Plant Physiol. (1980) 65, 1224–1228
0032-0889/80/65/1224/05$00.50/0

Incorporation and Degradation of ^{14}C and ^{3}H-labeled Thymidine by Sugarcane Cells in Suspension Culture[1, 2]

Received for publication June 19, 1979 and in revised form January 17, 1980

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ABSTRACT

Sugarcane cells growing in suspension culture degrade exogenous thymidine, releasing thymine. Thymine is not utilized for DNA synthesis. Thymine is rapidly catabolized to β-aminoisobutyric acid which is found within the cell. Thymidine in the medium is used for DNA synthesis. The label of [2-^{14}C]thymidine is lost as $^{14}CO_2$, but the label of [^{3}H]methylthymidine is found in the cell as [^{3}H]β-aminoisobutyric acid, some of which is used for the synthesis of other cell components. The degradation of thymidine can be partially inhibited by addition of certain substituted pyrimidines.

Radioactive plant cell DNA of high specific activity would be useful in some experimental approaches to the genetic modification of plants by the uptake of exogenous DNA at the cell and protoplast level (18). A specific precursor for DNA biosynthesis is TdR[4] (2) labeled with either ^{3}H or ^{14}C, but concomitant degradation found in bacteria (20), mammalian tissue (3), and plants (23) has often limited its use. In bacteria, it has been possible either to isolate mutant cells lacking the responsible degradative enzyme, thymidine phosphorylase, or, by adding AdR to the medium, to both inhibit phosphorylase and to supply deoxyribose-1-phosphate to reform TdR from T (2). Because similar mutant plants are not available and because the addition of AdR to plant tissues was only partly successful in reducing TdR degradation (26) the problem has been avoided in work with plants by using either very short exposure times (26) or by using large amounts (24 μg/ml) of relatively low activity labeled TdR (12). Under these conditions, only DNA of low specific activity can be synthesized. The use of high specific activity TdR is a relatively expensive alternative.

Although the first report was made in 1961 (5), the possibility of TdR degradation during radioactive labeling experiments in plants has often been ignored. Erroneous measurements of the duration and extent of DNA biosynthesis may occur unless care is taken to ensure, at least, that intact labeled TdR is continuously available during the experiment. Harland *et al.* (7) and King *et al.* (12) largely satisfied this requirement by showing that the rate of TdR uptake was linear over the experimental period. Tritium-labeled TdR has often been used to examine DNA synthesis in plant tissues by radioautography (4) but the possibility that labeled degradation products from [^{3}H]TdR were formed and used for synthesis of cell components other than DNA remains open. Degradation of TdR in buds of *Lilium* (9), root tips of *Vicia faba* (23), plumules of *Pharbitis* (26), and seedlings of *Pinus banksiana* (jack pine) (19) has been reported. Howland and Yette (10) found, using a suspension culture of wild carrot cells, that FUdR markedly reduced [^{14}C]TdR degradation and increased incorporation of TdR into DNA of these cells over a test period of 20 h. Here, we show the rate and extent of TdR degradation by sugarcane cells using TdR labeled either with ^{3}H in the methyl group, or ^{14}C in the ring at the 2 position. This experimental system removes problems of TdR transport and equilibration found with whole plants and also allows the isolation of metabolic intermediates and close control of some experimental variables not easily accomplished with intact plants or plant parts. Many compounds were tested for inhibition of TdR degradation but without complete success, since even a low rate of TdR degradation would be limiting in experiments where an extended labeling period of three to four generations (160 h) is required.

MATERIALS AND METHODS

Cell Propagation. Cells originally isolated from parenchymous tissue of sugarcane clone H50-7209 have been maintained in suspension culture since 1962 (16). The cells were cultured at 21 C in a modified White's basal medium containing yeast extract, vitamins, and sucrose, referred to as YE medium. Cultures were aerated on a rotary shaker at 260 rpm and routinely subcultured at 3-week intervals. When required, cells were transferred to growth in the completely synthetic medium, Syn II (16). Measurements of growth were made by determining the increase in dry weight of 10-ml aliquots of the culture.

Radioactive Materials. TdR labeled with ^{3}H in the methyl group (20 Ci/mmol), TdR labeled with ^{14}C in the second carbon position (62 mCi/mmol) and T labeled with ^{14}C in the second carbon position (57.3 mCi/mmol) were purchased from New England Nuclear Corp. The stock preparations of labeled precursors were chromatographically checked for possible contamination as found by others (4) and each was found to be >98% pure.

Chromatography. Whatman No. 4 paper was used with the solvent system No. 8 of Fink *et al.* (6) (upper phase of ethyl acetate-formic acid-water in the proportion 60:5:35, v/v) to separate labeled TdR, T, and BAIB in extracts of cultured cells. Suitable amounts of the respective unlabeled compounds were

[1] Chemistry and Biology Research Institute contribution No. 1077.

