

INHIBITION OF MYOBLAST FUSION AFTER ONE ROUND OF DNA SYNTHESIS IN 5-BROMODEOXYURIDINE

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ABSTRACT

The thymidine analogue 5-bromodeoxyuridine (BUdR) has a differential effect on the synthesis of tissue-specific products and molecules required for growth and division. Proliferating myogenic cells cultured in BUdR fail to fuse and fail to initiate the synthesis of contractile protein filaments. Conversely, BUdR has but a minor effect on cell viability and reproductive integrity. Low concentrations of BUdR result in an enhancement of cell number relative to the controls; higher concentrations are cytotoxic. Suppression of myogenesis is reversible after at least 10 cell generations of growth in the analogue. Cells that do not synthesize DNA, such as postmitotic myoblasts and myotubes, are not affected by BUdR. Incorporation of BUdR for one round of DNA synthesis was accomplished by first incubating myogenic cells, prior to fusion, in 5-fluorodeoxyuridine (FUdR) to block DNA synthesis and collect cells in the presynthetic phase. The cells were then allowed to synthesize either normal DNA or BU-DNA for one S period by circumventing the FUdR block with BUdR or BUdR plus thymidine (TdR). The cultures were continued in FUdR to prevent dilution of the incorporated analogue by further division. After 3 days, the cultures from the FUdR-BUdR series showed the typical BUdR effect; the cells were excessively flattened and few multinucleated myotubes formed. Cells in the control cultures were of normal morphology, and multinucleated myotubes were present. These results were confirmed in another experiment in which BUdR-³H was added to 2-day cultures in which myotubes were forming. Fusion of thymidine-³H-labeled cells begins at 8 hr after the preceding S phase. In contrast, cells which incorporate BUdR-³H for one S period do not fuse with normal myotubes.

INTRODUCTION

If DNA directs all cell metabolism, then there are probably two broad categories of DNA activity during differentiation. First, the DNA codes those essential molecules common to all cells: the respiratory enzymes and associated structural proteins, the enzymes synthesizing amino acids, common sugars, nucleotides, and all the species of molecules required for cell multiplication. The

second category involves the activation of different sets of nucleotide sequences in subpopulations of cells. It is this mechanism, the turning on or off of certain genes regulating tissue-specific "luxury" molecules, that is thought to control differentiation (Holtzer, Bischoff, and Chacko, 1968).

Modification of DNA by base analogue substitution appears to have differential effects on these

two classes of DNA function. 5-Bromodeoxyuridine (BUdR), a pyrimidine analogue, is incorporated into the DNA of dividing cells in place of thymine (Eidinoff et al., 1959; Djordjevic and Szybalski, 1960; Simon, 1963). The consequence of such replacement in differentiating cells is a rapid inhibition of the synthesis of tissue-specific components, while cell growth and proliferation are relatively unaffected. Skeletal muscle cells grown in BUdR do not fuse and do not synthesize contractile protein filaments (Stockdale et al., 1964; Okazaki and Holtzer, 1965; Coleman et al., 1969). BUdR also interferes with the synthesis of chondroitin sulfate by chondrocytes (Abbott and Holtzer, 1968; Coleman et al., 1968; Chacko et al., 1969; Lasher and Cahn, 1969) and the synthesis of hyaluronic acid by cultured amnion cells (Bischoff and Holtzer, 1968a). The effects of BUdR are reversible. When the analogue is removed and the cells are allowed to divide in normal medium, many of the progeny of the affected cells resume their specialized functions (Okazaki and Holtzer, 1965; Holtzer and Abbott, 1968; Holtzer and Bischoff, 1970).

The experiments to be described demonstrate that: (a) a variety of halogenated pyrimidines suppress myogenesis without interfering with cell replication, (b) exogenous thymidine blocks the action of the analogues, and (c) unifilar incorporation of BUdR suppresses myogenesis in the daughter cells.

MATERIAL AND METHODS

Myogenic cells were obtained from 10 day chick embryo breast muscle as described by Bischoff and Holtzer (1968b). The tissue was treated with 0.2% trypsin in Ca^{++} - and Mg^{++} -free Simms' solution for 45 min, transferred to growth medium, and dissociated by flushing through a pasteur pipette. Clumps and fibrous material were removed by filtration through a double thickness of lens paper. Cells were grown in either 35 or 60 mm plastic culture dishes (Falcon Plastics, Los Angeles) at an initial concentration of 5×10^5 cells/ml medium. The volume of the inoculum was adjusted to give the same initial density (7.5×10^4 cells/cm²) in each size dish. The medium consisted of Eagle's MEM with 10% horse serum, 10% embryo extract, 1% antibiotic-antimycotic solution and 0.2 mM L-glutamine. Embryo extract was prepared from 11-day chick embryos; other reagents were obtained from Grand Island Biological Company, Grand Island, N. Y. Cultures were incubated at 37.5°C in an atmosphere of 5% CO_2 :95% air and kept in the dark except for a 5

min period each day while the medium was changed. FUdR was donated by Hoffmann-La Roche, Inc., Nutley, N. J. All other analogues and nucleotides were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

For histological examination, cells were grown on coverslips, fixed in ethanol:formalin:acetic acid (20:2:1), and stained with hematoxylin or the Feulgen reaction. Glycerinated or fixed but unstained cultures were examined with a Zeiss polarizing microscope for the presence of birefringent myofibrils. The acetic acid-crystal violet technique of Sanford et al. (1951) was used for enumeration of nuclei in preparing growth curves. This method avoids the problem of loss of nuclei during myotube disruption in trypsin-dissociated preparations.

Labeling was carried out with 5-bromodeoxyuridine-6-³H (New England Nuclear, Boston; specific activity 8c/mmole). Growth and proliferation of labeled cultures appeared normal and there was no evidence of tritium toxicity. Radioautographs of cells grown on coverslips were prepared as described previously (Bischoff and Holtzer, 1969).

For determination of amino acid incorporation, cells were incubated in 5 $\mu\text{c}/\text{ml}$ leucine-³H (New England Nuclear, Boston, specific activity 5 c/mmole) for 4-hr periods. The cultures were washed with cold saline and fixed in cold 10% trichloroacetic acid. After thorough washing with water followed by absolute alcohol, the cells were scraped from the dish, dried in vacuo, and dissolved in N-chlorosuccinimide (NCS) (Amersham/Searle, Des Plaines, Ill.). One ml of the NCS solution was added to 10 ml scintillator fluid (5.0 gm 2,5-diphenyloxazole (PPO); 0.5 gm 1,4-bis [2-(5-phenyloxazolyl) benzene (POPOP) per liter toluene] and counted in a Beckman LS-200B liquid scintillation system (Beckman Instruments, Inc., Fullerton, Calif.). Background was less than 0.1% of the sample counts.

RESULTS

Behavior of Myoblasts During Continuous Exposure to BUdR

The developmental sequence and histology of myogenic cells in monolayer culture has been described (Holtzer, Abbott, and Lash, 1958; Bischoff and Holtzer, 1968b; 1969). A majority of the cells plated are in the division cycle and pass through at least one round of DNA synthesis during the first day. The cell generation time is about 10 hr. After completing mitosis, some of the cells withdraw from the cell cycle during G_1 and fuse to form multinucleated units. Multinucleated units appear during the second culture

