-4} \text{ M}$  NaCN,  $0.01 \text{ M}$  phosphate, at pH 7 for 16 hr at 35,000 rpm. Fractions were collected, diluted with water, and the absorbance at  $540 \text{ m}\mu$  was recorded. TCA-precipitable radioactivity in each fraction was determined after addition of  $0.1 \text{ mg}$  of bovine serum albumen to each sample.

were observed at 10 and  $20 \mu\text{g/ml}$ . At  $40 \mu\text{g/ml}$ , eight of nine cultures showed no erythropoiesis, while one of nine had 2.2% the control number of foci. No Hb staining nor foci were found at higher BUdR concentrations. The  $40 \mu\text{g/ml}$  concentration was chosen for all subsequent experiments; this is similar to the effective dose previously used with whole chick embryos and explants therefrom. This concentration is about an order of magnitude higher than that used with some monolayer tissue cultures (e.g., Coleman, Coleman, and Hartline, 1969).

In explants cultured continuously with 40 (or even  $100 \mu\text{g/ml}$ ) of BUdR, typical blood islands did not appear within 36 hr. There were, however, no obvious signs of necrosis, nuclear pycnosis, or tissue derangement (Fig. 3). Examination of

explants (cultured in BUdR) at times prior to 36 hr led to the same conclusion. Some groups of large, rather basophilic cells were observed; they are not unlike the cells which sort out to construct blood islands (Miura and Wilt, 1970), and it is possible that they represent aborted attempts at blood islands. These cultures also synthesize DNA, although the extent of replacement of BUdR for thymidine in the DNA is not known at present. Current experiments indicate that it may be extensive (cf. Stockdale et al., 1964).  $40 \mu\text{g/ml}$  of BUdR seems to be an effective level of the analog without introducing visible signs of toxicity.

#### *The Development of Insensitivity to BUdR*

Previous studies have shown that some time after attainment of the DPS stage, the erythro-

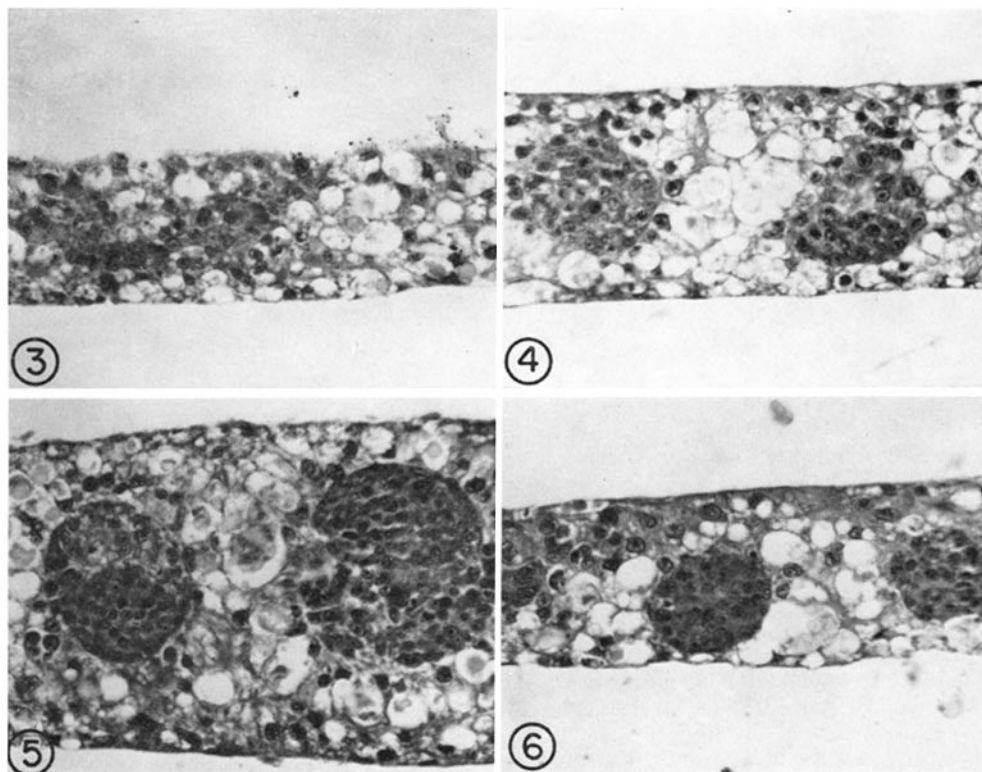


FIGURE 3 An example of tissue cultured continuously in the presence of BUdR ( $40 \mu\text{g}/\text{ml}$ ) for 36 hr is shown. Although clumps of basophilic cells may be seen, no blood islands or red cells are detectable. The vacuolated areas are endodermal cells.  $\times 280$ .

