A Review of Some Aspects of 5-Iododeoxyuridine and Azauridine*

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SUMMARY

A review has been presented of selected aspects of 5-iododeoxyuridine (IUDR) and of 6-azaunidine. Some of the basic problems which may be of pertinence to the successful use of IUDR clinically have been indicated. Although the role of IUDR as a chemotherapeutic agent in the treatment of neoplasms, either alone or in combination with other compounds or radiation, is still under investigation, its role as an anti-viral agent in the treatment of some established DNA-viral infections in animals as well as in man has been well substantiated.

I. IODODEOXYURIDINE

Prior to a discussion of the present clinical status of 5-iodo-2′-deoxyuridine (IUDR), it would be of value to review what is known of the chemistry of IUDR and the effect of this compound on biochemical and physiological mechanisms. Although the clinical usefulness of IUDR in the treatment of certain neoplasms in man has been demonstrated (12, 87), can anything be done to broaden its spectrum of activity? Is anything not being considered at present in the clinical evaluation of this compound?

Comparison of the molecular structures of IUDR and thymidine indicates that the methyl group on carbon-5 of the thymine moiety has been replaced by an iodine atom. Replacement by an iodine atom of the hydrogen on carbon-5 of a pyrimidine was first achieved by Johnson and Johns (58) in 1905 in an alkaline reaction mixture. Although this synthetic procedure can be used in the iodination of nucleosides of uracil, iodination in dilute nitric acid results in the direct formation of crystals of 5-iodouracil ribonucleoside (94) or of 5-iodouracil-2′-deoxyribonucleoside (89), a desirable feature for the preparation of large amounts of these compounds. Radioactive IUDR labeled with either iodine-131 or iodine-125 is readily obtained by this procedure or by appropriate modifications of it (68). The synthesis of the corresponding nucleotide, 5-iodo-2′-deoxyuridine 5′-phosphate, also has been accomplished by iodination in dilute nitric acid (40). The 5′-mono and -diphosphate derivatives of 5-iodouridine have been synthesized by Michelson et al. (80). Zemelhof et al. (128) and Dunn and Smith (923,924) have isolated IUDR from the DNA of microorganisms which grew in media supplemented with iodouracil. Friedkin and Roberts (30) presented evidence for the formation of IUDR by incubation of iodouracil, deoxyribose-1-phosphate, and thymidine phosphorylase.

The incorporation of iodine in place of the methyl group of thymidine 5′-phosphate results in an increase in the bulk volume, since the methyl group has a van der Waal’s atomic radius of 2.00, whereas that of iodine is 2.15. Although the size of the resultant halogenated molecule has been increased, any hindrance to its capacity to substitute for the thymidine 5′-phosphate component of the double helix of deoxyribonucleic acid (DNA) appears to be small. Indeed, consideration of the composition of this double helix, as proposed by Watson and Crick, clearly indicates that there should not be any problem for incorporation of IUDR into DNA because of steric hindrance. The iodine atom is well removed from the groups required for hydrogen bonding with the adenine moiety of the complimentary DNA strand of the double helix. Although the increased volume of the substituent on the 5-position of the pyrimidine ring does not appear to affect seriously the structure of the resultant DNA, another property of IUDR may have a significant effect. Titration of...
IUDR revealed that the molecule has a lower pKₐ than that of thymidine; whereas the pKₐ of thymidine is 9.8, that of IUDR is 8.25; thus, at pH 7.4, the proportion of the enolic form of IUDR is greater than that of thymidine by a factor of 34. This has obvious implications, not only in the forces binding the two strands of DNA together, but also in the potential problem of incorrect genetic coding. The genetic aspect will be discussed in more detail later. Incorporation of 5-bromo-2'-deoxyuridine (BUDR) into DNA in place of thymidine increases the forces binding the two strands of DNA together, since DNA that contains this analog denatures at a higher temperature than does normal DNA (55, 66, 1923). A DNA polymer that contains IUDR in place of thymidine would be expected to behave in a similar manner.

Studies in microorganisms.—Studies in microbial systems have shown that 5-iodouracil antagonizes the utilization of thymine or thymidine for growth and that the analog is incorporated into bacterial DNA (23, 24, 37, 49, 71–73, 122, 126–128).

The growth of Streptococcus faecalis (ATCC 8043) in media supplemented with either thymine, thymidine, or pteroylglutamic acid is inhibited by IUDR (37). A mutant of S. faecalis was selected that evolved in media containing 6-azathymine, an analog of thymine in which the carbon and hydrogen atoms in position-6 are replaced by a nitrogen atom (37). This mutant, as compared with the parent organism, not only had a 7,000-fold increased resistance to 6-azathymine, but also, for half-maximal growth, required either a 400-fold increase in the concentration of thymine or an eightfold increase in the amount of thymidine. Because this mutant possessed a more than 200-fold increased resistance to azathymine deoxyribonucleoside, studies with IUDR were initiated to determine whether cross-resistance existed to other analogs of thymidine. It was found, however, that the addition of low levels of IUDR to media supplemented with suboptimum amounts of thymidine resulted not in suppression of growth of this mutant, but rather in maximal stimulation of growth; however, with higher levels of IUDR, inhibition of growth was observed (37). Under those conditions in which IUDR caused a fivefold increase in cell number, the amount of incorporation of this analog into DNA was minimal; only about 8 per cent of the DNA-thymidine was replaced by IUDR. This could be explained on the basis that IUDR either stimulates the synthesis of thymidylc acid de novo or permits a more efficient utilization of the thymidine present in the media; the latter was shown to apply in this situation. Both parent and mutant cells or cell-free extracts have very active deoxyribonucleoside phosphorylase activities, which result in most of the thymidine's being cleaved to thymine. Whereas the parent cell utilizes such thymine as effectively as it does thymidine, in support of cell reproduction, the mutant strain is extremely inefficient in this respect. The mutant cells, as well as cell-free extracts of them, were grossly deficient in transdeoxyribosidation activity, a finding that may explain their incapacity to utilize efficiently free thymine. Any substance that can compete with thymidine, as a substrate for the nucleosidase, will increase the availability of thymidine for anabolic reactions that lead to its incorporation into DNA and thus to cellular reproduction.