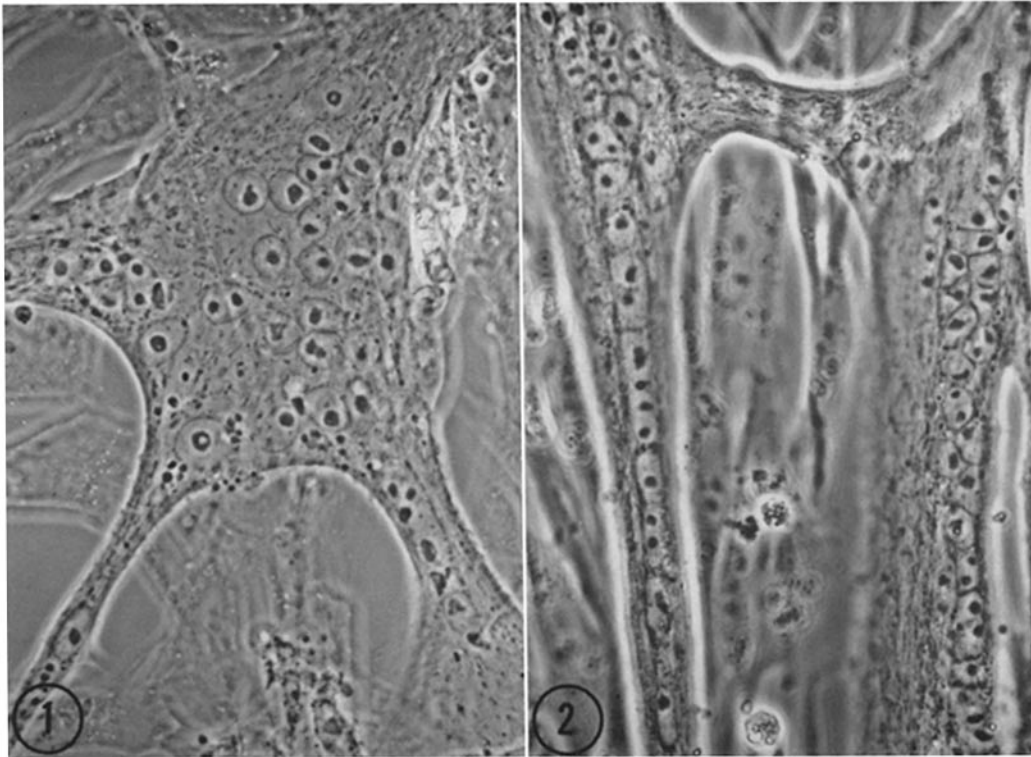


FIGURE 1 Phase-contrast micrograph of living 2 day culture, showing bifurcating myotube with four branches. Such myotubes change rapidly during subsequent days in culture to form the characteristic elongate myotubes. $\times 640$.

FIGURE 2 Segments of two adjacent myotubes connected by a bridge of sarcoplasm. More than 50 nuclei lying within a common sarcoplasm may be counted in this photograph. 3 day culture, phase-contrast. $\times 640$.

day and elongate rapidly to form myotubes (Figs. 1 and 2). The presence of contractile proteins can be detected on the second day (Okazaki and Holtzer, 1966), while fusion of additional mononucleated cells continues for about 4 days.

Cultures initiated in from 3.2×10^{-6} to 1.6×10^{-4} M BUdR differ little from untreated cultures during the first day (Fig. 3). Subsequently, however, myotube formation fails to occur and, instead of fusing, the cells move away from each other (Figs. 4 and 5). Concurrently, there is an increase in diameter and decrease in refractility. This effect is more pronounced with the higher concentrations of BUdR (compare Figs. 6 and 7). Excessive flattening has also been observed in HeLa (Hakala, 1959; Kajiwara and Mueller, 1964) and KD (Littlefield and Gould, 1960) cells

and chondrocytes (Holtzer and Abbott, 1968) grown in BUdR.

Formation of normal myotubes is suppressed in the presence of BUdR (Figs. 5-7). As the cultures reach confluence individual cells become tightly packed with considerable overlapping, but cytoplasmic fusion does not take place. Likewise, contractile protein formation cannot be demonstrated with polarizing optics or fluorescent anti-myosin (Okazaki and Holtzer, 1965, 1966). An exception to the absence of overt signs of muscle formation in BUdR is a small number (less than 1%) of highly elongate cells in the older BUdR cultures (Fig. 5). Some of these are multinucleated, with up to six nuclei, and occasionally exhibit spontaneous contractions and cross-striated myofibrils. Experiments described below demonstrate

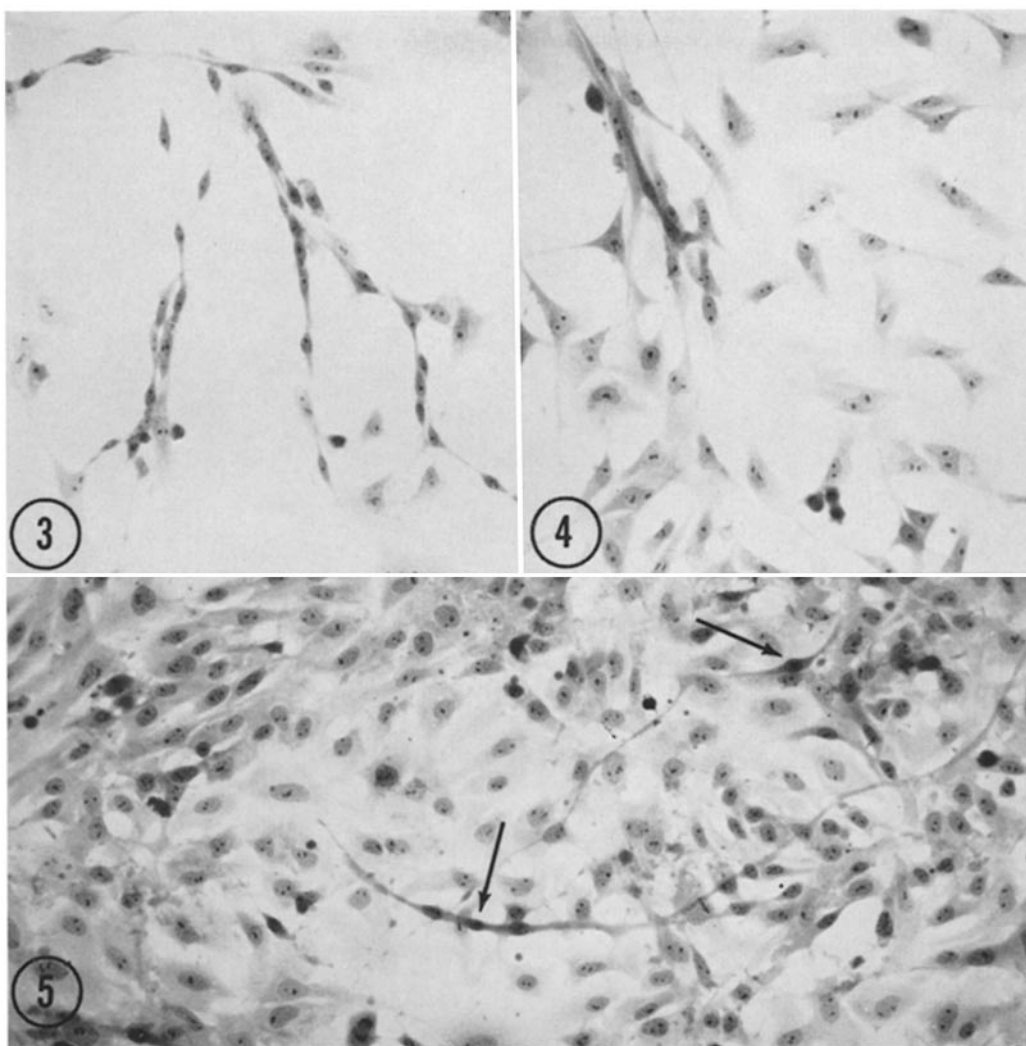


FIGURE 3 Cells grown in 1.6×10^{-4} M BUdR for 24 hr. The linear associations of spindle-shaped cells are characteristic of untreated cultures during the first day. $\times 256$.

FIGURE 4 Culture after 48 hr in BUdR. The aggregates have broken apart and large stellate cells predominate in the cultures. $\times 256$.

FIGURE 5 Culture after 72 hr in BUdR. The majority of cells are large and irregularly shaped. A small number of mono- and multinucleated cells with long twisted processes are present (arrows). $\times 256$.

that such elongate cells are the only ones in the cultures that have *not* incorporated BUdR.

