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The Effects of Incorporation of 5-Halogenated Deoxyuridines into the DNA of Eukaryotic Cells

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I. Introduction

The 5-halogenated deoxyuridines[1] are analogs of the naturally occurring nucleoside thymidine (dThd), which is a constituent of DNA. 5-Iodo-2′-deoxyuridine (IdUrd), one of the 5-halogenated deoxyuridine analogs, has been studied for several years because of its clinically important antiviral properties (reviewed in 64, 134, 139). Recently, interest in these analogs has been stimulated by their effects on eukaryotic cells. IdUrd and the other frequently used analog 5-bromo-2′-deoxyuridine (BrdUrd) are mutagenic, teratogenic, oncogenic and in sufficient concentration lethal or cytotoxic. At proper concentration, they can inhibit or reverse differentiation without apparent cytotoxicity. How do these diverse effects come about? Is there some common mechanism? Answers to these questions may help to illuminate basic mechanisms of differentiation and oncogenesis. In this review,[2] what is known to date about BrdUrd and IdUrd relative to these processes is discussed. The main points brought out are that almost all of the effects of BrdUrd and IdUrd probably derive from their incorporation into DNA in place of dThd, and that DNA containing

[1] This review deals with 5-bromo-2′-deoxyuridine and 5-iodo-2′-deoxyuridine. The 5-chloro and -fluoro derivatives are not discussed to any great extent, as little has been done on the former and the latter is not incorporated into DNA.

[2] Past reviews on the halogenated deoxyuridines have dealt mainly with BrdUrd (56, 76, 103, 144, 148, 162, 206), although W. H. Prusoff and the author have extensively reviewed the literature on IdUrd (139).

BrdUrd or IdUrd does not function normally. How the normal functioning of DNA is altered is discussed.

II. Biological Effects

A. Toxicity

In sufficient concentrations, both BrdUrd and IdUrd are lethal or toxic to cells, as shown most extensively in cultured cells. Nevertheless, striking differences in susceptibility are found between various cell types including a murine leukemia, L5178Y (113, 114), a mast cell tumor line, P8154 (118), rabbit KD cells (108), a human bone marrow cell line (47) and the human tumor cell lines HeLa (71) and HEp 1 (30, 48).

Some cell lines, however, become tolerant to the halogenated deoxyuridines (165, 195). In one case, lines of Syrian hamster melanoma cells have been developed that actually depend on the presence of BrdUrd for growth in the culture medium (41). One such line grows well with approximately all of its DNA dThd residues replaced by BrdUrd (14). These melanoma cells also require BrdUrd to maintain their transformed state of noncontact inhibition, *i.e.*, the ability of the cells to continue to grow when they have formed confluent monolayers in the culture flask (42, 77).

As a consequence of analog incorporation into DNA, some cells exhibit chromosomal aberrations in Drosophila (51), mouse cells (79–81, 84), and Chinese hamster ovary cells (85).

The 5-halogenated deoxyuridines are also toxic to whole animals as has been demonstrated with IdUrd. Daily intraperitoneal doses of about 250 mg of IdUrd per kg were lethal to mice (140). On the other hand, IdUrd, originally synthesized as an anticancer drug, significantly inhibited the growth of Sarcoma 180 and lymphoma L1210 and L5178Y cells in mice without observable host toxicity if a daily dose of 100 to 150 mg/kg was administered for 6 days (87). Post and Hoffman (132) found delay of DNA synthesis by IdUrd of rat lymphocytes and ileal and spleen cells. In man, Calabresi *et al.* (23) observed that daily intravenous infusions of 100 to 120 mg of IdUrd per kg for five or six days caused leukopenia, stomatitis and alopecia.

In both cultured cells and *in vivo*, the doses of analog that are toxic vary from case to case depending, among other factors, upon nucleotide metabolism and pools, analog metabolism and the rate of DNA synthesis. The concentrations of BrdUrd or IdUrd needed to induce oncogenic viruses (section II D) or inhibit or reverse differentiation (section II E) are usually not sufficient to cause appreciable toxicity.

B. Teratogenicity

Both BrdUrd and IdUrd are teratogenic. The first report of inhibition of embryonic development was by Karnofsky and Basch (89). BrdUrd prevented development of the sand dollar embryo beyond the blastula stage. Nemer (120) reported a similar block at the blastula state of Paracentrotus lividus by IdUrd. These initial studies were followed by the demonstration of teratogenesis in pregnant rodents by administration of either 5-chloro-2′-deoxyuridine (CldUrd) (29), BrdUrd (46, 119, 147, 170) or IdUrd (128, 168). In pregnant rabbits, IdUrd was teratogenic even when administered topically to the eye in a usual therapeutic regimen (86). In addition, BrdUrd was teratogenic in Drosophila melanogaster (58, 142). IdUrd was also capable of causing defective development in rats even if given to the newborns during the first 15 days postpartum (129) instead of, as is usually done, prepartum.

C. Mutagenicity

The mutagenic activity of the 5-halogenated deoxyuridine analogs have been well documented in prokaryotes and bacteriophage (reviewed in 139). It is believed that the mutations are caused by mispairing of 5-bromouracil or 5-iodouracil with guanine rather than the proper base, adenine, during DNA replication. The mispairing occurs when the halogenated base assumes an ionized anionic or enol form. In contrast to the

5-methyl group of thymine, the electrophilic 5-bromine or iodine is believed to increase the occurrence of the ionized or enol tautomer.

The mutagenic activity of BrdUrd in mammalian cells is a relatively recent finding (33, 82, 130, 175). Earlier attempts to demonstrate mutagenesis in human cells in culture (190) and in Drosophila (51) were unsuccessful. Thus far, only BrdUrd has been shown to be mutagenic in mammalian cells and only in human and Chinese hamster ovary cells. Aebersold (4) has proposed, on the basis of experiments with Chinese hamster ovary cells, that BrdUrd-induced mutants were not due to mispairing with guanine during replication. Chinese hamster ovary cells could be substantially substituted with BrdUrd without inducing mutations, yet concentrations higher than needed for full substitution induced mutations. In addition, Aebersold (4) cites the isolation of a mutant cell line by Bick and Davidson (14) that was capable of continued growth after all thymine residues in the DNA had been replaced by bromouracil. The complexity of mammalian DNA dictates caution in drawing conclusions based on lack of correlation between substitution and mutagenesis. As described in another section, there is a sizable portion of mammalian DNA that is assumed to have a regulatory function rather than actually coding for a specific protein. Since base mispairing could affect either or both types of DNA, it is difficult to reach secure conclusions based on total BrdUrd substitution in DNA.

D. Induction of Oncogenic Viruses

The induction of oncogenic viruses in a variety of eukaryotic cells is an example of an increased rather than decreased synthesis of specific proteins caused by BrdUrd or IdUrd. This phenomenon was first found in seemingly virus-free mouse embryo cells by Lowy *et al.* (110), and by Aaronson *et al.* (2) in various lines of virus-negative mouse cells such as BALB/3T3. Stewart *et al.* (181) found similar activation by IdUrd in a human sarcoma cell line. Many other similar reports have been published. Thus, the halogenated deoxyuridines will also induce the viruses, polyoma (54), SV40 (145, 201) and EB (57, 73, 187, 188), as well as herpes antigens in herpes virus-transformed cells (112).

In most studies of induction of putative oncogenic viruses, the particles have lacked *in vivo* bioactivity. For example, Schwartz *et al.* (158) found that C-type particles induced by BrdUrd in rat embryo cells were not oncogenic. However, Stephenson *et al.* (180) were able to produce lymphatic leukemias in mice with C-type virus particles induced by IdUrd treatment in a mouse embryo cell line, and Lazar *et al.* (102) found that oncornavirus induced in a mouse embryo cell line by IdUrd would infect other cells. The infected cells and the originally induced cells were carcinogenic when injected into syngenic mice. These findings contrast with those of Silagi *et al.* (163), in which BrdUrd treatment of tumor-producing melanoma cells induced oncogenic virus particles whereas at the same time the cells lost their tumorigenicity.