It is not inconceivable that a neoplasm may react similarly to a particular metabolic antagonist. As far as is known, a similar situation has not been observed in mammalian systems, but the possibility must be considered. The use of combination therapy, either sequentially or simultaneously, could result in selection of a neoplastic cell in which one of the antagonists functions to permit the more efficient utilization of the metabolite purported to be inhibited.

Since, in clinical chemotherapy, drugs are generally administered at levels near to those that are maximally tolerated, further increase in drug concentration, in order to achieve inhibition of cellular reproduction, might not be feasible.

Studies in mammals.—Earlier studies with radioactive 5-iodouracil-I³¹ and 5-iodouridine-I³¹ in mice bearing transplanted neoplasms showed no preferential uptake of radioactivity in the neoplasms (94). This is not surprising, because one would not expect iodouracil or its ribonucleoside to be converted readily into the deoxyribonucleoside triphosphate, necessary for incorporation into DNA, since thymine (7, 51, 86, 98) and thymine ribonucleoside (98) are very poor precursors of DNA-thymine in mammalian systems. More important was the observation that radioactive iodouracil is extensively dehalogenated to uracil and iodide; the iodine released is in a form that reacts with proteins, a finding that explains in part the nonspecific incorporation of the radioactive iodine into tissues (95).

Studies in the normal mouse of the fate of IUDR labeled with either H³ or I³¹ revealed that about three-fourths of the administered radioactivity appeared in the urine within 4 hours (95). Within 24 hours, 88 per cent of the radioactivity of the administered IUDR-I³¹ appeared in the urine as iodide, 3.8 per cent as 5-iodouracil, and 6.6 per cent as IUDR (95). Other studies are in agreement.
with these observations, and show that the initial degradation product of IUDR is 5-iodouracil, which is catabolized subsequently to uracil and iodide, not only in the mouse and rat, but also in man; iodide is the major radioactive metabolic product that occurs in blood, as well as in urine (98, 68, 125). There was no significant difference in the distribution of radioactivity between the tissues of normal and tumor-bearing mice (95). In addition to the tumor, many organs, especially the spleen, contained appreciable amounts of radioactivity. Fractionation of the tissues into the acid-soluble fraction, combined nucleic acids, and proteins revealed that in many tissues, including the neoplasm, the amount of radioactivity associated with protein was equivalent to or more than the amount in the combined nucleic acids (95). This finding raises a point that on occasion has been overlooked. Unless one performs adequate characterization, it cannot be assumed that the radioactivity derived from administered IUDR and present in a particular organ is either IUDR or an anabolic product of it, or whether it is even associated primarily with DNA. After the administration of IUDR many tissues that are known to concentrate iodide, such as thyroid or stomach, will contain a significant amount of radioactivity that is not associated with the DNA polymer. Thus, scanning technics used to locate areas of rapid nucleic acid biosynthesis by virtue of incorporation of IUDR labeled with I125 or I131 may be misleading. The protein fraction of most tissues has been shown to possess in many cases as much or even more radioactivity derived from IUDR-I131 than the DNA fraction (95). If the protein of a particular tissue does not have a rapid turnover, the radioactivity associated with the tissue will persist for a long period of time. Similarly, gross chemical separation into acid-soluble, lipid, nucleic acids, and protein fractions can be deceiving; thus, the hot trichloroacetic acid extract of a tissue may contain traces of hydrolyzed protein, and the aromatic amino acids may be labeled heavily with radioactive iodine. A preferred procedure is one in which polymerized DNA is isolated, freed of protein, and enzymically hydrolyzed to the nucleotides, which can be separated appropriately and their individual base composition determined. This will permit, in addition, an evaluation of the amount of DNA-thymidine that IUDR has replaced.

Eidinoff et al. (25) administered radioactive IUDR-I131 (820 µc., 9.6 mg.) intravenously to a patient with untreated chronic granulocytic leukemia and found that about 1 per cent was incorporated into the DNA of the leukocytes. On the 8th day after the administration of IUDR, the concentration of radioactivity in the white blood cells was about 40 times that of the red blood cells. Since the erythrocytes contain no DNA, the implication is that the protein (or some other) fraction of the red blood cells was labeled with I131. Body surface counting revealed that 6 days after the administration of IUDR-I131 the splenic area had the highest concentration of radioactivity, with an organ/thigh ratio of 27, whereas the ratio for liver was 11 and that for cecum and urinary bladder was 6. Krueger et al. (69), in a study of the metabolism of IUDR-I131 in the mouse, found that the radioactivity present in several organs corresponded to their proliferative activity. Gitlin et al. (31) found that incorporation of radioactivity derived from IUDR-I131 injected into mice is a function of the amount of x-radiation, as well as of the time between irradiation and dose. Thus, 20 hours after the injection of IUDR almost all radioactivity was present in the acid-insoluble fraction. Unfortunately, no attempt was made to fractionate the acid-insoluble fraction into its nucleic acid and protein components. Rotenberg et al. (101) studied the pattern of incorporation of IUDR-I131 into spontaneous C3H mammary tumors and found, in agreement with the studies of Hampton and Eidinoff (39) in rats, that 4 days after the administration of this compound the tumors had a higher specific activity than that of other tissues. This observation has been extended by these authors to the localization of human tumors by whole-body scanning with apparent success.1