FIGURE 4 The tissue shown here was cultured on normal medium for 6 hr and transferred to medium containing BUdR and cultivated an additional 30 hr. Note the blood islands containing erythroblasts.  $\times 280$ .

FIGURE 5 An example of tissue cultured for 12 hr on control medium and subsequently moved to BUdR media for 24 hr is shown. Blood islands are obvious.  $\times 280$ .

FIGURE 6 The tissue shown here was cultured continuously for 36 hr on control medium and has constructed "normal" blood islands.  $\times 280$ .

poietic system develops a resistance to BUdR. We have attempted to define the time of acquisition of insensitivity to BUdR in the culture system used here by transferring explants (prepared from DPS embryos) from control medium to BUdR medium at various times after initiation of the culture. Figs. 3-7 describe the results. The development of foci was almost completely suppressed with transfer occurring during the first 6 hr of culture (16% at 6 hr). Thereafter, there was a rapid development of insensitivity to BUdR, so that by 12 hr the number of foci/culture was at control levels. The few foci that did develop when transfers to BUdR occurred prior to 6 hr were

smaller than normal; but when transfer to BUdR occurred at later times, focus size and appearance (Figs. 4-6) were normal. The duration of the cell cycle is about 11 hr in this tissue (Hell, 1964; Wilt, unpublished).

The synthesis of Hb was monitored in similar experiments by the  $^{59}\text{Fe}$  labeling technique, and the results are shown also in Fig. 7. There was a low background level of incorporation in samples treated with BUdR at 0 time. With transfers to BUdR occurring at later times, the apparent extent of Hb accumulation paralleled the increase in the number of foci. In cultures transferred to BUdR prior to 6 hr there was little incorporation

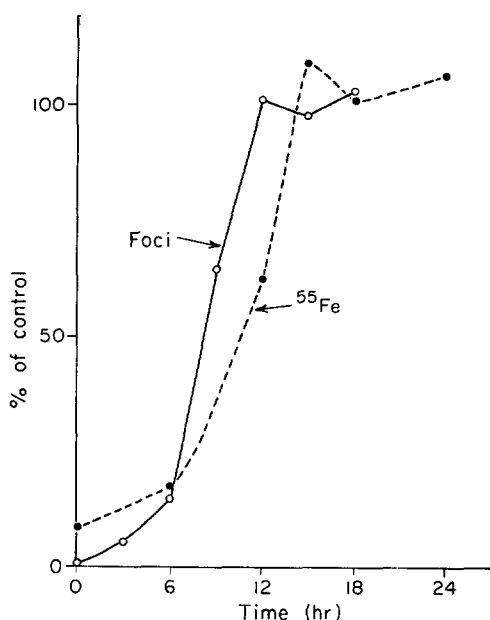


FIGURE 7 The number of erythropoietic foci (○—○) and the radioactivity due to incorporation of <sup>55</sup>Fe into heme (●—●) which can be detected in cultures of the same size after transfer from control to BUdR medium are shown. The time scale refers to the time of transfer from control to BUdR medium. The results are expressed as a comparison (in per cent) to the performance of tissue cultured continuously in control medium. All cultures were harvested after a total of 36 hr of culture before scoring for foci and radioactivity.

above initial levels. Transfer at later times showed a rapid increase in the ability of cultures to accumulate <sup>55</sup>Fe into Hb. Incorporation was about 60% of control levels by 12 hr, and 100% by 15 hr. Whether the 3 hr time difference between attainment of the control number of foci and of <sup>55</sup>Fe incorporation is significant is not known.

#### Reversibility of the BUdR Effect

Do cultures which have incorporated BUdR for a length of time sufficient to suppress all erythropoiesis (12 hr) have the ability to recover? This was tested by transferring cultures grown on BUdR (40 μg/ml) for 12 hr to control medium containing twice that molar level of thymidine. When these cultures were harvested 36 hr later and compared to controls cultured continuously on thymidine, it was obvious that considerable recovery had taken place. The BUdR-treated

cultures stained intensely for Hb, but the blood islands tended to be irregular and fused, which made counting of foci impossible.