In addition to the variation in bioactivity of the induced viral particles, there is variation in the inducibility of various cloned cell lines (104, 110, 206, 213). Clones isolated from cell lines and tested for virus inducibility by IdUrd varied by as much as 100-fold in the number of virus produced by each clone. Also, most mouse cells cease making viral particles about 4 to 5 days after a 24-hr IdUrd treatment. By contrast, cells cultured from embryos of C58 mice continued to produce viral particles for more than 3 weeks after a 24-hr IdUrd exposure (178). Furthermore, although the combination of IdUrd and dexamethasone induced C-type virus particle production in a mouse neuroblastoma cell line, the treatment had no discernible effect on the basal concentration of A-type particles present in these same cells (99).

Not a great deal is known about the mechanism of oncogenic virus induction by halogenated deoxyuridines beyond that

which may be inferred from studies of these analogs on cells in which differentiation is affected. These studies are dealt with in a later section (III, MECHANISM OF ACTION). However, there are some findings specific to virus induction. Several studies have demonstrated that induction of tumor viruses by IdUrd or BrUrd occurs even if the analog is present only during the S phase of the cell cycle (10, 72, 157, 192) and will not occur if DNA synthesis is inhibited (192) during incubation with the analog. Besmer *et al.* (10) studied induction of RNA oncogenic viruses by IdUrd in a mouse fibroblastic cell line. By using hybridization with a radioactive complementary DNA of induced virus prepared with reverse transcriptase, they demonstrated that concomitant with virus induction there was an increase in viral-specific hybridizable RNA in both the nucleus (5-fold increase) and cytoplasm (10-fold increase) of the treated cells. Interestingly, the untreated cells also contained tumor virus-specific RNA. Besmer *et al.* noted that this may indicate that what they regard as transcriptional derepression by IdUrd for virus synthesis may be a quantitative rather than qualitative change. However, this idea must be tempered with the probability that not all virus-specific RNA that may be synthesized in cells is represented by the complementary DNA that was prepared from the endogenous virus.

Yoshikura (212) has made the interesting observation that caffeine will block the induction by IdUrd of C-type viruses in a mouse cell line. On the strength of this observation, Wharton and Goz (205) tested the effect of caffeine as well as theophylline and 3-iso-1-methylbutylxanthine on the induction of alkaline phosphatase activity in HeLa cells by IdUrd. All three compounds inhibited the induction in a dose-dependent fashion. The most potent compound was 3-iso-1-methylbutylxanthine. One possible interpretation of these data (205, 212) is that the induction of oncogenic viruses and alkaline phosphatase activity by IdUrd or BdUrd or inhibition, or both of the induction involves cyclic AMP. Another possibility is that caffeine blocks virus induction by inhibition of DNA repair (212).

Wu *et al.* (211) found that cordycepin, which interferes with processing of nuclear heterogenous RNA and inhibits polyadenylate synthesis, blocked induction of murine leukovirus by IdUrd in cultured mouse cells. This same group (125) and another group (52) also observed that the induction of C and B particles by IdUrd was enhanced by adrenal corticosteroids. The corticosteroids added alone had no effect. The effect of cordycepin would seem to be logical in light of the results of Besmer *et al.* (10) and others of increased transcription after IdUrd treatment. How the corticosteroids act is not clear at this point, although many investigators have demonstrated that receptor protein-bound steroids bind to chromatin and, in one study, incorporation of BrdUrd into DNA enhanced the nuclear binding of radioactive dexamethasone in rat hepatoma cells (146). Wu *et al.* (210) further studied the enhancing effect and concluded that the steroids were acting posttranscriptionally on the basis of experiments on time course of induction, inhibition of induction by cordycepin and interferon and nucleic acid hybridization studies.

Other factors also enhance oncogenic virus induction by halogenated deoxyuridines. Increased virus yield was achieved by cotreatment with ultraviolet and visible light, X-irradiation (192) and dimethyl sulfoxide (182). The carcinogens 3-methylcholanthrene and 7,12-dimethylbenz[α]anthracene and other polycyclic hydrocarbons, after activation by microsomes, also increased oncogenic virus production by IdUrd and BrdUrd (55, 214).

A very important question concerning oncogenic virus induction by IdUrd or BrdUrd is whether the chromosomal loci for viral induction represent the viral genome or are actually regulatory regions that allow expression of the viral genome located elsewhere? Stephenson and Aaronson (179) have described two genetic loci for induc-

ibility of C-type virus in mouse embryo cells by IdUrd. Both are genetically dominant. Chattopadhyay *et al.* (27, 28) presented evidence from nucleic acid hybridization experiments and chromosomal mapping by mouse breeding that at least one locus for IdUrd induction of C-type virus, termed *Akv-1*, contained the viral sequences and not a regulatory sequence. If this is generally true, then IdUrd or BrdUrd induction represents a direct effect on the expression of the viral genes rather than an indirect one *via* a regulatory region. This in turn raises the issue of how to accomodate this with the current thinking that halogenated deoxyuridine analogs act by more tightly binding regulatory chromosomal proteins (see section III C on DNA protein binding for details). One possibility is that binding of the polymerase that transcribes the viral genome may be increased by IdUrd or BrdUrd substitution, without appreciably hindering its ability to copy these sequences once bound at the initiation site.

A possibly related phenomenon is the facilitation of cell transformation by tumorigenic viruses by treatment of cells with BrdUrd or IdUrd before exposure to the transforming virus. The first such report was by Todaro and Green (194) of an up to 9-fold increase in SV40 virus transformation of mouse 3T3 cells by prior cell exposure to BrdUrd or IdUrd. Ashkenazi (5) and Coggin (35) made similar observations for SV40, and Castro (26) used BrdUrd or IdUrd to increase hamster cell transformation by adenovirus. Also of related interest, cells transformed by SV40 and then treated with BrdUrd lost the ability to produce T antigen that is characteristic of SV40-transformed cells (95).

The facilitation or enhancement of productive infection with certain viruses is another effect that involves pretreatment of cells with IdUrd or BrdUrd. Thus, St. Jeor and Rapp (149) demonstrated that pretreatment of human embryonic lung cells with IdUrd increased the yield of human cytomegalovirus by 5-fold. Plummer and Goodheart (131) showed comparable results for IdUrd with murine cytomegalovirus. Murine teratocarcinoma cells are similarly made more susceptible to SV40 or polyoma infection by pretreatment with BrdUrd (173), as are Chinese hamster kidney cells to SV40 by pretreatment with IdUrd (186). This effect is not limited to DNA virus replication, as cited above. Green and Baron (68) showed that both RNA and DNA virus replication were enhanced by prior addition of IdUrd.

E. Inhibition of Differentiation

Numerous studies have been published describing the effects of BrdUrd or IdUrd on differentiation or synthesis of a particular cellular protein. This section reviews only some of these studies. Most of the studies to be presented were chosen either because they were representative of a particular effect, or because the effect of the halogenated analog had been examined in depth.