To determine the extent of BUdR incorporation during continuous growth in the analogue, cultures were initiated in 1.6×10^{-4} M BUdR to which was added $1 \mu\text{c}/\text{ml}$ of BUdR- ^3H (specific activity in medium $6.2\text{mc}/\text{mmole}$). The cultures were continued for 3 days with a daily

change of medium containing cold and labeled BUdR. Radioautographs showed that more than 99% of the cells incorporate the analogue during the 3 day exposure. All the labeled cells appear flattened and enlarged. Approximately 0.6% (33/5000) of the cells are unlabeled (Fig. 8) and correspond in appearance to the elongate cells described above. The persistence of unlabeled cells

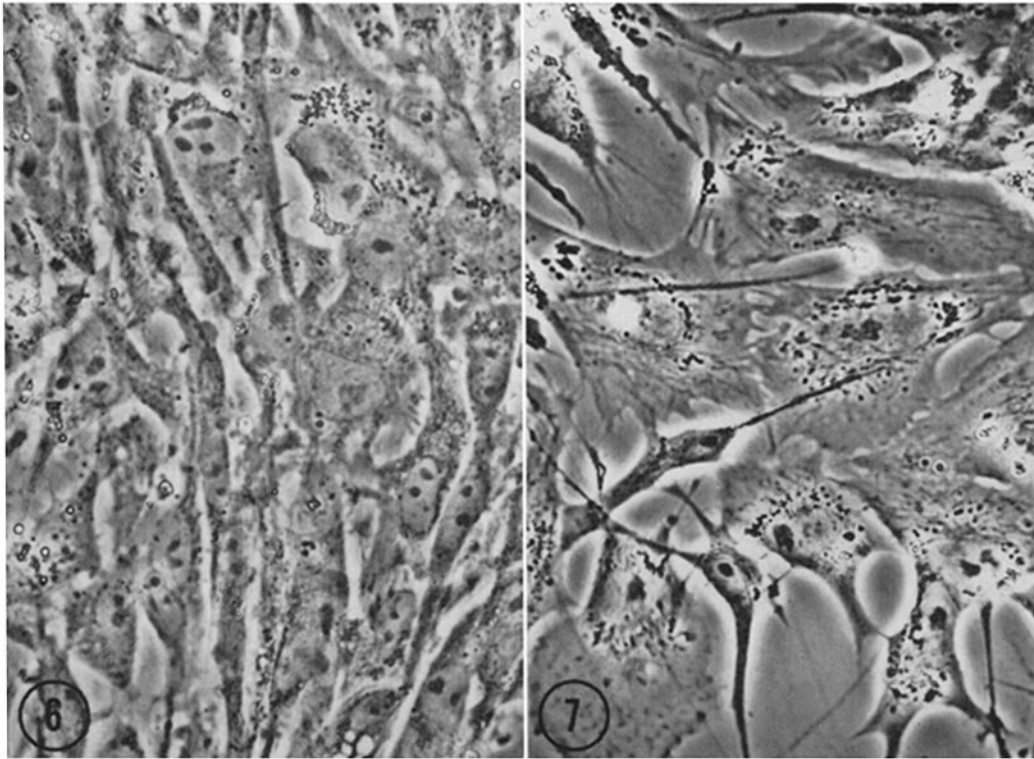


FIGURE 6 Cells grown in 1.6×10^{-5} M BUdR for 3 days, showing the moderate degree of cytoplasmic enlargement observed with this concentration of analogue. Fusion does not take place in such cultures. Living cells, phase-contrast. $\times 800$.

FIGURE 7 Cells grown in 1.6×10^{-4} M BUdR for 3 days. Note the extremely flattened, irregularly shaped cells with numerous cytoplasmic spikes. Living cells, phase-contrast. $\times 800$.

under these conditions is consistent with continuous thymidine- 3 H-labeling experiments performed earlier during which we found that a small number of the cells in the initial inoculum are postmitotic and fuse without synthesizing DNA in vitro (Bischoff and Holtzer, 1969). Evidently, these postmitotic cells escape the effects of the analogue since they do not replicate their DNA in culture.

There is additional evidence that the BUdR must be incorporated into the DNA to be effective. Since myotube nuclei do not synthesize DNA or divide (Holtzer et al., 1958; Konigsberg et al., 1960; Stockdale and Holtzer, 1961; Okazaki and Holtzer, 1966; Bischoff and Holtzer, 1969), the myotubes from older cultures provide a control for the effects of BUdR on dividing cells. Three- and 4-day cultures were incubated in 1.6×10^{-4} M BUdR for 7 days. Myotubes in these cultures

continued to show normal growth and maturation and began to contract spontaneously at the same time as did myotubes in control cultures. Increase in the quantity of myofibrils and alignment of contractile protein, as judged by birefringence between crossed polarizers, was similar to that found in the controls. There was no evidence of flattening or spreading of the myotube membrane. The dividing mononucleated cells lying between myotubes, however, became flattened and enlarged in the BUdR cultures.

Other Halogenated Pyrimidines

Several other halogenated pyrimidines were tested at the same concentration as the BUdR (1.6×10^{-4} M). Of these, 5-chlorodeoxyuridine, 5-iododeoxyuridine, and 5-bromodeoxycytidine (BCdR) inhibited myogenesis and produced similar alterations in cell morphology as did BUdR.

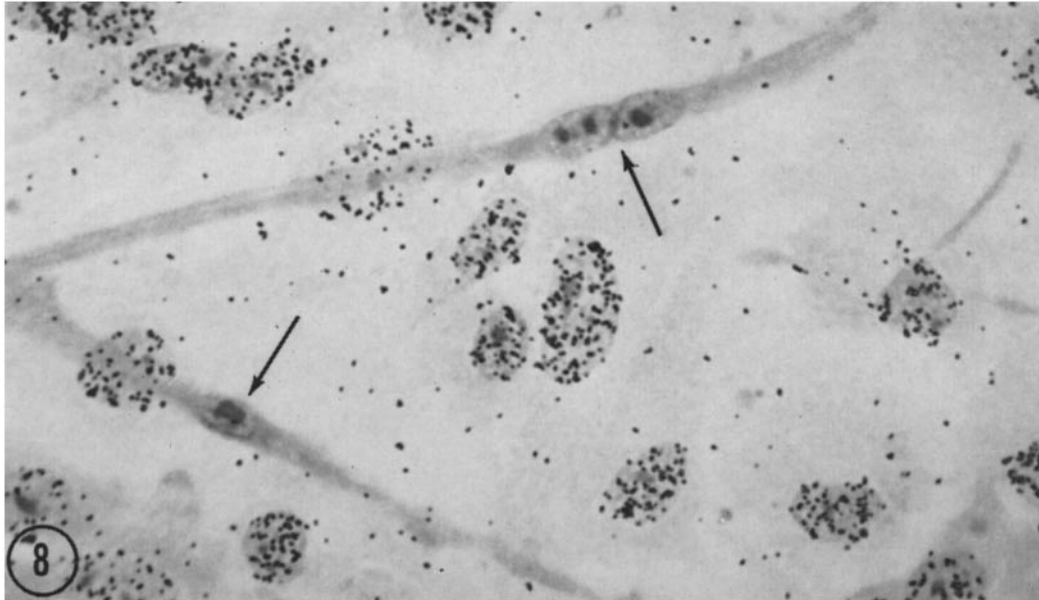


FIGURE 8 Radioautograph of culture grown continuously in $1 \mu\text{c/ml BUdR-}^3\text{H}$ (total BUdR concentration, $1.6 \times 10^{-4} \text{ M}$) for 3 days. Virtually all the mononucleated cells have incorporated the label and show the typical BUdR morphology. This field was selected to show two elongate bipolar cells (arrows) that have not incorporated the analogue. One of these is binucleated. Such unlabeled cells comprise about 0.6% of the population. $\times 840$.

It is likely that the BCdR is converted to BUdR by the enzyme deoxycytidylate deaminase (Cramer et al., 1961). Although cell production was not measured with these analogues, mitoses were common in the cultures and the amount of growth appeared to be comparable to that obtained with BUdR. 5-Fluorodeoxyuridine (FUdR) blocks DNA synthesis and mitosis but does not directly prevent myoblast fusion or produce extreme cell flattening (see below). 5-Bromouridine, 5-bromouracil, and deoxyuridine do not block fusion.

A series of experiments were carried out to assess the effectiveness of normal pyrimidines in preventing the action of the analogues. Cultures were initiated in either $1.6 \times 10^{-4} \text{ M}$ BUdR or bromodeoxycytidine (BCdR) and received in addition each of the following compounds at twice the molar concentration of the analogues: thymidine, deoxycytidine, deoxyuridine, or uridine. Of these, only thymidine was able to prevent the inhibition of myogenesis by BUdR or BCdR. Cultures grown in either analogue plus thymidine are indistinguishable from untreated control cultures.