The incorporation of <sup>55</sup>Fe could be monitored, however, and the "recovered" cultures had accumulated 40% of the level of <sup>55</sup>Fe of their companion controls. Within a day after removal from BUdR, inhibited cultures may carry out a high level of Hb synthesis and erythropoiesis.

#### The Synthesis of DNA

Hell (1964) has previously shown that explanted embryos of ages comparable to the culture period studied here contain many cells in the AOV engaged in DNA synthesis. This was examined in the present instance by pulse labeling with thymidine-<sup>3</sup>H. Cultures were exposed to thymidine-<sup>3</sup>H for 2 hr at various times during the culture period, and transferred to medium containing unlabeled thymidine at the conclusion of the pulse. All cultures were harvested at 36 hr of development. The radioautographic picture should be a reliable index of the proportion of cells in the DNA synthetic phase of the cell cycle if the cold thymidine chase was efficacious, i.e., that there was no reutilization of label, and if the cells were randomly distributed throughout the cell cycle. We presume that these conditions are satisfied: if explants were fixed at the conclusion of the pulse rather than after a chase, the same extent of labeling (per cent cells marked) was found.

About 80% of the erythroblasts were labeled if the cultures were exposed to 2-hr pulses of thymidine-<sup>3</sup>H anytime during the first 18 hr of culture. This labeling index declined to 70% if the pulses occurred during the latter 18 hr of culture (Fig. 8). Apparently about 3/4 of the erythroblasts and their precursors are engaged in DNA synthesis at any given moment during the culture period. Mitotic figures are observed at all times during the culture period. The labeling index is close to that reported by Hell (1964) for intact blastoderms. Contrary to the situation in medullary erythropoiesis, DNA synthesis occurs extensively before, during, and considerably after initiation of Hb synthesis. A representative radioautogram is shown in Fig. 9.

Some nuclei outside the blood islands are also labeled, regardless of the time during the culture period when the thymidine-<sup>3</sup>H pulse is applied. The very heavily labeled cells are probably endoderm; endoderm cell nuclei show this same promi-

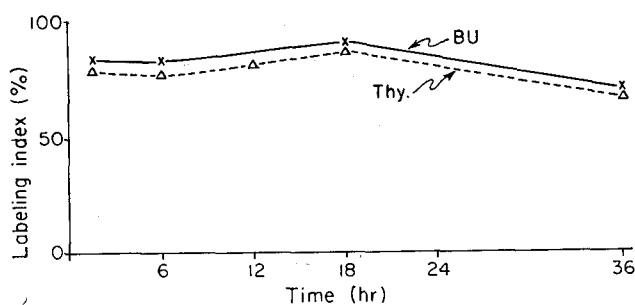


FIGURE 8 The labeling index of erythroblasts pulsed for 2 hr with thymidine-<sup>3</sup>H (Δ----Δ) and BUdR-<sup>3</sup>H (X—X) during various portions of the culture period is shown.

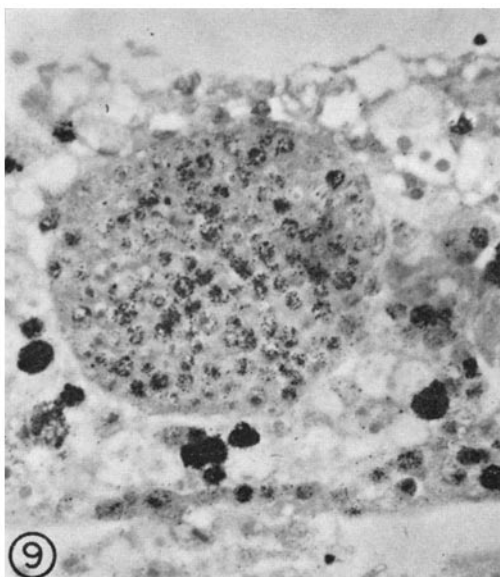


FIGURE 9 A radioautogram of a culture pulsed with thymidine-<sup>3</sup>H for 16–18 hr after beginning the culture period, followed by 18 hr of culture on unlabeled thymidine, is shown. The cells are out of focus, to display the silver grains.  $\times 355$ .

ment incorporation in cultures of intact tissue fragments.