The effects of 5-halogenated deoxyuridines on differentiated functions are not uniform. The effects that have been demonstrated in developing embryonic tissues, depending upon conditions, are not always reversible. Also, the time of analog addition during differentiation is critical. Analog addition beyond a certain time may have no effect. Generally, the inhibition of the expression of differentiated phenotypes in cultured tumor cells is completely reversible. This may relate to the important distinction made by Davis and Adelberg (44) that during differentiation a particular gene becomes expressed, whereas subsequently (as with cultured tumor cells) the activity of that gene in the differentiated cell is modulated. Different repressors may be involved in each process as well as possibly different changes in DNA structure. The changes in DNA structure may be in the nature of DNA amplification during embryonic differentiation as posited by Strom and Dorfman (185). The fact that the effects of the analogs are many times readily and quickly reversed, however, argues against their being caused by mutagenesis.

Many of the earliest findings were by Holtzer and his colleagues [see Holtzer *et al.* (76), for a review of these studies]. In 1964, Stockdale *et al.* (183) reported that chick myoblasts grown in culture with BrdUrd, although continuing to divide, did not differentiate to form myotubes. The treated cells also did not synthesize myosin (38, 122). Similarly, Wessells (204) found that BrdUrd blocked the *in vitro* differentiation of mouse pancreas. Later papers reported that BrdUrd inhibited *in vitro* differentiation and chondroitin sulfate synthesis of chick chondrocytes (3, 37) and red blood cell differentiation (116, 202). Silagi and Bruce (164) discovered in 1970 that BrdUrd not only caused the loss of pigmentation in a cultured line of mouse melanoma cells, but also the loss of the ability to form tumors when injected into mice.

When tested, IdUrd or CldUrd had an effect similar to that of BrdUrd. For example, Turkington *et al.* (198) found that both BrdUrd and IdUrd inhibited induction of casein and α-lactalbumin by prolactin in mouse mammary epithelial cells in organ culture. IdUrd and BrdUrd inhibited amylase activity accumulation in rat embryonic pancreas (200). Coleman *et al.* (37) reported that CldUrd and IdUrd as well as BrdUrd prevented chick embryo muscle cells from differentiating. Similarly, CldUrd, IdUrd and BrdUrd inhibited myogenesis in chick myoblasts (16) and chondroitin sulfate synthesis in chick chondrocytes (3).

Silagi has recently reviewed (162) the considerable findings that she and her collaborators have made on the effects of BrdUrd on mouse melanoma cells. BrdUrd (3.3–10 μM) affected this tumor cell line in several ways. Although the growth rate of the treated cells approximated the control cells' growth rate, the cells, which were originally rounded, became flattened and contact-inhibited after about 48 hr (164). Concomitantly, there was a loss of melanin, which was associated with the disappearance of tyrosinase, the enzyme that synthesizes melanin from tyrosine (209). Over a similar period of time in the culture medium as in the above studies, BrdUrd markedly reduced or eliminated the ability of these cells to form tumors when injected into mice. The treated cells, however, showed a marked increase in murine leukemia virus particles and, although these cells no longer produced tumors they retained immunogenicity so as to be able to protect mice against the growth of injected untreated melanoma cells (163). Correlated with the loss of tumorigenicity, BrdUrd-treated melanoma cells also largely lost the ability to produce active plasminogen activator, the protease that is responsible for the conversion of plasminogen to plasmin (32, 50). Silagi (162) reflected that, although the temporal correlation between loss of tumorigenicity and plasminogen activator activity may indicate a relationship between the two, more definitive evidence is required. Finally, in 10 of 13 amino acid pools studied in the melanoma cells, BrdUrd treatment caused a significant increase in pool size (151). All of the actions of BrdUrd were reversed upon removal of the analog.

All of the studies thus far described are of inhibition or failure to make a product. But the 5-halogenated deoxyuridines do not only cause inhibition of phenotypic expression. There are reports that BrdUrd or IdUrd will seemingly increase the amount or activities of certain proteins. Induction of oncogenic viruses, covered in the preceding section (II D), is a good example of this. In the case of oncogenic virus induction, however, as in the other examples to be mentioned, it is quite possible that the observed increases are secondary to or the result of decreased synthesis of a primarily affected protein. For example, inhibition of synthesis of a repressor protein by BrdUrd or IdUrd could secondarily result in increased synthesis of another protein. Another such example may be the induction of alkaline phosphatase activity in HeLa cells by IdUrd (61). The increased alkaline phosphatase activity appears not to be due to increased synthesis of enzyme molecules, but more likely is due to an altered enzyme

molecule (66). The alteration could conceivably be due to decreased synthesis of a modifier molecule. Several other studies have shown induction of alkaline phosphatase activity in other species by BrdUrd. Koyama and Ono (93), the first to report induction of alkaline phosphatase activity by halogenated deoxyuridine compounds, found that both BrdUrd and IdUrd (94) increased alkaline phosphatase activity in hybrid mouse-Chinese hamster cells. BrdUrd also induced alkaline phosphatase activity in rat embryo pancreas (59) and choriocarcinoma cells (31). Bulmer *et al.* (22) observed that, although BrdUrd induced alkaline phosphatase activity in HeLa cells, it decreased alkaline phosphatase activity in another human tumor cell line, HEp 2. A number of other enzyme activities are induced by BrdUrd. In human neuroblastoma cells, tyrosine hydroxylase and catechol-O-methyl transferase activities were increased (133). Inductions of enzyme activity were also reported for cyclic AMP phosphodiesterase in rat glial tumor cells (152), deoxycytidine (dCyd) deaminase in Chinese hamster ovary cells (38a) and calcium-stimulated adenosine triphosphatase in primary cultures of heart cells from neonatal hamsters (34). Nonenzyme proteins have also been induced by BrdUrd. Interferon production was increased in human lymphoblastoid cell lines (196), as was a surface glycoprotein in mouse neuroblastoma cells (21) and prolactin synthesis in rat pituitary tumor cells (17). Erythropoiesis was stimulated in chick blood cells by 0.66 μM BrdUrd, although 16 to 26 μM BrdUrd was inhibitory (199). Last, BrdUrd and IdUrd induced in human lymphoma cells the appearance of tubular structures in the endoplasmic reticulum that have been associated with autoimmune diseases, viral infection and cancer (69, 83).

Exposure to BrdUrd or IdUrd during a single round of DNA synthesis or cell replication was sufficient to elicit a maximal effect on the synthesis of particular proteins (16, 61, 96, 116, 176, 202, 208) or the induction of oncogenic viruses (10, 72, 157). Rutter *et al.* (148) have called this unifilar dominance. That is to say, incorporation of analog into only one strand gives a maximally inhibitory or stimulatory effect on synthesis of a protein rather than a 50% maximal effect, or less.

All of these observations are compatible with the view that the halogenated analogs exert their effect when incorporated into cell DNA. This concept is elaborated upon in the next section concerned with what is known about mechanism of action.

III. Mechanism of Action

Often, better understanding of the mechanism of action of a drug is related to advances in biochemistry and physiology. In many cases, drugs have been in use prior to the discovery of the cell entities affected. And probably just as frequently, new discoveries in cell physiology and biochemistry are a result of the use of drugs as molecular probes or the effort to understand the mechanism of drug action. An excellent example of the latter situation is the elegant work of Strominger and his colleagues on the mechanism of action of pencillin. So too, how BrdUrd and IdUrd affect eukaryotic cell function has been coupled with advances in the understanding of the synthesis and structure of DNA and RNA.

A. Incorporation into DNA

A basic question about how BrdUrd or IdUrd acts is whether these compounds have to be incorporated into DNA in order to affect a specific cell function. As is brought out, in most cases the answer appears to be yes.

The two types of studies that have been done are those that block analog incorporation into DNA by various experimental maneuvers, and those that seek to correlate the extent of incorporation of analog into DNA with the extent of the phenotypic effect.