There have been several attempts to increase the amount of IUDR that is incorporated into the DNA and to minimize the incorporation of radioactive iodine derived from IUDR into non-nucleic acid fractions. Hampton and Eidinoff (39), through the administration of amethopterin, a drug that prevents the formation of thymidylic acid, increased by threefold the amount of radioactivity derived from IUDR-I131 that was incorporated into tumor tissue in the rat. Kriss, Tung, and Bond (68) increased the uptake of IUDR-I131 into tissues of the rat by a factor of 2-5 by the concurrent injection of 5-fluorodeoxyuridine. Hampton and Eidinoff (39) also studied the effect of a 60-fold molar excess of 5-iodouridine, but this did not influence the uptake of radioactive IUDR into tumor or intestinal tissue, although the radioactivity taken up by the liver was increased fivefold. These authors also found that the vehicle in which IUDR was administered could play an important role; thus, injection of IUDR in an oil emulsion

1 W. R. Bruce, personal communications.
increased the uptake by a factor of 3 above that observed when 0.9 per cent of solution of sodium chloride was used as the vehicle. 5-Iododeoxyuridine 5'-phosphate, although less susceptible to the degradative enzymes that attack IUDR, nevertheless did not cause any greater uptake than that seen with IUDR alone (39).

In an attempt to obtain a more stable form of IUDR, Chang and Welch (16) synthesized 5-iodo-2'-deoxycytidine (ICDR), but, although this compound has greater heat stability and solubility than does IUDR, its biological applications have given somewhat disappointing results (19). This is not because ICDR per se, in contrast to IUDR, has not been as resistant to nucleosidase cleavage of the essential deoxribose moiety as had been envisaged (124), but rather because of its limited conversion to ICDR 5'-phosphate and then to IUDR 5'-phosphate as a result of deamination by deoxycytidine deaminase (19). Rather, ICDR, at the nucleoside level, is rapidly converted enzymatically to IUDR. The enzyme responsible for this deamination, which has been purified and the substrate specificity examined by Creasey, is probably identical with the cytidine deaminase studied by Greenstein, Carter, and Chalkley (33), although the affinity of cytidine for the enzyme is far less than that of various halogenated nucleosides. Dependent upon the variable distribution of this nucleoside deaminase in tissues, ICDR can be disadvantageous, as compared with IUDR, due to the stability of ICDR to nucleosidases, since ICDR cannot be incorporated into DNA in place of thymidine until it is converted to IUDR and its phosphorylated derivates (19).

Preferential localization into DNA of iodinated deoxyribonucleosides should be greatly enhanced by the prevention of the initial catabolic cleavage of IUDR to iodouracil. Metabolic dehalogenation of IUDR per se does not appear to occur (95); however, after cleavage, the 5,6-double bond is enzymically reduced and the 5-iodo-5,6-dihydrouracil formed undergoes dehydrohalogenation with the formation of uracil and iodide ion (92, 36, 39, 68, 84, 93). Recent studies with Mr. J. Perkins have been concerned with elucidation of the mechanism of this initial catabolic reaction and an attempt to prevent the cleavage of IUDR. A soluble enzyme preparation was made by homogenizing mouse liver with 4 volumes of 0.25 m sucrose and subjecting the homogenate to centrifugation at 100,000 X g for 1 hour at 4° C. The major enzymic activity of the liver was present in the particulate-free supernatant fraction. Various derivatives of thymine and thymidine listed in Table 1 have been used in an attempt to inhibit the cleavage of IUDR. Radioactive IUDR-I25 was incubated for 10 minutes with the soluble enzyme preparation in the presence and absence of the same molar concentration of the potential antagonist. The reaction products were separated by thin-layer chromatography, and the amount of radioactivity in the iodouracil and IUDR areas was determined in a well-type gamma scintillation counter. The most effective inhibitor of the cleavage of IUDR so far found is thymine ribonucleoside, but the mechanism of its action has not yet been clarified. A preliminary study has been made of the effects of thymine ribonucleoside in vivo. Mice were given injections intraperitoneally of either IUDR-I25 or IUDR-I35 plus a 100-fold molar excess of thymine ribonucleoside. Mice were sacrificed 2 hours after injection, and the urine from the urinary bladder, as well as that expelled, was subjected to ion exchange chromatography. The results indicate that the inclusion of thymine ribonucleoside resulted in a threefold increase in the total amount of radioactivity that appeared in the urine within 2 hours, of which 91 per cent was in the form of IUDR, whereas in the absence of thymine ribonucleoside 50 per cent was as

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Per cent Iodouracil formed in 10 minutes</th>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>88</td>
</tr>
<tr>
<td>5-Fluorodeoxyuridine</td>
<td>65</td>
</tr>
<tr>
<td>5-Iodouracil</td>
<td>60</td>
</tr>
<tr>
<td>5-Iodouridine</td>
<td>65</td>
</tr>
<tr>
<td>Thymine</td>
<td>54</td>
</tr>
<tr>
<td>Thymidine</td>
<td>60</td>
</tr>
<tr>
<td>Thymine ribonucleoside</td>
<td>30</td>
</tr>
</tbody>
</table>

Compounds with essentially no activity: 5-Iododeoxyuridine; 5-hydroxyuridine; 5-fluorouracil; 5-aminoaracil; 5-hydroxymethyluracil; uracil; 6-azathymidine; 6-azathymine; 1-cyclopentanethymine; 1-n-butyl thymine; 1-phenyl thymine; 1-(cis-3-hydroxycyclopentyl) thymine; 1-(cis-3',5'-dihydroxy cyclopentyl) thymine; fluoro-4-bromo-tri-0-acetyl glucose; 5-arnabinofuranosyl thymine; 5-arabinofuranosyl thymine; 5-sylofuranosyl thymine; 5-sylofuranosyl thymine; 5-sylofuranosyl thymine; 5-sylofuranosyl thymine.