Effect of Prolonged Growth in BUdR and Reversibility of the BUdR Suppression

The descendants of cells grown in BUdR for 5 days are capable of forming myotubes when subcultured and replated in normal medium (Okazaki and Holtzer, 1965). Myotubes appeared in the subcultures about 4 days later than did myotubes in normal primary cultures. These results were confirmed. In addition, we tested the ability of BUdR-suppressed cells to express their myogenic phenotype after prolonged growth in the analogue.

Freshly-isolated myogenic cells were grown in BUdR ($1.6 \times 10^{-4} \text{ M}$) for a total of 15 days. The cells were subcultured twice during this period and replated at low density ($7.5 \times 10^4 \text{ cells/cm}^2$) to maintain a high rate of division (the effect of BUdR on cell production is considered below). Thus, the average amount of BUdR incorporated into the DNA should approach the maximum possible for the external concentration supplied to the cells. A few highly attenuated myotubes formed in the BUdR primary cultures, but there were no myotubes or elongated cells in the two

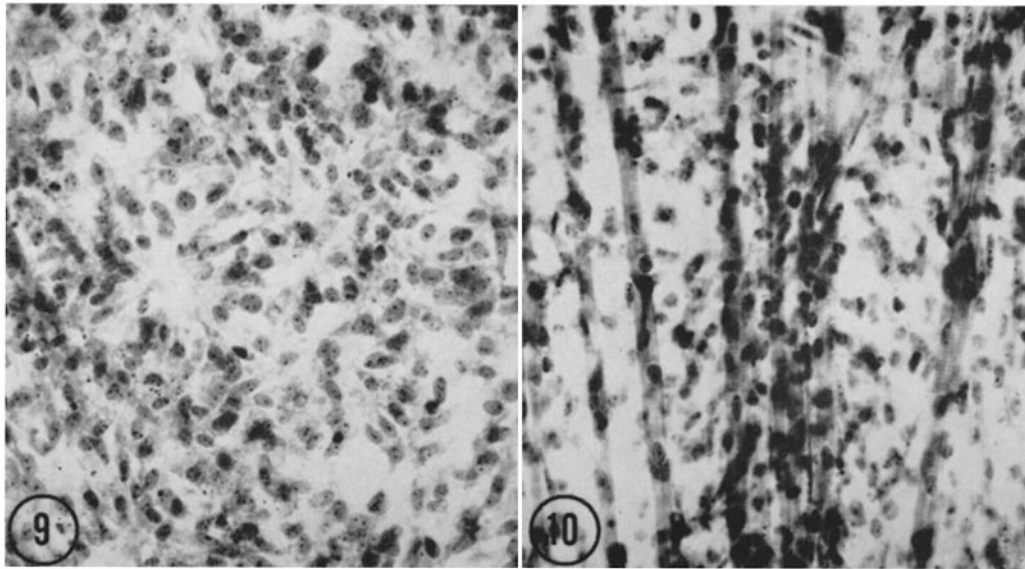


FIGURE 9 Cells after 10 days' growth in 1.6×10^{-4} M BUdR. After the first 5 days, the cells were subcultured, replated at low density (7.5×10^4 cells/cm²), and grown for a second 5 day period in BUdR. No elongate cells were present in the subculture. $\times 320$.

FIGURE 10 Myotube formation after prolonged growth in BUdR. Cells were subcultured twice in 1.6×10^{-4} M BUdR for a total of 15 days, then subcultured and replated in normal medium. No myotubes formed during this subculture generation. After 10 days, the cells were again subcultured in normal medium and myotubes began to form at 3 days. The photo shows a field of such a culture at 7 days. $\times 320$.

BUdR subcultures (Fig. 9). After 15 days the cells were again subcultured and this time were replated in normal medium. During the first 5 days of growth, the cells retained the appearance typical of BUdR cells, but by 10 days a high proportion of elongate cells arose in the cultures. No multinucleated cells were observed. At 10 days the cells were again subcultured in normal medium. Multinucleated myotubes appeared at 3 days and increased in frequency with continued culture (Fig. 10). The proportion of nuclei in myotubes was approximately the same in these cultures as it was with fresh myogenic cells. Thus, myogenic precursor cells are able to reproduce themselves with high efficiency during a total of 25 days of growth in vitro, even though overt muscle differentiation has been suppressed by BUdR. Under these conditions there has been no "overgrowth" of myogenic cells by fibroblasts. These results are in contrast to the loss of presumptive myoblasts that occurs in less than 1 wk when myogenic cells are allowed to fuse in normal medium (Bischoff and Holtzer, 1969).

Similar experiments have been carried out with cells grown for up to 3 wk in 1.6×10^{-5} M BUdR. The lag period between removal from 1.6×10^{-5} M BUdR and formation of myotubes is comparable to that obtained with 1.6×10^{-4} M BUdR.

Effect of Unifilar Incorporation of BUdR

The fact that BUdR is incorporated into the DNA of myogenic cells has been established by the use of density gradient centrifugation and by radioautography with tritiated BUdR (Stockdale et al., 1964; Okazaki and Holtzer, 1965). It has not been determined, however, whether incorporation of the analogue during one round of DNA synthesis is sufficient to block fusion of myoblasts during the following G₁. Since replication in BUdR is accompanied by semiconservative distribution of the newly synthesized DNA (Simon, 1963; Haut and Taylor, 1967), growth in the analogue for one S period should produce daughter cells that have one normal and one BU-containing polynucleotide strand in the DNA duplex.

The generation time of myogenic cells is 10 hr

and the S phase, 5 hr (Bischoff and Holtzer, 1969). Exposure of 1-day cultures to BUdR for one cell generation does not block fusion but merely delays its onset for about 12 hr. This suggested that the BUdR had a transient effect but was rapidly diluted from the DNA by subsequent division in the absence of the analogue. An experiment was designed to permit incorporation of BUdR into a single strand of the DNA duplex, block further DNA synthesis, and then challenge the cells to display their capacity for fusion and for contractile protein synthesis. The thymidine analogue 5-fluorodeoxyuridine (FUdR) inhibits the enzyme thymidylate synthetase and brings DNA synthesis and mitosis to a halt when thymidylic acid is exhausted (Cohen et al., 1958; Taylor et al., 1962). The efficacy of FUdR in these cells was determined by adding 10^{-6} M FUdR to 24-hr cultures and measuring the mitotic index at 2-hr intervals. The mitotic index began to drop sharply after 2 hr and was essentially 0 by 10 hr after addition of FUdR (Fig. 11). Exogenous thymidine or BUdR can circumvent the inhibition of DNA synthesis by FUdR (Paul and Hagiwara, 1962). FUdR also serves to induce partial synchrony by collecting cells at the beginning of S (Erikson and Szybalski, 1963; Whitmore et al., 1967). When the FUdR block is relieved by BUdR, most of the cells should begin to synthesize BU-DNA.

Cultures were initiated in medium containing 10^{-6} M FUdR. After 12 hr, BUdR (1.6×10^{-4} M) or BUdR plus thymidine (3.2×10^{-4} M) was added to the inhibited cultures; other cultures were maintained as FUdR controls. The concentration of thymidine used prevents the suppressive effects of BUdR on myogenic cells. The cultures were incubated for 7 hr, and then the cells were washed, suspended with trypsin, and plated in fresh dishes to facilitate removal of residual BUdR. All dissociating and rinse solutions contained FUdR, and the cells were maintained in this inhibitor throughout the remainder of the period. Thus, the cells were subject to inhibition of DNA synthesis for the entire experiment except for a 7 hr period during which the block was relieved with either BUdR or BUdR plus thymidine. At the end of the 7 hr reversal period, the mitotic index of the cells in FUdR + BUdR and in FUdR + BUdR + TdR was about twice the normal value, while the mitotic index of the FUdR cells remained essentially zero. Cell counts made at this time indicated that about 75% of the cells had divided during the

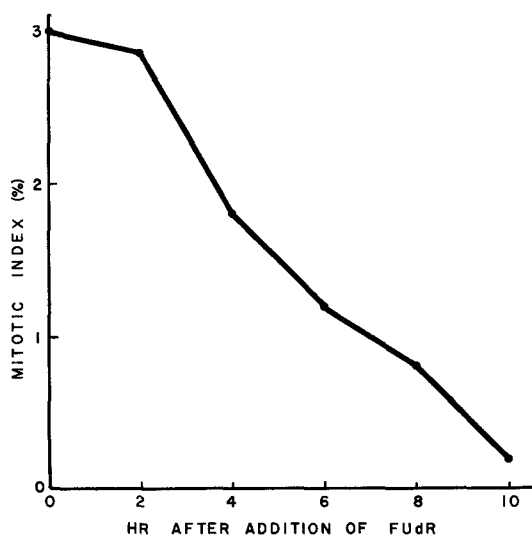


FIGURE 11 Effect of FUdR on the mitotic index. 24-hr cultures were grown in 10^{-6} M FUdR and sacrificed at 2-hr intervals. The mitotic index was determined by counting 500 cells in each of two duplicate cultures per time period.