Cotreatment with a higher concentration of dThd, the most frequently used means of blocking analog incorporation into DNA, has prevented the phenotypic effects of

BrdUrd or IdUrd (3, 37, 38, 61, 164, 183, 192, 198). Alternatively, mutant cells lacking dThd kinase activity have been tested to see if BrdUrd or IdUrd effects are prevented since the analogs, like dThd, must be phosphorylated prior to imcorporation into DNA. Cells lacking dThd kinase activity are resistant to the effects of the analogs (61, 123). The common shortcoming of both approaches is that neither distinguishes whether the prevention of the inhibitory effects of the analogs is due to blocking the formation of 5-halogenated nucleotides, or is due to the secondary consequence of this inhibition, prevention of the subsequent incorporation of these nucleotides into DNA.

Another means of blocking analog incorporation into DNA is by inhibiting DNA synthesis with drugs. The shortcoming of this approach is the obvious caveat that since drugs have multiple effects, the prevention of the analog action may be due to an effect of the compound used other than inhibition of DNA synthesis. This reservation aside, in several studies inhibition of DNA synthesis by drugs (37, 65, 161, 192, 207) has resulted in prevention of the effects of BrdUrd or IdUrd.

In other studies the extent of analog incorporation into DNA has correlated with the extent of the effect on differentiated functions. Correlations exist between the extent of BrdUrd incorporation into DNA and loss of tyrosine aminotransferase activity in rat hepatoma cells (176), inhibition of mouse adrenal tumor cell steroid production (207), loss of amylase activity in embryonic pancreas (200) and inhibition of development of creatine phosphokinase activity in chick myogenic cells (117). Such correlations, however, do not exist over the whole range of possible BrdUrd substitution for dThd. For example, Walther *et al.* (200) found that in embryonic pancreas 5% BrdUrd substitution resulted in 50% inhibition of amylase accumulation and at about 20% substitution the accumulation of the enzyme was inhibited by 90%. Goz and Walker (65) presented evidence that incorporation of IdUrd into HeLa DNA was apparently necessary for induction of alkaline phosphatase activity but the extent of incorporation did not correlate with the extent of enzyme induction. Such findings may reflect differential incorporation of analog into certain regions of DNA and is discussed subsequently (section III D).

Although most studies of the above type have indicated that 5-halogenated deoxyuridine inhibitory effects are correlated with analog incorporation into DNA, there are notable exceptions. The first exception was reported by Schubert and Jacob, (150) and others have offered similar data (143, 152). In some instances (143, 152), prevention of the action of BrdUrd by coadministration of dCyd has been offered as supporting evidence for a mechanism not involving incorporation of analog into DNA. Experiments involving nucleotide biosynthesis and pool sizes, however, have offered an alternative explanation for the effects of dCyd, which is discussed in the next section.

B. Nucleotide Pools

The size and possible compartmentalization of nucleotide pools may play a significant part in the actions of BrdUrd and IdUrd. Hauschka (74) and Elliott and Fitzsimmons (49) have reviewed the literature on nucleotide pools and related enzymes in animal cells. In particular, the pool of deoxythymidine triphosphate (dTTP) is of interest for the obvious reason that it would compete with the triphosphate derivative of BrdUrd or IdUrd for incorporation into DNA.

The dTTP pool varies appreciably from one cell type to another (74) and can change dramatically in the same cell type with changing conditions. For example, the addition of serum to serum-starved cultured mouse embryo cells caused about a 12-fold increase in the dTTP pool (171). Of importance to this discussion are the data summarized by Hauschka (74) of work with several cell lines that demonstrated the change in dTTP as a function of exogenous dThd. The addition of 1 mM dThd may

increase the dTTP pool by as much as 750-fold over the basal level. On the other hand, dTTP can inhibit dThd kinase and thus decrease the rate of dThd phosphorylation and consequently the rate of dTTP formation. 5-Iodo-2′-deoxyuridine triphosphate (IdUTP) and 5-bromo-2′-deoxyuridine triphosphate (BrdUTP) are even more potent inhibitors of this enzyme (138), and as such would probably affect the phosphorylation of their respective nucleoside precursors quantitatively differently than dTTP. As a consequence, a smaller than expected BrdUTP or IdUTP pool might form, if the expected pool size was calculated on the basis of data for addition of an equivalent concentration of dThd.

BrdUTP and IdUTP also are better inhibitors than dTTP of the enzyme ribonucleotide reductase (115 and cited in 139), which is important in the synthesis of deoxyribonucleotides. In this regard, Meuth and Green (115) have demonstrated that in a number of cell lines, BrdUrd will stop cell replication by this mechanism. They further showed that addition of dCyd will relieve the BrdUrd block by serving as a precursor for the formation of dCyd nucleotides that cannot be synthesized from cytidine diphosphate by the inhibited ribonucleotide reductase. Horn and Davidson (78), as well as Bick (11), more recently presented data that showed that the action of dCyd is more complicated than just bypassing the block of ribonucleotide reductase, which is not unexpected, given the intertwining regulation in nucleotide metabolism. For example, dTTP is an allosteric inhibitor of deoxycytidylate deaminase and, again, IdUTP is a better inhibitor (137). This issue is important because of a few studies that have shown reversal of BrdUrd effects by dCyd (143, 150). In these papers, it was argued that this was evidence against BrdUrd acting as a result of incorporation into DNA.

Horn and Davidson (78) examined the effect of dCyd on several actions of BrdUrd on Syrian hamster melanoma cells. dCyd prevented BrdUrd inhibition of pigmentation and tumorigenicity as well as other BrdUrd-induced effects. Most importantly, however, this antagonism by dCyd of BrdUrd action was accompanied by a decreased incorporation of BrdUrd into DNA. Schubert and Jacob (150) did not measure BrdUrd incorporation. Horn and Davidson argued that Rogers *et al.* (143) were measuring low levels of incorporation (1–2%) even without addition of dCyd and that the method of analysis, CsCl gradients, would be too insensitive to accurately determine a change in analog incorporation. This effect of dCyd on analog activity is, however, not universal. Others have reported no effect in other cells of dCyd on similar actions of BrdUrd (200, 208) on differentiated functions, and Davidson and Kaufman (43), in a paper discussed below, have published data that indicate that inhibition of BrdUrd incorporation into DNA may not be the site of the dCyd effect on melanoma pigmentation.

Bick (11) has offered more insight into this issue with his studies of BrdUrd inhibition of induction of erythroid differentiation in Friend leukemia cells. Bick's study was facilitated by a procedure that he developed (12) to measure the pool of BrdUTP and the dTTP pool. In essence, Bick has shown that dCyd most likely reduces BrdUrd incorporation into DNA by increasing the cellular concentration of dTTP as a result of dCyd conversion to dTTP. Inhibition of deoxythymidylate (dTMP) synthetase, one of the steps in the conversion route, prevents the effect of dCyd on BrdUrd inhibition of induction in Friend leukemia cells. It should be noted that a much earlier study of mouse leukemia cells, L5178Y, foreshadowed Bick's findings. Delamore and Prusoff (45) discovered that dCyd greatly increased utilization of radioactive formate (used for the methyl group in dTMP biosynthesis), and offered as one possible explanation that dCyd was increasing the pool of 2′-deoxyuridylate with subsequent increased dTMP synthesis.

Last, Davidson and Kaufman (43), after

more critically examining the effect of dCyd on BrdUrd inhibition of pigmentation in Syrian hamster melanoma cells, concluded that the reduction of pigmentation is not due to the reduction in the incorporation of BrdUrd into DNA. The maximal reduction of BrdUrd incorporation into DNA was achieved with about 8 μM dCyd. At this concentration of dCyd, there was no significant prevention of the decrease in pigmentation by BrdUrd. At concentrations of 0.4 to 1.0 mM dCyd, a range in which there was no further decrease in BrdUrd incorporation into DNA, the inhibition of pigmentation was prevented. Aminopterin, which prevented conversion of dCyd to dThd nucleotides, prevented the effect of dCyd on pigmentation. This suggests that dCyd must be converted to dThd nucleotides to affect the inhibition of pigmentation by BrdUrd.