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3 W. A. Creasey, unpublished data.
4 J. Perkins and W. H. Prusoff, unpublished data.
5 We are grateful to Dr. J. J. Fox of the Sloan-Kettering Institute, to Dr. K. C. Murdock of the American Cyanamid Co., and to Dr. P. Kent of Oxford University for supplying us with many of these compounds.
IUDR. Thus thymine riboside resulted in a sixfold increase in the excretion of IUDR per se. Whether thymine riboside increases the incorporation of IUDR into DNA or increases inhibition of the growth of neoplasm also is under investigation.

IUDR, when injected into mice in amounts of 100 or 150 mg/kg daily for 6 days, inhibited significantly the growth of Sarcoma 180 and of lymphomas L1210 and L5178Y without evidence of host toxicity (37). When the drug was administered to mice in combination with azaserine, 6-azathymine, 6-azauridine, 5-fluorodeoxyuridine or amethopterin, only additive inhibitory effects on the growth of lymphoma L1210 were seen (57). Other investigators have observed, however, that IUDR can potentiate the activity of 5-fluorodeoxyuridine in mice or rats bearing various neoplasms (9, 45). Similarly, in mice bearing leukemia P815, the effects of combinations of IUDR and 2-chloro-terephthalanilide-4,4′-bis (imidazolin-2-yl), as well as of the latter with 5-iodouridine, 5-bromouridine, or 5-bromodeoxyuridine, were more than additive (10). The results obtained by Jaffe and Prusoff (57) were not significantly different whether the drug was administered daily in a single dose or in divided parenteral doses; oral administration of IUDR in amounts equivalent to those administered parenterally was much less effective. In no instance was a “cure” obtained, but marked inhibition of tumor growth was observed. Tumor viability was retained even under conditions that resulted in over 95 per cent inhibition of tumor growth, accompanied by marked host toxicity. Goldin et al. (39) evaluated several antileukemic agents in advanced leukemia L1210 in mice and found that IUDR is only 20 per cent as effective as amethopterin. Thus, on the basis of experiments in mice eradication or “cure” of a neoplasm does not appear feasible by the administration of IUDR alone. In this respect, however, it is of pertinence to cite the following two encouraging experiments. Sartorelli5 has observed that IUDR in combination with uracil mustard (5-(bis-2-chloroethyl) aminouracil) resulted in a potentiation of anti-tumor activity in mice bearing the transplanted neoplasm, Sarcoma 180. This combination of an alkylating agent with IUDR is in contrast to the additive effects seen in this laboratory when IUDR was combined with known antimetabolites (57). The desirability of extending these studies in man is obvious. Other encouraging experiments are those of Berry and Andrews (4, 5), who demonstrated a marked potentiating effect on the growth of the ascitic P-388 lymphocytic leukemia in mice by combinations of IUDR and x-radiation, as compared with either type of therapy alone. The capacity of halogenated uracil derivatives to reduce the threshold of cells to the damaging effects of radiation had been described previously for bacteria (34—36) and for mammalian cells in culture (19, 22).

These results indicate two possible clinical applications, and one of these—i.e., sensitization of cells to radiation injury through prior exposure to IUDR, has been productive of encouraging results (67, 125). Studies in experimental animals indicate clearly that the administration of IUDR alone to patients bearing neoplasms should produce some degree of inhibition, but not eradication. The relatively limited number of clinical studies so far carried out appear to support this contention (12, 13, 87). Although all the pharmacological avenues of administration of IUDR have not been explored, because of severe limitations on the availability of this very costly compound, it would appear that efforts should be directed toward combinations of IUDR and either radiation or alkylating agents.

A competitive relationship between thymidine and IUDR has been observed in mammalian systems in vivo. The successful prevention of the toxic effects of an otherwise lethal sequence of daily doses (18 μmoles; about 250 mg/kg) of IUDR, when administered intraperitoneally to mice repeatedly, was obtained by the prior administration of thymidine (100 μmoles) (95). The amount of IUDR required to produce a toxic effect in the absence of thymidine appeared to be critical, since a decrease in the quantity of IUDR administered to half of the dose that produced 100 per cent lethality caused no discernible toxic effects. Thymidine may have saturated either the transport mechanism involved in the penetration of IUDR into the cell or the pyrimidine deoxyribonucleoside kinase within the cell for a sufficiently long period of time that catabolism and excretion of IUDR resulted in a diminution of the size of the pool of IUDR to a non-toxic level. In any case, a toxic intracellular concentration of IUDR or more probably phosphorylated derivatives of IUDR can be prevented by thymidine. Since thymine ribonucleoside increased the urinary excretion of IUDR in mice,5 the specificity of thymidine protection should be reinvestigated.

The ability of thymidine to protect an animal from the lethal effects of IUDR was extended to man by Calabresi and Mark (11, 75). These workers could prevent some of the toxic manifestations of IUDR in man, such as alopecia and stomatitis, by infusion of a very small amount of thymidine.