7 hr after resumption of DNA synthesis. The cultures were maintained in FUdR for 4 days with daily feedings. As seen in Fig. 12, the cells from the FUdR + BUdR series showed the typical BUdR effect, i.e. enlarged and excessively flattened, with little evidence of fusion. Cells in the other series were of normal morphology, and considerable fusion had occurred in the FUdR + BUdR + TdR cultures (Fig. 13). There was definite fusion in the FUdR series also (Fig. 14), although fewer myotubes were present, probably as a result of the smaller number of cells in these cultures.

These results were confirmed in an experiment designed to assess the effect of unifilar BUdR incorporation on fusion of mononucleated cells with preformed myotubes. In normal medium, fusion begins at the end of the first culture day, and many immature myotubes are present by 48 hr. When cultures of this age are pulse labeled with thymidine- 3 H and sacrificed at intervals, fusion of labeled myoblasts begins at 8 to 10 hr after the preceding S phase, and many labeled nuclei appear in myotubes by 20 hr postlabeling (Bischoff and Holtzer, 1969). Since the duration of the cell cycle is 10 hr, the number of fused labeled nuclei present at 20 hr represent those cells that have undergone not more than one division cycle prior

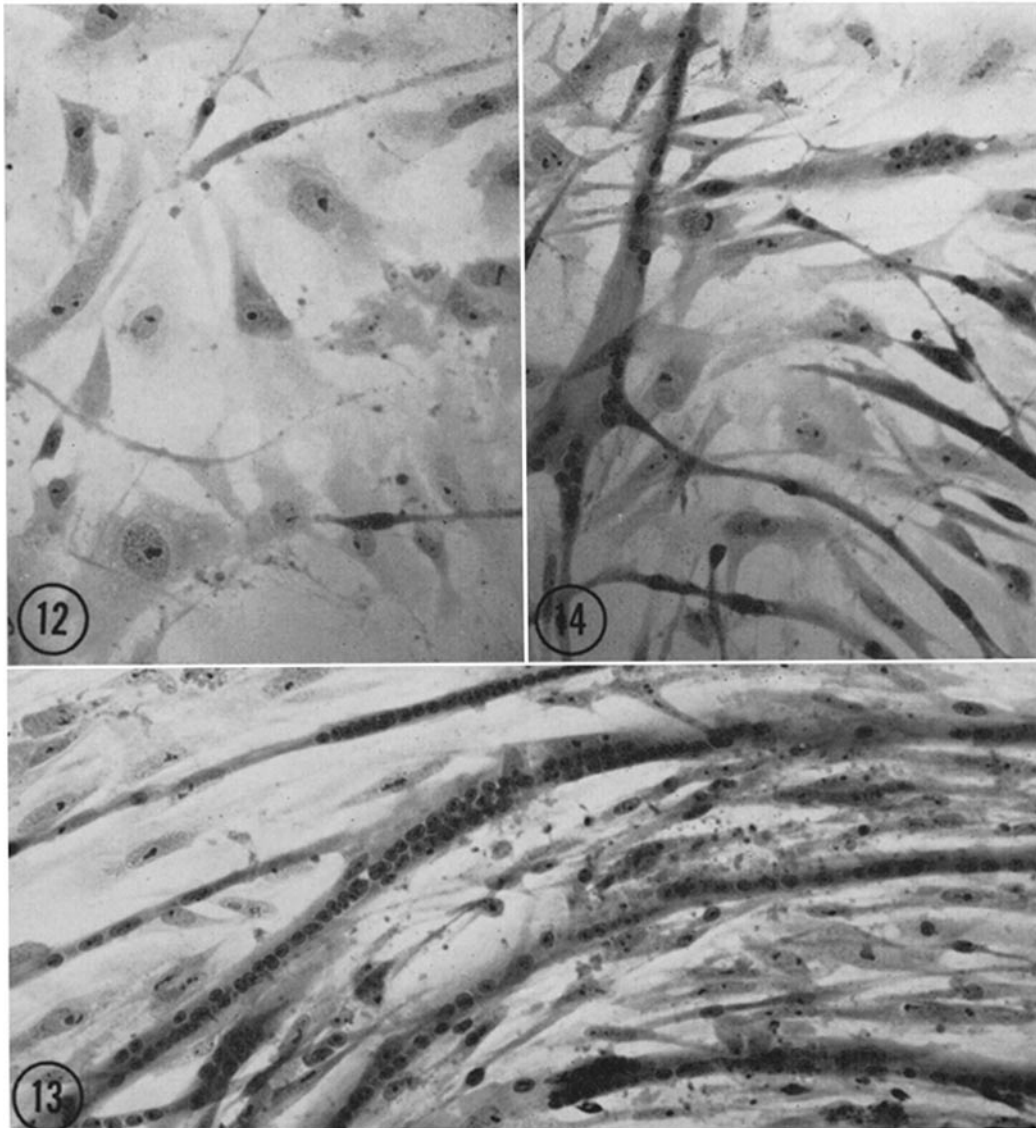


FIGURE 12 Cells grown continuously in 10^{-6} M FUdR for 4 days. The block to DNA synthesis was relieved for 7 hr on day 1 with 1.6×10^{-4} M BUdR. $\times 280$.

FIGURE 13 Same as Fig. 8; block was relieved with BUdR plus thymidine. $\times 280$.

FIGURE 14 Cells grown continuously in 10^{-6} M FUdR for 4 days. $\times 280$.

to fusion (10 hr cycle + 8–10 hr from end of S to fusion = 18–20 hr). Similar experiments were conducted with BUdR- 3 H instead of thymidine- 3 H to determine whether those cells that normally fuse following an S period would do so if DNA synthesis took place in the presence of BUdR. Two-

day-old cultures were incubated in a total of 1.6×10^{-4} M BUdR which included $0.75 \mu\text{c}/\text{ml}$ of BUdR- 3 H (specific activity in medium $4.6 \text{ mc}/\text{mmole}$). After 30 min the label was removed and the cultures were continued in unlabeled BUdR. Triplicate cultures were sacrificed at intervals up

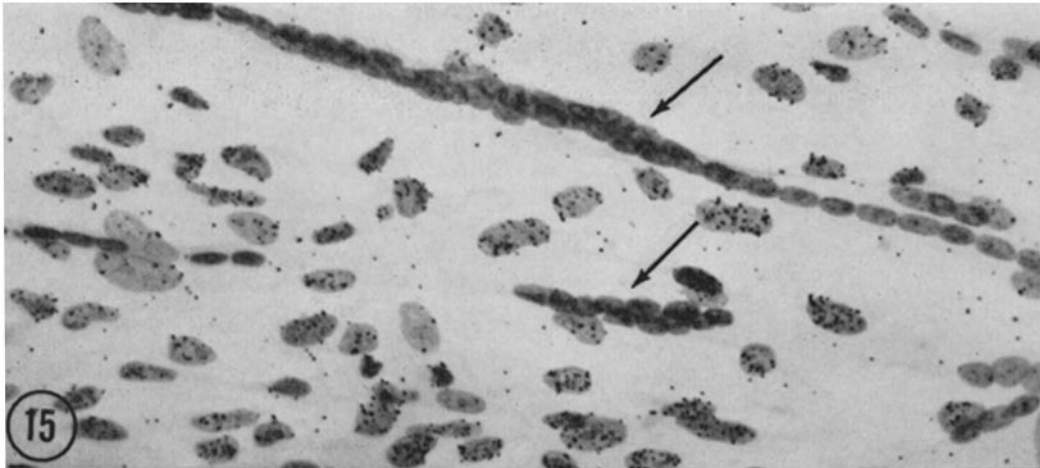


FIGURE 15 Radioautograph of 2 day culture sacrificed 24 hr after exposure to BUdR-³H. The majority of the mononucleated cells are labeled but have not become incorporated into myotubes. The myotube nuclei (arrows) present at the time of labeling remain unlabeled. Nuclei were stained with the Feulgen reaction. $\times 380$.

to 3 days and the radioautographs were examined for the presence of labeled nuclei within myotubes. The labeling index in cultures sacrificed immediately was 22%, or about the same as that found with thymidine-³H (Bischoff and Holtzer, 1969); only the mononucleated cells took up the label. In contrast to the thymidine label, however, the BUdR-labeled cells were not incorporated into myotubes during subsequent incubation (Fig. 15). Labeled division figures were common in the cultures. In a few instances (<1%) lightly labeled nuclei were found within myotubes. These may represent cells in which only a final few minutes in S remained when the BUdR was applied. In this context, it would be of interest to determine what portion of the S period is most sensitive to BUdR incorporation with respect to fusion and contractile protein synthesis.