In light of this just cited study by Davidson and Kaufman (43), it is necessary to be cautious in attributing an effect of eukaryotic differentiation of BrdUrd or IdUrd to incorporation into DNA. More data are needed on the effect of relatively high concentrations of dCyd in other sensitive cell lines to ascertain if the effect is universal. The results of Goz (62), Goz and Prusoff (63) and Aamodt and Goz (1) are relevant here even though the studies were done with a bacterial virus. In these studies, purified virus with IdUrd-substituted DNA was used to infect bacterial cells. These viruses with IdUrd-substituted DNA were unable to induce normal levels of viral proteins. This effect, albeit in a bacterial virus not a eukaryote, could only have been due to the IdUrd in the viral DNA, since no analog was in the medium for maintaining the infected cells.

Some studies on compartmentalization should be cited before leaving the topic of nucleotide pools. As is discussed in detail in section III, D, there appears to be preferential incorporation of BrdUrd relative to dThd into certain DNA regions. Compartmentalization of nucleotide pools is one possible explanation for the preferential incorporation. Hauschka (74) has reviewed some of this literature. There is evidence for compartmentalization of dTTP in various types of cells. For example, three groups have presented evidence for dThd nucleotide pool compartmentalization in HeLa cells (9, 98, 172).

C. DNA Protein Binding

If it can be agreed that BrdUrd and IdUrd exert their effects on differentiation as a consequence of incorporation into DNA, the next question would be how the properties of the substituted DNA differ from normal DNA so as to lead to the observed effects on cell function? The principal idea that has come from the studies of BrdUrd-or IdUrd-substituted eukaryotic DNA is that the affinity of chromosomal proteins for DNA is increased.

It has been known for several years that the physical properties of BrdUrd- or IdUrd-substituted DNA are different than those of normal DNA. These differences include greater sensitivity to hydrodynamic shearing forces (189) and increased temperature (91, 109) as well as decreased pH (8) required for DNA denaturation. For more details, the reader is referred to the review by Prusoff and Goz (139). Most of these studies were done with purified DNA from prokaryotes or from bacterial viruses. A seminal series of experiments also using prokaryotic DNA, but with important implications for how the 5-halogenated deoxyuridine analogs may be working in eukaryotes, was reported by Lin and Riggs. After having established that poly[d(A-BrU)] was approximately 40-fold more effective than poly[d(A-T)] as a competitor for binding to the *Escherichia coli lac* repressor (105), Lin and Riggs demonstrated that the *lac* repressor would bind 10-fold more tightly to the *lac* operator that had about 90% of its dThd residues replaced by BrdUrd than it would bind to unsubstituted *lac* operator DNA (106).

The obvious extension of the findings of Lin and Riggs was to examine the binding of eukaryotic chromosomal proteins to normal and 5-halogenated deoxyuridine sub-

stituted DNA. Several of these studies were published in 1974 of mouse (6, 100), rat (39) and human (166) chromatin. Both Augenlicht *et al.* (6) and Simpson and Seale (166) reported a blue shift and increased positive ellipticity in the 250 to 300 nm region of circular dichroism spectra of BrdUrd-substituted chromatin. Lapeyre and Bekhor (100) also reported a change in circular dichroism spectra of BrdUrd-substituted chromatin but in the opposite direction. Positive ellipticity was decreased. Nicolini and Baserga (121) attempted to resolve this discrepancy by demonstrating that the changes in spectra are related to the extent of dThd replacement. In addition, the well documented increase in the melting temperature of DNA caused by 5-halogenated deoxyuridine substitution was shown to be more pronounced in analog-substituted DNA in chromatin (6, 39).

Subsequent reports have focused on which specific chromatin protein(s) may be interacting differently with BrdUrd-substituted DNA as compared with unsubstituted DNA.

Lin *et al.* (107) discerned a stronger binding of certain pea seedling histones to 90% BrdUrd-substituted *lambda* phage DNA than to control DNA.

Schwartz (154), in his studies of BrdUrd induction of oncornavirus in rat embryo cells, ascertained that isolated, radiolabelled nonhistone chromosomal proteins were bound more avidly to BrdUrd-substituted DNA than to control DNA. Acrylamide gel analysis of the nonhistone chromosomal proteins that bound to control or substituted DNA revealed a complex mixture of proteins which may have differed from each other in composition. However, this could not be concluded with certainty because of the method of analysis used. Apparent differences in amino acid composition between the proteins bound to the two types of DNA were also reported, but Schwartz chose not to amplify this finding.

Gordon *et al.* (60) compared the interaction of both histone and nonhistone chromosomal proteins with normal and BrdUrd-substituted DNA. Histones showed a greater affinity for BrdUrd-substituted DNA (one strand was 80% substituted, the other not). Differences in affinity for substituted DNA existed among the various histones. They occured if measured either by DNA retention on nitrocellulose filters or on hydroxylapatite. Histone-depleted chromatin (assumed to contain only nonhistone chromosomal proteins) also caused greater retention of BrdUrd-substituted than unsubstituted DNA, and this was equated with a greater affinity of nonhistone chromosomal proteins for substituted DNA.

Bick and Devine (15) studied the interaction of chromosomal proteins with BrdUrd-substituted DNA of a Syrian hamster melanoma line and also concluded, as others have, that BrdUrd-substituted DNA competes better than control DNA for binding of nonhistone chromosomal proteins. In addition they showed that the greater the BrdUrd substitution, the greater the effectiveness of the DNA to compete for binding of nonhistone chromosomal proteins.

The extension of these *in vitro* binding studies to what may be happening in the intact cell is somewhat tenuous, an inherent problem in any extrapolation from *in vitro* experiments. For example, the order of histone-binding affinities to BrdUrd-substituted DNA varies somewhat according to the technique employed for measuring binding (60). More to the point, the studies of Lapeyre and Bekhor (101) illustrate the complicated situation that exists in the binding of histone and nonhistone chromosomal proteins, since each class of proteins affected the binding of the other. When BrdUrd was substituted into the DNA of the murine Krebs II ascites cells to about 33%, there was a tendency, in the absence of histone binding, for the substituted DNA to bind more nonhistone chromosomal proteins per unit input than the unsubstituted DNA. Binding of histones to control DNA caused an order of magnitude increase in binding of nonhistone chromosomal proteins. Strikingly, under these latter conditions BrdUrd substitution in DNA

reduced rather than increased, by 50 to 60%, the binding of most nonhistone chromosomal proteins.

It should also be mentioned at this point that incubation of cells with BrdUrd does not appear to alter the synthesis or degradation of DNA-binding proteins (25, 166).

D. Regional Incorporation of Analog into DNA

A second line of attack on the mechanism of action of the 5-halogenated deoxyuridines has been to study the incorporation of analog into particular regions of eukaryotic DNA. This approach was presaged by the work on bacteriophage DNA by Szybalski *et al.* (191), who reported that bacteriophage DNA contained tracts of pyrimidines. They suggested that these oligopyrimidines functioned as binding sites for RNA polymerase. In agreement with this idea, Shishido and Ikeda (160) reported preferential binding of RNA polymerase to dThd-rich fragments of bacteriophage F1 DNA, and Jones and Berg (88) reported that the polynucleotide, poly(dT), inhibited the binding of RNA polymerase to T7 bacteriophage DNA. Incorporation of BrdUrd into such sites might then alter RNA polymerase activity. Subsequent to the findings of Szybalski *et al.*, Britten and Kohne (20) and Britten and Davidson (19) published their now classical papers summarizing their initial work on unique and repeated sequences of the DNA of higher organisms. Briefly stated, eukaryotic DNA contains three classes of nucleotide sequences, (1) those that appear only once per genome, single copy; (2) those repeated 10^3 to 10^5 times, moderately repetitive; and (3) those repeated about 10^6 times, highly repetitive. In addition, the moderately repetitive sequences, which are believed to have a regulatory role, are interspersed and contiguous with the single-copy sequences (40), which are believed to code for the proteins made in the cell. The essence of the work to be described at this point is, with two exceptions, that BrdUrd is preferentially incorporated into intermediate repetitive sequences (10^3–10^5 copies) of eukaryotic DNA when compared with incorporation of dThd.