#### *Incorporation of BUdR*

Labeling of cultures with BUdR-<sup>3</sup>H was carried out in experiments of the same design and gave almost identical results. The labeling indices associated with pulses at various times in culture are shown in Fig. 8. BUdR is incorporated into the majority of erythroblasts throughout the cul-

ture period. It is important to note that the cultures during the BUdR insensitive period (12 hr after explantation) incorporate BUdR-<sup>3</sup>H extensively. Fig. 10 shows an example of tissue pulsed for 2 hr with BUdR-<sup>3</sup>H during the insensitive phase; erythroblasts of the blood island are labeled as well as some presumed endodermal cells. Similar labeling indices were obtained when labeled BUdR was used at concentrations of 40  $\mu\text{g}/\text{ml}$ . The predominance of the nuclear location of the grains makes it likely that BUdR is in DNA, and the grains are eliminated by prior digestion of tissue sections with DNase, but not by RNase. The concentration of BUdR-<sup>3</sup>H used in this labeling experiment is much lower (by a factor of  $10^3$ ) than the level needed for inhibition of erythropoiesis. If the BUdR-<sup>3</sup>H pulse is followed by unlabeled BUdR during the insensitive portion of the culture period, labeled erythroblasts are easily detectable (Fig. 11).

#### DISCUSSION

The tissue of the area opaca vasculosa (DPS embryo) possesses a period of sensitivity to BUdR which prevents erythroblast production. After approximately 12 hr in culture the tissue is completely insensitive to the analog. The target cell(s) for BUdR have not been definitively identified. While it is likely that the erythroblast precursors are the sensitive elements, endodermal cells cannot be rigorously eliminated. Endoderm does improve the erythropoietic performance of the tissue (Miura and Wilt, 1969). BUdR is incorporated into nuclei of both endoderm and mesoderm during both the sensitive and insensitive portions of the culture period. We shall assume that the erythroblasts and their precursors are the cells

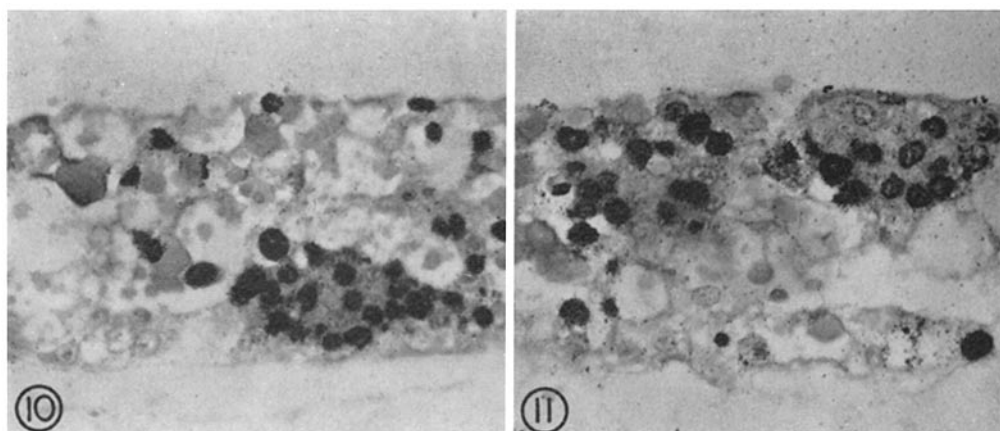


FIGURE 10 A radioautogram of a culture pulsed with BUdR-<sup>3</sup>H for 16–18 hr after beginning the culture period, followed by cultivation for 18 hr on medium containing unlabeled thymidine. Many erythroblasts are clearly labeled.  $\times 355$ .

FIGURE 11 A radioautogram of a culture pulsed with BUdR-<sup>3</sup>H from 16 to 18 hr, followed by cultivation in 40  $\mu\text{g}/\text{ml}$  of unlabeled BUdR until 36 hr total had elapsed. Notice that the erythroblasts which had formed in the presence of BUdR are clearly labeled.  $\times 355$ .

primarily sensitive to BUdR, but the argument applies even if endodermal cells are involved.

Cells that have incorporated BUdR during their sensitive period may undergo rather quickly (not more than two cell divisions) the completion of differentiation upon transfer to control medium. This recovery may be due to excision of segments of DNA containing BUdR, followed by repair, or to dilution of the analog by DNA replication; we have no direct evidence to distinguish the hypotheses.