In summary, unifilar substitution of BU for thymine prevents fusion and contractile protein synthesis in the progeny of affected cells. Cells with BU-DNA neither fuse with each other nor with normal myotubes. Incorporation during one S period is effective even when thymidylate synthesis is not blocked.

Effect of BUdR on Growth

Since the generation of competent myoblasts from myogenic precursor cells depends upon continued proliferation (Bischoff and Holtzer, 1969;

Holtzer and Bischoff, 1970), inhibition of fusion by BUdR might result from interference with cell divisions. Although cells continued to proliferate through repeated subcultures for at least 15 days in a high concentration of BUdR (1.6×10^{-4} M), the cultures yield fewer cells than do untreated control cultures. To obtain quantitative data on the effect of BUdR on cell production and cell survival, cultures were initiated in either 1.6×10^{-4} or 1.6×10^{-5} M BUdR and the increase in total nuclei was compared with that found in untreated cultures (Fig. 16). Replication occurs in both the BUdR and control cultures during the first day. The slight drop in cell number is the result of plating loss. After the first 24 hr, cultures in 1.6×10^{-4} M BUdR failed to keep pace with the controls and by 4 days produced 75% fewer nuclei than the myotube-containing control cultures. Despite the reduction in growth, there was no difference in the mitotic index at 2, 3, or 4 days. In contrast, cell production is slightly enhanced in 1.6×10^{-5} M BUdR. At 3 days the nuclear count of cultures grown in 1.6×10^{-5} M BUdR is about 120% that of the control cultures. Nuclear count is an accurate estimate of cell number in the BUdR cultures since all the cells are mononucleated.

To further investigate the effect of BUdR concentration on growth, cultures were initiated in from 0 to 1.6×10^{-4} M BUdR and maintained with daily feeding for 3 days. Nuclei were isolated

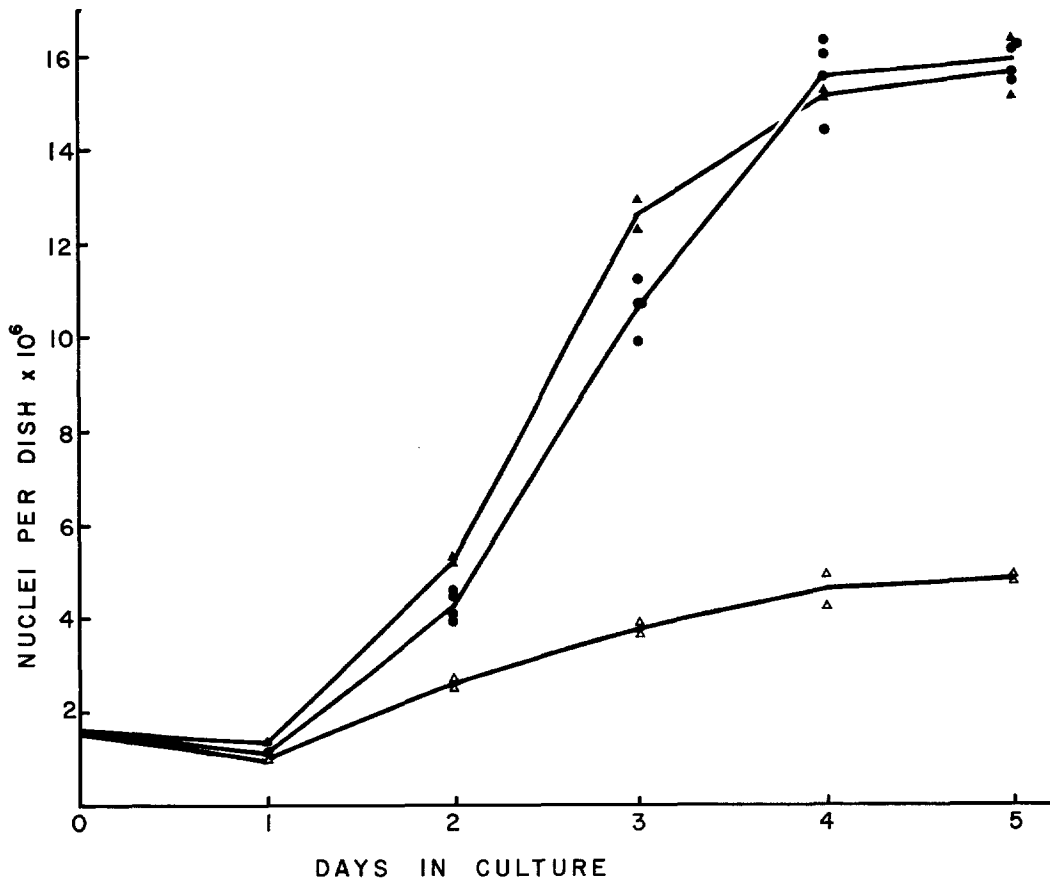


FIGURE 16 Inhibition of growth by BUdR. Cultures were initiated in normal medium (closed circles) and in 1.6×10^{-5} M (closed triangles) or 1.6×10^{-4} M BUdR (open triangles). The total number of nuclei per 60 mm dish was determined each day by using the citric acid-crystal violet technique.

in citric acid and counted. From about 4 to 16×10^{-5} M BUdR there is a progressive inhibition of total numbers of cells (Fig. 17). At lower concentrations, however, there are more cells in the BUdR cultures than in the untreated controls. The lowest concentration of BUdR used (3.2×10^{-6} M) results in a 40% increase in cell number. Alteration of cell morphology in BUdR is also influenced by concentration. The greatest degree of flattening and membrane expansion occurs at 1.6×10^{-4} M, while cells grown in 3.2×10^{-6} M are spindle shaped or elongate and exhibit no excessive spreading (Fig. 18). Intermediate concentrations produce intermediate effects on morphology.

Myoblast fusion is blocked throughout the entire range of BUdR concentration. Despite the normal appearance of cells in 3.2×10^{-6} M BUdR, fusion does not take place during at least 10 days of

continuous culture. In older cultures many cells become highly elongate and aligned in closely packed swirls. Formation of contractile protein filaments in these cells is currently under investigation with the electron microscope. Preliminary results indicate that after 10 days in 3.2×10^{-6} M BUdR some mononucleated cells form moderate amounts of thick and thin filaments.