Baker and Case (7, 24) published findings on the incorporation of BrdUrd into sea urchin DNA which, although they did not interpret it as such, probably reflected preferential incorporation of the analog into intermediate or moderately repetitive DNA sequences. These workers found that DNA extracted from sea urchins grown in the presence of BrdUrd (41% substitution) accumulated low-molecular weight fragments of duplex DNA (30–60 S) as compared with control DNA (>60 S). They attributed this to incorporation of BrdUrd into what they considered naturally occurring regularly spaced single-stranded regions of sea urchin DNA. The existence of these single-stranded regions was determined by analysis of DNA treated with the single-strand-specific nuclease from Aspergillus oryzae. The data of Schwartz (153) have indicated that these single-stranded regions may not be naturally occurring but rather formed by depurination by the acid conditions needed for the Aspergillus nuclease.

Schwartz and Kirsten (156) ascertained that when rat embryo cells were grown with either radioactive 10^{-7} M dThd or BrdUrd (this concentration of BrdUrd yielded less than 5% substitution), the BrdUrd was preferentially incorporated into intermediate repetitive over single-copy sequences as compared with dThd, which was relatively uniformly incorporated into intermediate repetitive and single-copy sequences. At a higher dosage of BrdUrd, 10^{-4} M, that gave greater than 90% substitution, distribution of BrdUrd was, like that of dThd at the lower dosage, uniform into all sequence frequencies of DNA. This same phenomenon, at the lower concentration, also occurred when the cells were synchronized and the nucleosides were present only during portions of S phase, (157). Moreover, in contrast to their earlier report (156) in which 10^{-4} M BrdUrd was reported to be the optimal concentration for latent C-type virus expression, in synchronized cells 10^{-7} M BrdUrd added during S phase was sufficient for expression of oncogenic viral func-

tions such as a virus-specific antigen. Thus, the biological effect of BrdUrd was obtainable at a concentration that gave preferential incorporation into intermediate repetitive sequences. Schwartz (153) further extended these findings by employing nucleases along with hybridization studies to obtain data that suggested that the radiolabelled BrdUrd was incorporated preferentially into adenosine-thymidine-rich clusters, and particularly into such clusters in intermediate repetitive sequences in rat embryo DNA.

Strom and Dorfman (184) made similar observations of preferential BrdUrd incorporation into intermediate repetitive sequences of the DNA from cultured dissociated chick limb bud mesenchymal cells. BrdUrd inhibited differentiation of these cells into cartilage (3).

Grady and Campbell (67), on the other hand, did not find preferential incorporation of BrdUrd into the intermediate repetitive sequences of polyoma virus-transformed mouse cells. However, these studies used a relatively high concentration of BrdUrd, 10^{-5} M, that resulted in 30% substitution of dThd as compared with the 10^{-7} M and less than 5% BrdUrd substitution for dThd reported by Schwartz and Kirsten (156). Singer *et al.* (167), however, found that at all levels of substitution from 2 to 95%, BrdUrd did not preferentially substitute for dThd in the intermediate repetitive sequences of rat hepatoma DNA. This study also revealed no difference in the frequency of methylcytosine residues in BrdUrd substituted *vs.* control DNA. It may be that the discrepancy here between the data of Singer *et al.* and of Schwartz and Kirsten (156) and Strom and Dorfman (184) is attributable to the use of aminopterin by Singer *et al.* to block *de novo* dTMP synthesis. The effect of inhibition of *de novo* dTMP synthesis is discussed further later in this section. Also, Singer *et al.* studied a tumor line as contrasted to the other two studies that employed normal embryonic cells.

In summary, the main line of thought developed here and reflective of the data and hypotheses of the authors cited above is that BrdUrd and IdUrd are preferentially incorporated into intermediate repetitive sequences of DNA. These sequences are believed to serve as regulatory sites on DNA (19) and, as such, probably interact with specific chromosomal proteins (126, 159, 193). Chromosomal proteins in turn have been shown to have a higher affinity for BrdUrd-substituted DNA. Thus, it is conceivable that if a regulatory protein binds more quickly to a BrdUrd-substituted regulatory region, the adjacent gene will not be transcribed. Thus far, specific nonhistone chromosomal proteins have not been identified that interact differentially with substutited DNA, although there are reported differences in the relative affinities of specific histones for BrdUrd-substituted DNA (60, 107). Allowance must be made in this scheme for the report of nonpreferential substitution into intermediate repetitive sequences (167), and the fact that selective inhibition of differentiated functions have routinely been observed under conditions of considerable BrdUrd substitution for dThd. Thus, it is possible that, although there is preferential substitution, it may not be related to the selective effect of BrdUrd or IdUrd on differentiation or oncogenic virus induction. Rather, it may be that selective inhibition of differentiated functions lies in differential sensitivity of function of the region of DNA that is substituted. That is to say, BrdUrd or IdUrd substitution into a region of DNA may be effective only if that particular region binds a regulatory protein, and if the binding or release of that protein becomes critical to expression of the gene and its product.

Mention should be made at this point of the ideas of Strom and Dorfman (184, 185) regarding gene amplification, differentiation and a possible mechanism of action of BrdUrd. The system that they studied was dissociated chick stage 24 limb bud mesenchymal cells, cultured at high cell density, which differentiate into cartilage. Treatment with 32 μM BrdUrd during the first 48 hr of culturing irreversibly inhibited differentiation. Analysis of the DNA by

reassociation kinetics showed that the amount of moderately repetitive sequences were proportionately higher in differentiated cartilage cells than in undifferentiated cells, suggesting that amplification of some moderately repetitive sequences was associated with differentiation. Strom and Dorfman (184) observed that BrdUrd was preferentially incorporated into moderately repetitive sequences and that under culture conditions where there was little DNA synthesis, little radiolabelled dThd previously incorporated into DNA was lost from the DNA over 2 days but that almost half of similarly incorporated radioactive BrdUrd was lost. These data taken together suggested to Strom and Dorfman that BrdUrd-substituted amplified sequences were produced but were subsequently lost due to increased degradation and if these amplified sequences were lost, differentiation was thus blocked. By way of support, the data of Skalko and Packard (169) showed an 11-fold decrease in the half-life of radiolabelled BrdUrd in mouse embryo DNA compared with radiolabelled dThd, and Krider and Blake (97) described a decrease in the number of moderately repetitive ribosomal DNA sequences in Drosophila larvae after administering BrdUrd. As stated above, the effect of BrdUrd under the conditions used by Strom and Dorfman was irreversible. More data and understanding are needed to comprehend how this hypothesis would fit the situation where the effect of BrdUrd is reversible, or how such a hypothesis could account for the reversal rather than the prevention of differentiation by BrdUrd reported in some studies. Does this difference in the half-lives of radioactive dThd and BrdUrd occur only under conditions of little or no DNA synthesis or does it require such a state to be measurable, or both? Also, how would this fit in with the notion that perhaps BrdUrd acts by changing the binding of regulatory chromosomal proteins? A similar experiment with IdUrd in HeLa cells under conditions that induced alkaline phosphatase activity revealed no measurable difference in half-life in DNA between radioactive dThd and IdUrd (Goz, unpublished observations). The HeLa cells were actively synthesizing DNA and perhaps, as mentioned above with active DNA synthesis, the removal process did not occur or was not measurable.