5 J. J. Jaffe, and W. H. Prusoff, unpublished data.
6 A. Sartorelli, personal communications.
into the external carotid artery while IUDR was administered intravenously; however, the effects of IUDR on bone marrow and on neoplastic tissues were not abolished by this regimen. This inability to control the toxicity of IUDR to the bone marrow in man by the regional administration of thymidine has prevented any increase in the amount of IUDR administered to a patient. This is a major clinical problem that must be solved before any significant increase in the amount of IUDR administered can be accomplished. Important progress in the area of protection of bone marrow against the toxic effects of IUDR by the judicious infusion of thymidine has been described recently by Mark and Calabresi (75).

Of pertinence is the observation by Jaffe and Prusoff (57) that a 10–30 M excess of thymidine administered simultaneously with IUDR reduced the activity of the latter by only one-half against the growth of lymphoma L1210 in mice. Whether this implies that sites in addition to those involving inhibition of the utilization of thymidine or its phosphorylated derivatives are susceptible to inhibition by IUDR will be discussed later.

It has been demonstrated in several laboratories that IUDR is incorporated into the DNA of mammalian cells in vitro as well as in vivo (17, 25–27, 41, 76–78, 90, 93). A comparison was made of the relative uptake of thymidine-2-C^14 and IUDR-I^131 into the DNA of Ehrlich ascites cells in vivo (92). Two groups of mice were given equimolar amounts of either thymidine-C^14 or IUDR-I^131 daily during a period of 4 days, and a utilization of thymidine 40-fold greater than that of its analog, for the biosynthesis of DNA, was observed. This may be attributed to a variety of circumstances that have not yet been investigated; these include possible differences in the rate of transport into the cell, in the rate of phosphorylation, in the rate of egress from the abdominal cavity, as well as in the rate of excretion from the organism. Nevertheless, by a twofold increase in the amount of IUDR-I^131 administered, and by dosage at 12-hour rather than 24-hour intervals, the extent of incorporation into DNA approached that of thymidine. Other means whereby the amount of IUDR incorporated into DNA can be increased have already been discussed.

An important problem relates to the conditions required to obtain maximum incorporation of IUDR into the DNA of a neoplasm. It has been demonstrated with mammalian cells in culture that cells exposed to a critical concentration of IUDR are capable of only one cell division prior to death of the cell (17, 77, 78). Is it possible to attain in vivo a similar concentration without concomitant adverse effects on normal tissues? Is it possible to dissociate increased uptake of the analog into the neoplasm from increased host toxicity? Will it be possible to protect susceptible normal tissues from the toxic manifestations of IUDR by an approach of the type initiated by Calabresi and Mark (11, 75)? Since both thymidine and IUDR inhibit the enzyme aspartate transcarbamylase, as shown by Bresnick (6), and hence the de novo biosynthesis of pyrimidines, is it possible that, in addition to thymidine, other substances, such as either uridine or cytidine, or both, should be infused into the bone marrow in order to obtain protection?

Another important question is concerned with the possible necessity for the incorporation of a critical amount of IUDR into DNA in order to inhibit cellular reproduction. Zamenhof (128) found a lack of correlation between incorporation of bromouracil into the DNA of E. coli and its inhibitory effect. Previous studies in a microbial system with another analog of thymine, 6-azathymine, also revealed that there is no apparent relationship between the extent of incorporation of the unnatural base into the DNA and either its lethal effect or its inhibition of growth (88). Whether a similar lack of relationship exists between the incorporation of IUDR into DNA and inhibition of reproduction of a neoplastic cell has not been elucidated.

What other biochemical effects have been observed with this analog? IUDR inhibits competitively the utilization of radioactive thymidine for the biosynthesis of DNA-thymine in mammalian cells, as well as in microbial systems and under these conditions is utilized in lieu of thymidine for the formation of the DNA-polymer. The appearance in the thymidylate of the DNA of mouse Ehrlich ascites tumor cells in vitro of such precursors as radioactive orotic acid, formate, or thymidine is inhibited by IUDR, but, as might be expected, the utilization of orotic acid for the biosynthesis of DNA-cytosine or of RNA-pyrimidines is not affected (91). Thus, both the exogenous and the de novo pathways concerned with the formation of phosphorylated derivatives of thymidine were affected by IUDR (or its derivatives). In collaboration with Delamore (21), studies of the specific enzymic reactions affected by IUDR in various murine and human neoplastic tissues were carried out; thus, the decreased incorporation, in the presence of IUDR, of formate-C^14 or of thymidine-H^3 into DNA-thymine was found to be a reflection of an inhibition of the utilization of thymidine, thymidylate, thymidyl acid, or thymidine triphosphate, presumably by IUDR acting in the form of the corre-
sponding phosphorylated derivatives of IUDR. The specific metabolic site primarily affected in the various tissues studied is a characteristic of the individual tissue; thus, DNA-polymerase was preferentially inhibited in murine Ehrlich ascites carcinoma and in human chronic granulocytic and acute monocytic leukemia, whereas studies with murine L5178Y leukemia cells, with thymidine-H7 and formate-C14 indicated a primary inhibition of thymidylate kinase and thymidylic acid kinase, respectively. At concentrations of IUDR that exerted no inhibition of the formation of thymidylate from formate-C14 by Ehrlich ascites cells, a significant inhibition was observed in L5178Y leukemia cells; the former finding is in agreement with that of Hartman and Heidelberger (44) with Ehrlich ascites cells in which IUDR, under conditions in which IUDR 5'-phosphate would be formed, caused no inhibition of thymidylate synthetase. Studies are in progress in collaboration with Y. S. Bakhle to determine whether the decreased formation of thymidylate by L5178Y cells in the presence of IUDR is related causally to an inhibition of thymidylate synthetase or of a more obscure metabolic reaction. It is possible that a difference in affinities of the thymidylate synthetase enzyme for IUDR 5'-phosphate exists in these two neoplastic cells; this possibility is under investigation. It is clear that it is not essential for IUDR to be incorporated into DNA in order for cellular reproduction to be inhibited by this compound, since deprivation of an essential precursor required for the formation of DNA-thymine may be adequate.