As another index of the influence of BUdR on growth, we measured the rate of amino acid incorporation at several points during a typical culture period. Preliminary experiments established that incorporation of labeled leucine proceeds at a linear rate for at least 4 hr in these cells. The experiment was designed to allow 5 days in primary culture for incorporation of the BUdR. Then the cells were subcultured and leucine- ^3H ($5 \mu\text{c}/\text{ml}$) uptake was measured for 4-hour periods

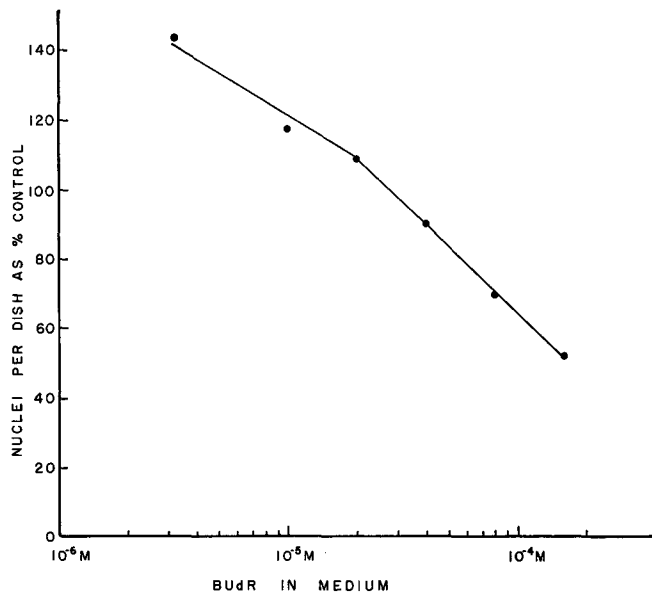


FIGURE 17 Effect of BUdR on growth as a function of concentration. 60 mm dishes were seeded with 1.5×10^6 cells (density = 7.5×10^4 cells/cm²) and grown continuously in BUdR for 3 days. The total number of nuclei in duplicate dishes was counted by using the citric acid-crystal violet technique and expressed relative to control cells grown in untreated medium. Control culture produced an average of 10.5×10^6 nuclei during 3 days. Fusion was prevented throughout the entire range of BUdR concentration.

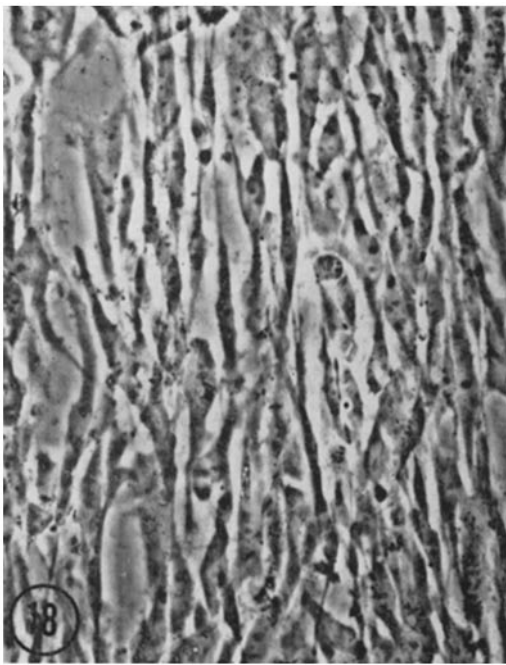


FIGURE 18 Phase-contrast micrograph of living cells grown for 3 days in 3.2×10^{-6} M BUdR. No myotubes are present. $\times 320$.

on days 1, 3, and 5 of the secondary cultures. The leucine-³H was added to the cells with the daily change of fresh medium containing BUdR. A suitable control for this experiment is not a normal

primary culture forming myotubes, for, in such cultures, much of the total protein synthesized would be myosin or actin. The appropriate control should consist of replicating, mononucleated cells reared in normal medium. Accordingly, a subculture of replicating cells from a 5 day old primary muscle culture was used for controls. The cells in these control cultures replicate vigorously but form few myotubes (Bischoff and Holtzer, 1969).

In BUdR-suppressed and control cultures there is a striking drop in the rate of amino acid incorporation between 1 and 3 days and a less pronounced decrease from 3 to 5 days (Fig. 19). Such an effect has been reported elsewhere and is presumed to be a consequence of density-dependent inhibition of growth (Eagle, 1965).

The modest difference in amino acid incorporation between the BUdR and control cultures is probably not significant. Indeed, experiments of total protein synthesis are difficult to interpret. The species of proteins made in the BUdR cells may be very different from the species of protein made in the control cells although the total may be the same.

DISCUSSION

Fusion is the result of interactions between competent myogenic cells. Its component processes include recognition, membrane rearrangement, and cytoplasmic confluence (Holtzer, Bischoff, and Chacko, 1968). Heterologous cells do not partici-

pate in myotube formation (Okazaki and Holtzer, 1965; Yaffe and Feldman, 1965), and myoblasts themselves fuse only in the G_1 phase of the cell cycle (Bischoff and Holtzer, 1969). If the S period prior to fusion includes incorporation of BUdR, fusion of the daughter cells is prevented.

That BUdR incorporation for one S period is effective in blocking fusion and contractile protein synthesis has been demonstrated in two types of experiments. First, we attempted to obtain unifilar incorporation into a majority of the cells by releasing a previously established FUdR block for one S period with either BUdR or BUdR + thymidine (TdR). The cells were then continued in FUdR for 4 days to prevent further DNA synthesis and to allow ample opportunity for fusion and contractile protein synthesis. The degree of fusion that took place in the BUdR + TdR control series (Fig. 13) should be a measure of the number of cells that underwent a terminal division after release of the FUdR block. The same amount of division oc-

curred in the BUdR series; however, fusion of these cells during the 4 day period was negligible (Fig. 12). The use of FUdR raises the possibility that the quantity or quality of BUdR incorporation into DNA may be different from that obtained when endogenous thymidylate synthesis is not prevented. BUdR incorporation has been reported to be enhanced in the presence of FUdR (Dvordjevic and Szybalski, 1960), although Simon (1963) and Littlefield and Gould (1960) did not observe this effect. Also, thymidylate deprivation may produce DNA lesions that would be susceptible to nonsemiconservative BUdR incorporation via a repair mechanism (Pauling and Hanawalt, 1965). At present, however, there is no evidence for FUdR-induced repair in tissue cells. In the second type of experiment FUdR was not used. Instead, we utilized the observation that in 2-day cultures there is a sizeable population of cells that synthesize DNA and subsequently fuse without undergoing another round of the cell cycle

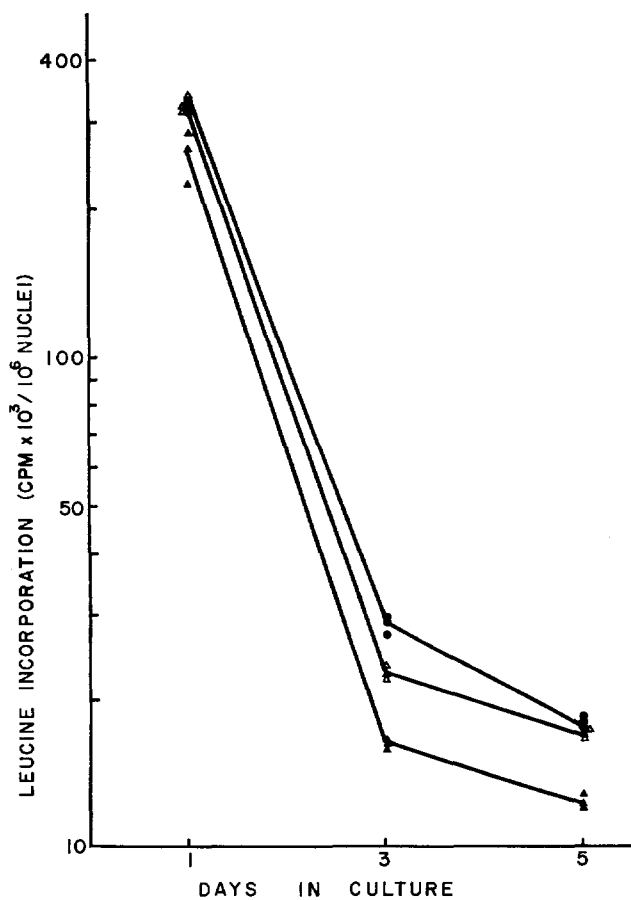


FIGURE 19 Effect of BUdR on incorporation of leucine- ^3H . Cells (initial density = 7.5×10^4 cells/cm 2) were grown continuously in either 0, 1.6×10^{-5} , or 1.6×10^{-4} M BUdR, subcultured after 5 days, and triplicate dishes were pulsed with leucine- ^3H ($5 \mu\text{C}/\text{ml}$) for 4-hr periods on day 1, 3, and 5 of the second generation culture. Nuclei were counted with the citric acid-crystal violet method. Closed circles, untreated control cells; open triangles, cells in 1.6×10^{-4} M BUdR; closed triangles, cells in 1.6×10^{-5} M BUdR.

(Bischoff and Holtzer, 1969). When this period of DNA synthesis takes place in the presence of BUdR, these cells are prevented from fusing.