Whether or not the effects are due to preferential incorporation of analog into particular portions of the DNA molecule and subsequent interaction with chromosomal proteins, how is such preferential incorporation accomplished? Thus far, the studies of the enzymes of nucleotide and DNA synthesis have revealed no preference for analog over endogenous substrate. The only difference observed has been that the triphosphate forms of the analogs are more potent feedback inhibitors (see section III B on nucleotide pools). One possibility is that the relevant enzyme(s) has not been studied yet. For example, the eukaryotic DNA polymerases have multiple forms and functions (203). Perhaps one will interact with BrdUTP or IdUTP differently than with dTTP. Also, it may be that different forms of polymerase may make different parts of the DNA molecule. Another possibility is compartmentalization of nucleotide pools. The halogenated analogs are incorporated into DNA only after first being phosphorylated by thymidine kinase of the salvage pathway. By contrast, dTMP may be formed by dThd kinase phosphorylation of dThd or *via* the *de novo* pathway, culminating with conversion of deoxyuridylate to dTMP by dTMP synthetase. Kuebbing and Werner (98) have demonstrated that, at least in HeLa cells, there may be separate pools of dTTP; one formed *via* the salvage pathway, another from *de novo* synthesis and a third, replication precursor pool. It is worth noting again that Singer *et al.* (167) failed to find selective incorporation of BrdUrd into the repetitive sequences of HTC (rat hepatoma) DNA. Their protocol, which employed aminopterin to block *de novo* dTMP synthesis, could alter the relation between compartmentalized dTTP pools if such existed also in rat hepatoma cells. This effect on pool

compartmentalization could be the reason why preferential incorporation was not found at any degree of substitution from 2.1 to 95.0%. Although no preferential incorporation was observed, selective decreases in the activity of certain enzymes were still observed. Thus, the data of Singer *et al.* present an apparent contradiction to the current direction of thinking that needs to be resolved. Their data indicate a selective effect in the absence of preferential analog incorporation. Last, in this vein, Rizki and Rizki (141) reported that, although coadministration of 5-fluorouracil (an inhibitor of dTMP synthetase after conversion to fluorodeoxyuridylic acid) with radioactive dThd made no difference in the distribution of dThd in various chain lengths of pyrimidine oligonucleotides isolated from Drosophila melanogaster DNA, it did alter the incorporation pattern of radioactive BrdUrd.

E. Effects on RNA Synthesis

If the primary site for selective inhibition of differentiated functions by BrdUrd or IdUrd occurs in chromatin, there should be observable effects on the next step in the sequence leading to protein synthesis, mRNA synthesis. Two probable possibilities exist. BrdUrd or IdUrd incorporation into DNA results either in selective inhibition of one or a few specific mRNAs or there is a general inhibition of mRNA synthesis. Stellwagen and Tompkins (176, 177) used the latter possibility as a basis for explaining the differential inhibition of synthesis of certain proteins in HTC hepatoma cells. If each species of mRNA has a different rate of degradation, then a uniform slowdown of the rate of synthesis of all mRNA would differentially change the available pool of particular mRNAs. Such an argument could also be extended to a differential rate of protein degradation. The data of Gurr *et al.* (70) for Reuber hepatoma cells do not support this idea. The half-lives of mRNA and protein for phosphoenolpyruvate carboxykinase and tyrosine aminotransferase are similar, yet BrdUrd induced the activity of the former and inhibited the activity of the latter. Kasupski and Mukherjee (90) reached the same conclusion by studying enzymes in mouse L cells that were sensitive or resistant to inhibition by BrdUrd.

The direct measurement of specific eukaryotic mRNAs, until relatively recently, has been a difficult task and few have been isolated (18). The discovery of polyadenylate [poly(A)] on the 3′ end of most mRNA (reviewed in 18), the use of RNA-dependent DNA polymerase to make complementary, highly radioactive DNA from purified mRNA (135) and certain immunological approaches (124) have greatly facilitated this type of experiment.

Preisler *et al.* (135) have done the only study to date correlating inhibition of synthesis of a specific protein with inhibition of specific mRNA production. In Friend mouse leukemia cells, BrdUrd inhibited the synthesis of globin and globin mRNA that was stimulated by treatment with dimethyl sulfoxide. This was measured by hybridization studies between total cell RNA and radiolabelled DNA synthesized complementary to purified globin mRNA by RNA-dependent DNA polymerase. An intriguing finding of this study is that, although BrdUrd inhibited the increment in globin mRNA caused by dimethyl sulfoxide, when BrdUrd was added in the absence of dimethyl sulfoxide, globin mRNA increased by 3.5- to 5-fold over that of the control. Preisler *et al.* posit that there may be two different mechanisms for the effects of BrdUrd. They cite the paper of Ostertag *et al.* (123), which reported that BrdUrd slightly stimulated globin synthesis in the presence of dimethyl sulfoxide in one mutant line of Friend leukemia cells that lacked dThd kinase activity. Thus, Preisler *et al.* theorized that one effect of BrdUrd, stimulation of globin synthesis, is accomplished *via* a mechanism not requiring incorporation of analog into DNA.

Lykkesfeldt and Andersen (111) compared the inhibition by BrdUrd of synthesis of 17 and 25 S ribosomal RNA with the

inhibition of synthesis of all other RNAs (tRNA, 5 S RNA and some mRNA) in Tetrahymena pyriformis. There was an apparent greater inhibition of synthesis of 17 and 25 S RNA than of the other RNAs. On the other hand, Fausto-Sterling *et al.* (cited in 36) found increased ribosomal RNA synthesis in chick myoblasts. Krider and Blake (97) reported that in Drosophila virilis, BrdUrd treatment of larvae significantly reduced the redundancy of ribosomal RNA cistrons in the adult but not in the larvae. The effects on Tetrahymena ribosomal RNA (111) occurred within one generation time, whereas those on Drosophila ribosomal RNA cistron redundancy were evident only after maturation. It appears unlikely that these two effects of BrdUrd are related, although the reduction in ribosomal RNA cistrons in Drosophila by BrdUrd may be explained by the amplification hypothesis of Strom and Dorfman (185) discussed previously.

Neither Preisler *et al.* (135) nor Stambrook and Williamson (174), who studied error frequency in 5 S RNA of the Chinese hamster, found a higher frequency of errors in the RNA nucleotide sequence in cells grown in BrdUrd, as compared with control cells. This is in contrast to the results of Hill *et al.* (75) with mouse 3T6 cells. These investigators found an alteration in the base composition of RNA transcribed from 20% BrdUrd substituted DNA both *in vivo* and *in vitro*. In the *in vitro* experiments, RNA was transcribed from either a chromatin or DNA template with a highly purified RNA polymerase. The RNA transcribed from BrdUrd-substituted DNA had a higher proportion of guanine and a lower adenine proportion than transcripts from control DNA. Stambrook and Williamson (174) suggested that the apparent discrepancy between their data and that of Hill *et al.* (75) may be attributed to the fact that Hill *et al.* analyzed nascent transcripts, whereas they were analyzing functional 5 S RNA from ribosomes. If the cell can recognize and remove faulty transcripts before they reach the ribosomes, this would then account for the difference in results. Preisler *et al.* (135) extracted RNA from whole cells, which would contain a mix of nascent and functioning RNA. Presumably, the functioning RNA would contain the major portion of the globin mRNA.