That one should not restrict one's thinking to inhibition of obvious pathways is indicated by the studies of Bresnick (8), previously referred to; these describe an inhibitory effect of IUDR on the aspartate-transcarbamylase system of mammalian cells, a reaction which is essential for the synthesis of pyrimidines de novo. Thymidine exerts also a feedback control over this reaction. Phosphorylated derivatives of thymidine have been shown by Reichard, Canellakis, and Canellakis (99) to inhibit the conversion of cytidylic acid to deoxycytidylic acid, the reduction apparently taking place at the diphosphate level. Evidence for an inhibitory effect by a phosphorylated derivative of thymidine was obtained in whole cells (81–83). Recently, thymidinetriphosphate has been demonstrated by Ives, Morse, and Potter (56) to exert a feedback inhibition of thymidine kinase, and a similar effect on deoxycytidylic deaminase has been reported by Maley and Maley (74). Since IUDR has been shown not only to compete with various phosphorylated derivatives of thymidine for the kinase as well as the polymerase (81), but also to replace thymidine in the DNA polymer, it is logical to assume that the appropriate phosphorylated derivative of IUDR also may exert pseudo-feed-back inhibitory control over these enzymic reactions just described in which thymidinetriphosphate has been shown to exert a regulatory influence. One may wonder, therefore, whether each of these several definite or hypothetical effects of IUDR is concerned with the observed biological effects of IUDR, and to what degree.

What is the consequence of the partial replacement of the thymidine component of DNA by IUDR? Mantsavinos, when associated with this department, found no decrease in the primer activity in a polymerase system of a preparation of DNA derived from Ehrlich ascites tumor cells that contained IUDR. Since DNA represents a heterogeneous population of polymers, it is not clear whether the activity observed was associated with the IUDR-containing DNA (I-DNA), the IUDR-free DNA, or both. Even if the iodinated DNA were inhibitory it may not have been present in a concentration sufficiently high to manifest itself. If the iodinated DNA is not inhibitory to DNA replication, is the product formed biologically active?

The presence of IUDR in DNA has exerted no inhibitory effect on the enzymes concerned with the catabolism of I-DNA to IUDR 5'-phosphate and subsequent dephosphorylation to IUDR. Similarly, Michelson et al. (80) observed that homopolyiodouridylate did not interfere with the activities of ribonuclease, venom phosphodiesterase, 5'-nucleotidase, or semen phosphomonoesterase. Szybalski et al. (118) reported that transforming DNA retained its activity, as assessed with three genetic markers, even after extensive incorporation of 5-bromouracil into one or both strands of DNA. That I-DNA may not be innocuous completely is indicated by the preliminary note of Schimizu and Swafuchi (107), which describes a 30 per cent inhibition of the incorporation of glycine-14C into several protein fractions of Ehrlich ascites tumor cells in vitro by I-DNA or by I-DNA treated with deoxyribonuclease (DNase). Whereas the addition of DNA, but not of DNase-treated DNA, prevented the inhibition produced by I-DNA, the inhibition exerted by DNase-treated I-DNA was prevented by DNase-treated DNA, but not by DNA. More details concerning, as well as confirmation of, this provocative paper, will be of much interest.

It was stated earlier that the pKo of IUDR is sufficiently lower than that of thymidine that, at pH 7.4, 34 times as many units of IUDR may be

R. Mantsavinos, unpublished data.
in the enolic form, as is the case with thymidine; thus, the probability of incorrect base-pairing, with subsequent formation of either a genetically altered cell, a mutant, or a cell that forms incorrect messenger RNA is presumably increased by a factor of 34. Lawly and Brooks (70) have calculated that at neutral pH 5-bromodeoxyuridine (BUDR) exists in the anionic form to a degree about 80 times greater than that of thymidine. If one enolic unit of IUDR in DNA is responsible for an incorrect “message,” the probability of such occurrences with increased replacement of thymidine by IUDR in DNA may be increased exponentially. Does the fact that DNA incorporates IUDR constitute a hazard clinically? How much concern should one have for this possibility? It would be of value to know what the potential genetic hazard is from incorporation of IUDR into the DNA of man or, for that matter, any mammalian species.

If IUDR, or any other mutagenic agent, were to be really efficacious in the treatment of neoplastic diseases, or of potentially lethal infections with adenovirus type 192(53), the efficacy of IUDR was extended by Kaufman and his colleagues (60—85). Several reports have appeared in vivo by Berry and Zamenhof (34, 35) and later in mammalian cells in vitro by Djordjevic and Szybalski (9292) and in vivo by Berry and Andrews (4, 5). Whereas 5-bromodeoxyuridine which contain bromouracil in the DNA show increased sensitivity to visible light. Recently a report has appeared (38) that describes the treatment of herpes simplex viral infections of the eyelids, lips, and cheek in man that were successfully resolved in 2–5 days by topical therapy with IUDR, whereas the normal duration in these patients was claimed to be 2–3 weeks. Unfortunately, controls were not run, let alone a double-blind study. The studies reported previously in rabbits are unequivocal, well controlled, and “blindly” evaluated; indeed, studies at Yale have not indicated that the topical application of solutions to dermal lesions caused by herpes simplex is of value.8 Of particular importance is the incapacity of the ribonucleoside of iodouracil to exert any beneficial effect in the control of herpes simplex infection of the rabbit eye under conditions in which IUDR was very efficacious (85). Since iodouridine is catabolized in a similar manner to IUDR, with similar release of the iodine atom, specificity of action of IUDR is indicated. The viral infection of the cornea, in rabbit and man, is almost ideal from the point of view of topical therapy in that the virus and host tissue are in a localized area and susceptible to the direct penetration of the IUDR applied in solution. Although IUDR has been shown9 not to have a direct inhibitory effect on the virus particle per se (50), the corneal epithelial cells in which the virus propagates can be subjected to a very high concentration of IUDR. The mechanism whereby viral replication is prevented is under investigation in these laboratories, and from previous studies one might postulate that either (a) IUDR, after phosphorylation to the monophosphate derivative inhibits the phosphorylation of thymidine monophosphate to thymidine triphosphate, or (b) IUDR-triphosphate inhibits the utilization of thymidine triphosphate for the biosynthesis of new viral DNA, or (c) the incorporation of IUDR into viral DNA renders it noninfective; in addition, any one of the previously described potential actions of IUDR may apply.