The results are formally similar to those on which is based the widely reported phenomenon of insensitivity to drugs that derange nucleic acid metabolism prior to the detectable onset of overt differentiation (for example, see Davidson, 1968). The afore-mentioned studies are usually interpreted to mean that expression of genes essential for the terminal phases of differentiation has already occurred prior to the period of insensitivity to drugs or analogs. This is probably the case here; what is unusual is that the analog chosen is incorporated into DNA and somehow modifies the expression of genes. How does incorporation of BUdR perturb the completion of differentiation? Why are there phases of insensitivity, and how is the molecular action of BUdR manifested at the cell and tissue level?

#### *Is the Primary Site of Action of BUdR at the Level of DNA?*

As mentioned before, results similar in some respects to those reported here have already been obtained in a variety of other cell types completing or maintaining their overt differentiated state. A site of action for BUdR other than DNA is possible. In the present instance the radioautographic evidence demonstrates incorporation of BUdR into DNA, and we have recently shown that the DNA of these cells has the expected increase in buoyant density characteristic of DNA containing BUdR (Fabian and Wilt, unpublished). A recent search for BUdR in cell surface precursors was negative (Holthausen et al., 1969). The effect of BUdR in this system is reversed by thymidine but not by uridine (Wilt, 1967). The development of insensitivity requires almost the same length of time as the length of time required by the population to complete a cell cycle (Hell, 1964; cf. Bischoff and Holtzer, 1970). We believe that the evidence is compatible with the idea that the analog is incorporated into DNA which is the sole primary site of action. Other alterations of the cell surface, etc. would be a secondary manifestation of analog incorporation.

By definition, the effect of BUdR is not mutagenic since the action of BUdR is an all-or-none



effect and occurs in the absence of light (where BUdR is a more effective mutagen). Nor does an ill-defined toxic effect seem to be the case. Treated cultures recover quickly, the cells having incorporated BUdR become erythroblasts, and histological signs of toxicity are absent. Nor does BUdR grossly affect the rate of synthesis and sedimentation distribution of newly synthesized RNA, nor alter the rate of incorporation of amino acid into protein (Wilt, 1967).

### *The Consequences of BUdR Incorporation on Gene Activity*

If the sole primary site of incorporation of BUdR is DNA, how does this lead to the observed derangements? BUdR might act by virtue of its effects on the information encoded in DNA, *per se*. In other words, substituted DNA may fail to transcribe accurate messages (RNA), or might interfere with saltatory gene replication (Cahn and Lasher, 1969). In view of the all-or-none effect and nonmutagenic action of BUdR, we believe that it is more likely that BUdR affects regulatory events dependent on the *whole chromosome*. In other words, we propose that it is the physical presence of BUdR and its effect on chromosome organization (or reorganization at mitosis or S) rather than perturbations of coding properties that are involved. It is known that sand-dollar embryo chromosomes containing BUdR display gross abnormalities (Mazia and Gontcharoff, 1963). The probability of disturbances in chromosome functional morphology is high; disturbances at this level might result in perturbations of important regulatory events in the differentiation process.

The target cells could become insensitive after 12 hr in culture because the regulatory event(s) that BUdR perturbs has already occurred, and need occur no more. A consequence of this hypothesis is that the regulatory event (s) need not be completed at a set chronological time. Overt differentiation may be postponed, at least for a while, by maintaining tissue in the analog and then transferring it to normal media. Effects on myoblasts may also be postponed (Bischoff and Holtzer, 1970).

### *How Is the Sensitivity to BUdR Manifested?*

The suggestion that presence of the analog secondarily results in disturbances of cell surface

behavior (Holtzer and Abbott, 1968) is consistent with our findings. We have previously shown that sorting out of precursor cells occurs in these cultures (Miura and Wilt, 1970) within 6 hr after initiation of culture. Since cultures are still sensitive to BUdR by 6 hr after culture initiation, it is unlikely that the inhibition of Hb synthesis by BUdR is primarily due to interference with this initial sorting out process, i.e., it is unlikely that BUdR induces cells to forget who they are. But tight blood island-like clusters never appear when there is early continuous exposure to BUdR, and, even in cultures recovering from BUdR, the erythroblasts are reticulate and fused in their arrangement rather than being the punctate foci of control cultures. BUdR may be affecting the development of important cellular associations after the initial sorting out process has occurred. An ultrastructural study of the condition of these cells would be worthwhile.

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