The BUdR suppression of fusion suggests that some activity of the DNA essential for fusion takes place during or shortly after the final round of DNA synthesis. Unifilar BU insertion may interfere with transcription from both nucleotide strands or, alternatively, perhaps it is the newly synthesized gene that is active in preparing the cell for fusion. The latter possibility is consistent with earlier experiments which suggest that the terminal cell cycle leading to the final "quantal" mitosis is essential for subsequent fusion and contractile protein synthesis (Bischoff and Holtzer, 1969; Holtzer and Bischoff, 1970).

Although fusion usually precedes initiation of contractile protein synthesis, the relation between the two is not obligatory. Early somite musculature consists entirely of mononucleated myoblasts engaged in contractile protein synthesis (Holtzer et al., 1957; Okazaki and Holtzer, 1966). A small proportion of cultured breast muscle cells can also synthesize myofibrils without fusing (Okazaki and Holtzer, 1965; Coleman et al., 1966). Thus, the failure of BUdR-treated cells to initiate the synthesis of myofibrils does not result from their inability to fuse. Unfortunately it is not possible to study the effect of BUdR on contractile protein synthesis in progress. Since DNA synthesis and myosin synthesis are mutually exclusive events in skeletal muscle (Stockdale and Holtzer, 1961), there is no opportunity to introduce the analogue into the DNA of overtly differentiated muscle cells.

All the available evidence indicates that BUdR suppresses myogenesis by virtue of its incorporation into DNA. Especially convincing is the demonstration that the first cells to recover the capacity to fuse following exposure to BUdR are the ones in which the BU has been the most highly diluted by subsequent divisions in normal medium (Okazaki and Holtzer, 1965). Nevertheless, it has not been conclusively demonstrated that DNA is the primary site of action of the analogue. Kajiwara and Mueller (1964) showed that the reduction in cloning efficiency of HeLa cells grown in 8×10^{-5} M BUdR did not occur when DNA synthesis was suppressed during exposure to BUdR. Similar experiments with myogenic cells have not yet been performed.

Of the three types of cells studied in this laboratory, muscle, cartilage, and amnion, the same

phenomenon results in each after exposure to BUdR. The synthesis of tissue-specific, luxury molecules, myosin, chondroitin sulfate, and hyaluronic acid, respectively, is inhibited, while growth and division are relatively unimpaired for extended periods of culture (> 10 cell generations). Suppression of specialized function by BUdR has been observed in several other cell types also. Immunologically competent cells fail to synthesize antibodies when exposed to BUdR during periods of DNA synthesis (O'Brien and Coons, 1963; Dutton et al., 1960). Cell survival and growth, however, were not examined in these experiments. BUdR also prevents the appearance of zymogen in pancreatic cells (Wessels, 1964) and the formation of echinochrome by sea urchin cells (Gontcharoff and Mazia, 1967). In the latter two cases, proliferation continued after exposure to BUdR, although there was some reduction in the growth rate.

There is ample evidence documenting the toxicity of BUdR in cultured cell lines (Littlefield and Gould, 1960; Hakala, 1962; Simon, 1963; Kajiwara and Mueller, 1964; Kim et al., 1967). The amount of growth inhibition and cell killing depends upon the concentration (Kim et al., 1967) and the number of divisions in the analogue (Littlefield and Gould, 1960). HeLa and KD cells exhibit a progressive inhibition of growth from about 0.1 to 100 $\mu\text{g/ml}$ BUdR (Kim et al., 1967; Littlefield and Gould, 1960). The toxicity of a given concentration of BUdR is amplified by preventing formation of endogenous thymidylate with FUdR or aminopterin (Kajiwara and Mueller, 1964).

With myogenic cells, there is definite growth inhibition at higher analogue concentration. At 1.6×10^{-4} M, about 75% fewer cells are produced during a 3 day period. Even at this concentration, however, proliferation continues for at least 15 days of continuous exposure. The low rate of cell accumulation in 1.6×10^{-4} M BUdR is probably a result of cell death since the mitotic index is the same as that found in control cultures. The resistance of these cells to BUdR toxicity, when compared with results from cell lines, may be related to the rich medium employed here. Pyrimidines in the medium would compete with BUdR for insertion into the DNA. In addition, plating efficiency is often used to measure BUdR toxicity; cells grown in high density, however, are better able to survive in BUdR than are cloned cells (Littlefield and Gould, 1960; Holtzer and Abbott, 1968).

It is worth emphasizing that the toxic effects observed at high concentration of BUdR are not responsible for the inhibition of fusion and contractile protein synthesis. 1.6×10^{-6} M BUdR does not interfere with cell production (Figs. 16 and 17), yet myotubes fail to appear until the cells are removed from the analogue and allowed to divide for a time in normal medium. As yet there is no explanation for the apparent growth stimulation at low analogue concentration (3.2×10^{-6} M).

Loss of expression is quite reversible even after many rounds of division in the analogue. During normal development of muscle the proportion of myogenic stem cells declines as the tissue matures. Subcultures prepared from older muscle cultures consist primarily of mononucleated cells; presumably these are fibroblasts (Bischoff and Holtzer, 1969; Hauschka, 1968). In contrast, there is no evidence of overgrowth by nonmyogenic cells in BUdR. After many generations of growth in BUdR, the cells are still capable of forming myotubes comparable to those obtained in normal primary cultures. The absolute number of stem cells generated during growth in BUdR is far greater than that found during myogenesis in normal medium. The retention of the myogenic commitment in BUdR-suppressed cells stresses (a) the differentiated nature of replicating presumptive myoblasts, and (b) the fact that the progeny of "proliferative mitoses," in contrast to "quantal mitoses," display the same synthetic behavior as that of the parent cell (Holtzer, Bischoff, and Chacko, 1968; Holtzer and Bischoff, 1970).

BUdR prevents the formation of postmitotic myoblasts but does not affect the continued propagation of myogenic stem cells. The mechanism responsible for myogenic determination in these cells is stable through many divisions in BUdR. Indeed, BUdR might force cells to remain in the mitotic cycle. The effects of BUdR on the timing of the cell cycle phases is currently being investigated.

The differential effect of BUdR on synthesis of specialized products seems paradoxical in view of current dogma on control of cellular activity. On one hand, we have no reason to believe that the instructive activity of DNA is different in controlling different cellular processes. Yet BUdR appears to block preferentially those processes not essential for cell growth and proliferation. The paradox is that BUdR prevents the formation of actin for myofibrils, but not the formation of the presumably related mitotic spindle protein. In experiments to be reported elsewhere it has been found that

BUdR-suppressed myogenic cells do not translate for myoglobin (Mochan and Holtzer, unpublished). On the other hand many of the cytochromes of the BUdR-suppressed are normal.

In bacterial cells BUdR is reported to have a mutagenic effect by causing errors in base pairing (Zamenhof et al., 1958; Shapiro and Chargaff, 1960). In our experiments BUdR is not acting primarily as a mutagen since (a) virtually all cells that incorporate the analogue are affected and (b) the inhibition is reversible when the cells are allowed to divide in normal medium.

There are several possible mechanisms that could explain the effect of BUdR. For example, certain sites in the DNA are responsible for initiating gene transcription (Jacob, 1966). These regions may be especially sensitive to analogue substitution by virtue of a high pyrimidine content (see Szybalski et al., 1966). The selectivity of the BUdR effect may indicate that genes coding for metabolic functions essential to cell viability and growth are active at all times and do not include an initiator region, whereas genes coding for differentiated functions are more susceptible to BUdR disruption owing to their pyrimidine-rich initiator sites. Another possibility is that genes controlling essential processes may be highly redundant so that it would take much longer for a given concentration of analogue to block transcription in the entire array of genes. Attempts to saturate the DNA with 5-bromouracil by prolonged growth in BUdR, however, did not result in complete loss of viability or capacity to recover when the cells were replated in normal medium. Finally, in certain nucleotide sequences, or regions of the DNA, BUdR may be a poor competitor with thymine for insertion into the DNA. The thrust of all these speculations is that the transmission of information from DNA is qualitatively or quantitatively different for essential and "luxury" (Holtzer and Abbott, 1968; Holtzer and Bischoff, 1970) molecules. Whatever the mechanism, further study of the BUdR effect should provide useful information on the genetic control of cell differentiation.

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