Another very important property of the halogenated derivatives of uracil, in general, is their capacity to increase the sensitivity of cells to the lethal effects of UV- or x-radiations. This was first observed in bacteria by Greer and Zamenhof (34, 35) and later in mammalian cells in vitro by Djordjevic and Szybalski (92) and in vivo by Berry and Andrews (4, 5). Whereas 5-bromodeoxyuridine increased the sensitivity of mammalian cells to UV-radiation to a greater extent than IUDR, the

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8 P. Calabresi, personal communication.
9 J. F. McCrea, Y. S. Bakhle, and W. H. Prusoff, unpublished data.
latter resulted in increased sensitivity to x-radiation (22). Although the mechanism whereby halogenated uracil derivatives exert their effect has not been completely clarified, a correlation has been found between the degree of replacement of DNA-thymidine by IUDR and x-ray sensitization of mammalian cells by Erikson and Szybalski (28). A study (88) was made of the relative photochemical reactivity of IUDR and thymidine to UV-radiation, since a prime effect of UV-radiation of DNA is the formation of dimers of thymine. Under conditions in which thymidine showed UV-photosensitivity, as measured by a decrease in absorbency, IUDR reacted less markedly. Thus, the mechanism whereby IUDR sensitizes the cell to UV-radiation appears to be related not to the extent of reaction, but rather to either the actual products formed or the subsequent inability of the irradiated I-DNA to be reactivated (34, 592, 1092, 106). Smith (116) has recently written an excellent review in which various aspects of this problem have been discussed.

In conclusion, the main objective in the clinical use of IUDR as an antitumor agent appears to be to expose neoplastic cells to as high as possible a concentration of IUDR per unit of time. Enzymic cleavage to the free base is the primary catabolic reaction that, in conjunction with urinary excretion, serves to prevent the accumulation of a high intracellular concentration of phosphorylated derivatives of IUDR, in which form it is not susceptible to nucleosidase inactivation or cleavage of iodine. The relative insolubility of IUDR limits the “concentration per unit time” to which the neoplastic cell is exposed, since there is a maximum at which a large volume can be injected into the vascular system of man. The greater the rate of injection the higher will be the “concentration per unit time” exposure the neoplastic cell receives. It would be of value to decrease or prevent the cleavage of IUDR. The uptake of IUDR into the DNA of murine neoplastic cells in vivo could be increased about 40-fold by augmenting the dose, as well as the frequency of administration. Nevertheless, the administration of IUDR once daily for 6 days to mice bearing neoplasms was adequate to obtain inhibition of the growth of tumors (57). If an effective inhibitor of the nucleosidase enzyme were to be made available it would be of interest to explore clinically the effectiveness of a combination of this agent with IUDR.

Since the data in mice, as well as the relatively few that have been obtained so far in man, do not indicate complete regression of the neoplasm by the administration of IUDR alone, more imaginative use of this compound is indicated, as in the studies of Sartorelli, as well as continued studies of IUDR in combination with radiation. If biologically effective levels of IUDR were to be attained, the problem of bone marrow toxicity may become more acute; accordingly, continued efforts in this direction, as in the studies of Calabresi and Mark (11, 70), are indicated.

Even if IUDR were shown to be of limited value in the treatment of neoplasms, its present exciting role as an antiviral agent, in both the topical and the systemic therapy of established infections, has proved its clinical usefulness and has justified the time and effort that have been devoted to it by workers in several laboratories. It is not unreasonable, therefore, to close on a note of optimism and to indicate that, with continued efforts, related compounds of greater solubility and stability and perhaps, in combination with other agents, of greater efficacy will be found.

II. AZAURIDINE

A summary of the current information on 6-azauridine (AzUR) by Handschumacher et al. (43) has just appeared. The mechanism of action of AzUR in biochemical systems, as reviewed in (43), has been reported to correlate well with the clinical response (15, 29), although variability has been reported (8). The primary site of inhibition is the enzyme, orotidylate decarboxylase, for which azauridine 5'-phosphate and orotidine 5'-phosphate compete. Those cells that develop a resistance to the inhibitory effect of AzUR show little or no inhibition of the decarboxylation of orotidine 5'-phosphate. The resistant cells appear to be those that survive in the presence of AzUR because of a fortuitous lack of uridine kinase, the enzyme that appears to be responsible for the conversion of AzUR to azauridine 5'-phosphate.