When Kotzin and Baker (92) measured general incorporation of [^{3}H]-uridine into total cell RNA of gastrula state Strongylocentrotus purpuratus sea urchins, they observed an appreciable diminution in the rate of incorporation within about 5 min after addition of 162 μM BrdUrd. Hybridization experiments indicated that the composition of the radiolabelled RNA from the BrdUrd-treated embryos differed from RNA of control embryos. In a subsequent paper with Fitzmaurice (35), Baker described experiments that indicated that the decrease in [^{3}H]-uridine incorporation into RNA caused by BrdUrd was not due to a decrease in the gross rate of RNA or DNA synthesis, but rather was due to a decrease in the uptake of exogenous [^{3}H]-uridine. These investigators believed that BrdUrd competes with radiolabelled uridine for incorporation into the cell. Another possibility, not considered by them in their paper, is that BrdUrd or one of its metabolites had an effect on the cell membrane that caused the inhibition of [^{3}H]-uridine uptake. Tsuboi and Baserga (197) reported that 163 μM BrdUrd inhibited cellular uptake of both radiolabelled deoxyglucose and cycloleucine in 3T6 mouse fibroblasts. In HeLa cells, 3 μM IdUrd also inhibited cellular uptake of radiolabelled amino acids (B. Goz, unpublished observations). Neither of these effects are likely consequences of analog incorporation into cellular DNA, because they ensue too quickly (within minutes of addition of analog to the culture medium in the study with mouse fibroblasts) for there to be any effect from incorporation of analog into DNA.

Schwartz and Kirsten (155) also examined RNA synthesis in embryonic tissue. In this case, mesenchymal cells from 16-day-old rat embryo maxillary and mandibular tooth germs were treated after culturing in

monolayers in the presence or absence of 33 μM BrdUrd for 24 hr. A gradual (15–20%) decrease in total RNA synthesis occurred in treated cells over a period of 5 days. This correlated with a 20% decrease in [^{3}H]-uridine incorporation into RNA during a 1-hr pulse performed at 5 days. When [^{14}C]-adenosine was used to label poly(A)-containing sequences, quantitative but not qualitative differences in the sucrose gradient profile of the RNA from cells treated with BrdUrd for 24 hr were noted 24 hr after BrdUrd removal, but not immediately after removal of the analog.

Pawlowski (127) found a similar effect with 30 μM BrdUrd on synthesis of poly(A)-containing cytoplasmic RNA of primary cultures of sternum cells from 11-day-old chick embryos. Although BrdUrd after 24 hr had little effect on the synthesis of total cytoplasmic RNA, the rate of synthesis of poly(A)-containing cytoplasmic RNA was significantly reduced in the treated cells.

By contrast, Price (136) found that in the murine B16 melanoma cell line BrdUrd increased rather than decreased the transcription of certain poly(A)-containing RNA. The B16 melanoma line may be maintained in continuous culture with BrdUrd. Under this condition the cells are amelanotic, do not make plasminogen activator and contain C-type RNA tumor viruses (reviewed in section II E). Price compared the polyadenylated RNA synthesized by a clone of these cells with the polyadenylated RNA in a clone from the parental line. To do this, he used heterologous hybridization to complementary DNA made from isolated polyadenylated RNA from both clones, BrdUrd-maintained and control. His data indicated that less than 2% of the mRNA that contained poly(A) and that was made by the untreated cells was not made by the BrdUrd-treated cells. On the other hand, growth in BrdUrd caused the appearance of mRNA (equal to about 15% of the total mRNA) in the treated cells that was not present in the untreated cells. These sequences were part of the moderately repeated group of mRNAs. Price believed that some of these sequences of mRNA unique to the BrdUrd-treated cells may be related to the induced C-type viruses. Thus, by a more refined technique, apparent increase as well as a possible decrease in mRNA synthesis was observable. Price's work, therefore, does not necessarily contradict the results of Schwartz and Kirsten (155) or Pawlowski (127). It is also worth bearing in mind that the latter two papers were on work with embryonic cells whereas Price studied a tumor cell line.

Colbert and Coleman (36) also employed DNA-RNA hybridization to study primary myogenic cultures from 11-day-old chick embryos. In this instance, BrdUrd treatment decreased by 4 to 6% the amount of RNA hybridizable to nonrepetitive DNA. As Colbert and Coleman point out, these data are quantitative in nature and do not reveal whether there were any changes in the composition of the mRNA population. In an earlier study, Grady and Campbell. (67) also found a reduction in transcription of nonrepetitive DNA by BrdUrd. In this study by Grady and Campbell with polyoma virus-transformed mouse cells, the 10 μM BrdUrd did not substitute preferentially into either repetitive or nonrepetitive sequences of DNA.

IV. Concluding Comments

What may be concluded from the literature reviewed on the actions of halogenated deoxyuridine analogs of dThd in eukaryotic cells? The lethal, toxic, teratogenic and mutagenic activities of BrdUrd and IdUrd are well documented. There are also many examples of these compounds preventing or reversing embryonic and cell differentiation and inducing oncogenic viruses. The mechanism for the effects on oncogenic virus induction and differentiation is not understood, although there are data that may eventually lead to the mechanism of action.

Incorporation of the analog into DNA seems in most cases to be necessary for an effect on differentiation. But a sufficient number of studies have been published with data to the contrary to warrant caution in

fully accepting the incorporation of analog into DNA as essential to the analog effect, and there is an alternative hypothesis that the halogenated deoxyuridine compounds produce their effects by an action on plasma membranes.

The reservations about analog incorporation into DNA aside, the next point is that low (10^{-7}–10^{-6} M) concentrations of BrdUrd and IdUrd are incorporated to a greater extent into the intermediate repetitive sequences of DNA than into the other sequence frequencies. How such differential incorporation occurs is not known, although some possible means have been proposed. Whether this differential incorporation is causally related to the effects of the analogs on differentiation is not certain. In one publication, no differential analog incorporation was found but synthesis of specific proteins was inhibited.

Chromosomal proteins have greater binding affinities for BrdUrd-substituted DNA than for unsubstituted DNA. Some chromosomal proteins are believed to regulate DNA transcription by binding to regulator regions of DNA. Tighter binding of regulator proteins to BrdUrd- or IdUrd-substituted regulator regions of DNA could affect transcription of mRNA. This is an attractive idea in light of the reported differential incorporation of analog into intermediate repetitive sequences which are believed to be regulatory regions of DNA. Again, this idea must be viewed with caution because of the one report in which differentiation was affected without differential incorporation.

Finally, synthesis of specific mRNA species is inhibited, which then leads to reduced synthesis of particular proteins. More work is needed in this area since the synthesis of globin is the only instance in which both a specific mRNA and protein have been studied. The possible effects of the deoxyuridine analogs on processing or cleavage of high-molecular weight precursor RNA species and tRNA synthesis and function could also be profitable areas to investigate.

Much has been learned about the effects of the 5-halogenated deoxyuridines but many questions remain to be answered. What specific base sequences in DNA are most sensitive to analog substitution? Are they in fact regulatory sequences and if so which specific proteins interact with them? Do the regulatory regions that are affected control the synthesis of only one or a few proteins, or are they "master switches" which turn on synthesis of a battery of proteins? In any instance, do the 5-halogenated analogs act by inhibiting synthesis of repressor molecules? What are the physicochemical interactions between analog and proteins that underlie these effects? And most importantly, how can these actions of 5-halogenated deoxyuridines be used therapeutically in addition to the already known antiviral activity? The work of Silagi and colleagues is encouraging in this direction; BrdUrd-treated tumor cells injected into mice afforded protection against a challenge with untreated tumor cells. Looking further into the future, some day the ability of these compounds to prevent or reverse differentiation might be used to encourage limb or organ regeneration in humans. There is a great deal to be done and learned, and the prospects are exciting.

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