Although orotidylate decarboxylase is the logical site of the inhibitory action of AzUR, a more difficult question to answer is whether this is the only biochemical site that is affected. Škoda and his collaborators (111, 112) have shown, for example, that the exchange of orthophosphate-P\textsuperscript{32} with uridine 5'-diphosphate and adenosine 5'-diphosphate catalyzed by a polynucleotide phosphorylase from Escherichia coli was inhibited by 6-azauridine 5'-diphosphate. The synthesis of polyuridylic acid from uridine 5'-diphosphate by polynucleotide phosphorylase derived from Micrococcus lysodeiticus was also completely inhibited by 6-azauridine 5'-diphosphate at a molar ratio of 3 to 1 (110). Simon and Myers (108) have also observed an inhibition of polynucleotide phosphorylase by azauridine diphosphate. Thus, the 5'-diphosphate derivative of 6-azauridine not only is a potent in-
inhibitor of the biosynthesis of ribonucleic acids catalyzed by polynucleotide phosphorylase of these bacterial species but in addition can not be utilized as a substrate in the formation of RNA by this enzyme (1). Both the 5'-diphosphate and the 5'-triphosphate of azauridine have been observed in microbial systems (42, 113); however, this site of inhibition may not be important in explaining the action of AzUR in the inhibition of the reproduction of neoplastic cells for two reasons: (a) these polyphosphorylated forms of azauridine have been observed only in microbial, but not in mammalian, systems, and (b) polynucleotide phosphorylase may be concerned with degradation of RNA, rather than with its synthesis. 6-Azauridine 5'-triphosphate has been observed by Hurwitz et al. (54) not to exert any inhibitory effect on the DNA-dependent synthesis of RNA that requires for substrates the 5'-triphosphate of adenosine, guanine, uracil and cytosine nor was it utilized in lieu of uridine triphosphate. Although neither the 5'-diphosphate nor the 5'-triphosphate of 6-azauridine has been observed in mammalian systems, the possibility of a small pool size or marked lability to catabolism by phosphorytase has not yet been ruled out unequivocally. Of more pertinence perhaps is the report by Rychlik (109), who observed that 6-azauridine 5'-diphosphate, but not 6-azauracil, AzUR or 6-azauridine monophosphate, inhibits the incorporation of amino acids into the soluble RNA in the rat liver enzyme system of Keller and Zamecnik (65). Although the relative importance of these two systems in the biological inhibition of various types of neoplasms may need further study, it should be noted that Handschumacher could not detect conversion of AzUR 5'-phosphate to higher phosphorylated derivatives in the mammalian systems studied. Whether enzymes derived from AzUUR-sensitive neoplasms have a greater sensitivity to the inhibitory effects of azauridine diphosphate or azauridine triphosphate than do those derived from normal tissues will also be a point of interest.

Recent studies by Škoda and Handschumacher (109) have shown that AzUR inhibits the incorporation of orotic acid into pseudouridine excreted in the urine of mice and into pseudouridylic acid of rat liver S-RNA. The absolute amount of pseudouridine excreted in the urine was not affected. Because there is a relationship between viruses and tumor induction, it is pertinent to discuss the antiviral activities of AzUR. Rada et al. (97) reported that AzUR (1.2 × 10⁻² M), but not azauracil, inhibited by 75 per cent the multiplication of vaccinia virus, using Dulbecco's plaque method; a similar result was observed in membrane culture (96). The ratio of the concentration required to produce tissue toxicity to that inhibiting viral reproduction was 14. AzUR had no direct inactivating effect on vaccinia virus and had no inhibitory effect on influenza virus type A strain PR 8, on Newcastle disease virus or on Eastern equine encephalomyelitis virus. Several derivatives of 6-azaunidine were even more effective than AzUR in inhibiting the multiplication of vaccinia virus in monkey kidney cell cultures (114): these are 3-methyl-2,4-dithio-6-azaunidine, 3-methylmercapto-4-thio-6-azaunardin, and 3,5-dimethylmercapto-1,2,4-triazine. Šmejkal and Sorm (115) found that AzUR, in doses usual in human therapy, exerted an inhibitory effect on the reproduction of vaccinia virus in rabbits. Jasinska et al. (104) investigated the effect of AzUR alone and in combination with urethan on the mortality of mice infected intracerebrally with a neurotropic variant of vaccinia virus or infected systemically with a strain adapted to mouse lungs (104). Although AzUR or urethan alone exerted no effect on the intracerebrally infected mice, combination of these two compounds delayed mortality by 1 log₁₀ unit; with the systemically infected animals, both compounds exerted an effect, but combination therapy was better. Rosenbergová and Rada (100) investigated the synthesis of cellular nucleic acids during inhibition of vaccinia virus multiplication by AzUR and demonstrated that AzUR inhibited in parallel the synthesis of both cellular RNA and vaccinia virus. It must be stated, however, that in rabbits infected intradermally with a strain of vaccinia virus, large subcutaneous doses of AzUR, Calabresi, McCollum, and Welch (14) caused no suppression of the development of the lesions, under conditions in which 5-iododeoxyuridine was so highly inhibitory that 10,000–100,000 times as much virus was required to produce lesions comparable to those seen in untreated animals.

The use of AzUR in the treatment of human neoplasms has indicated that this compound is essentially free of any toxicity on normal tissue. If AzUR or a derivative thereof were to be of clinical values as an antiviral agent, then the effect of AzUR on embryonic tissue must be given consideration. Sanders, Wiesner and Yudkin (105) have reported that AzUR has a selective action on developing embryos in mice. Single doses of 1 mg/gm given for 3 days from the 4th to the 6th day interrupted every pregnancy. Whether AzUR offers any hazard to the fetus in the human female, therefore, remains to be established.

Although this paper can hardly be considered an adequate review of azauridine, it was consid-
er, in view of the recent appearance of such a paper (48), that certain aspects not covered therein might be of more interest.

REFERENCES


77. Prusoff—5-Iodo-deoxyuridine and Azauridine


A Review of Some Aspects of 5-Iododeoxyuridine and Azauridine

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