[2] A partial report of this work was presented previously (14).

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[4] Abbreviations: TdR: thymidine; T: thymine; AdR: deoxyadenosine; FUdR: 5-fluorodeoxyuridine; AR: adenosine; BAIB: β-aminoisobutyric acid.

added to each sample as internal markers, and after development, TdR and T were located by UV light absorption and BAIB by spraying with ninhydrin. The entire chromatogram was cut into segments and the ^3H or ^{14}C content of each segment was determined after wetting and adding each to 10 ml of toluene-based scintillation cocktail containing 10% BBS3 as solubilizer (Beckman Inst. Inc.).

Incorporation of Labeled TdR or T. Suspension-cultured cane cells used in these experiments had been transferred to fresh YE medium 5 days previously and were at about the midpoint of the exponential growth phase (8). Unless otherwise stated, the cell concentration was adjusted to provide 1.2 mg/ml, dry weight. Labeled T or TdR in 0.1 ml was added to 50 ml of cells in replicate 200-ml flasks, and the cultures were incubated at 29 C with aeration by shaking at 260 rpm. Aliquots (5 ml) were removed at intervals and the cells collected by suction filtration on tared filter paper discs. Filtrates were retained for measurement of extracellular radioactivity, and cells were immediately washed with cold medium and either dried and weighed as a measure of growth or were extracted for 45 min with cold 0.2 N $HClO_4$, filtered, and the radioactive content of the filtrate (cold acid-soluble cell fraction) measured. The acid-washed cells were resuspended in 5 ml of 0.5 N $HClO_4$, heated to 90 C for 20 min, cooled, and the cellular debris removed by filtration. The filtrate, (hot acid-soluble cell fraction) contained all of the cellular DNA. The remaining cellular debris was solubilized for counting with hyamine hydroxide but was never found to contain more than traces of radioactivity. An aliquot of each cell fraction was neutralized with KOH and added to 10 ml of a toluene-based scintillation cocktail containing 10% BBS3 as solubilizer. The radioactive content of each sample was measured by liquid scintillation spectrometry.

In some experiments, $^{14}CO_2$, formed by degradation of [^{14}C]-TdR or [^{14}C]T added to cane cells in a closed system, was trapped in a filter paper wick saturated with 0.2 N NaOH and placed in a small glass well fixed on the bottom of each culture flask. Dilute HCl was added to these culture flasks to stop the reaction and ensure release of CO_2 at each sample time. The wick was eluted in water and an aliquot taken for measurement of radioactivity.

RESULTS AND DISCUSSION

[^{3}H]TdR Utilization. Preliminary results indicated that a large proportion of the added [^{3}H]TdR label had been taken into the cells, and was found to be present in the cold acid-soluble cell fraction. A small amount was present in the hot acid-soluble cell fraction. In one experiment the amount of TdR added to the culture medium was varied over a wide range to determine the concentration of TdR required to saturate the incorporation system. The distribution of intracellular label between the cold and hot acid-soluble cell fractions was measured as a function of time. For this experiment, the initial cell density was adjusted to 0.46 mg/ml, dry weight, to allow normal cell growth for 120 h.

The results (Table I) show that the amount of TdR, as represented by the ^{3}H label, in the cold acid-soluble fraction increased with the concentration of TdR in the medium over the range 0.0415 to 415 $\times 10^{-7}$ M, (although proportionality fell off at 41.5 $\times 10^{-7}$ M) so that at 121 h the intracellular content of TdR appeared to be 0.0017 nmol and 15.66 nmol/ml cultured cells, respectively, an increase of 9,200-fold. It appeared that the amount of [^{3}H]Tdr contained in the cold acid-soluble cell fraction could therefore increase to very high levels before partial saturation of the system occurred at 83 μM. However, this result is deceiving, since as shown later, most of this label is present as BAIB, a breakdown product of TdR. It would appear prudent to identify tritium labeled intermediary metabolites after supplying [^{3}H]TdR to plants or plant cells. There were no significant differences (due to added TdR) in growth rates of the six cultures; cell mass increased on the average from 0.46 mg/ml to 1.67 mg/ml in 121 h.

Table I. *^{3}H-Thymidine Utilization with Time by Sugarcane Cells in Suspension Culture with Various Concentrations of Added Thymidine*

A constant amount of ^{3}H-TdR (51,000 cpm/ml) was added to each culture and the concentration of unlabeled TdR was varied.

Time	Initial TdR Concentration ($\times 10^7$ M)					
	0.0415	0.415	4.15	41.5	415	830
h	*nmol/ml cultured cells*[a]					
Cold acid-soluble cell extract						
50	0.0008	0.0087	0.092	0.946	5.54	6.61
97	0.0014	0.0136	0.149	1.58	10.33	15.79
121	0.0017	0.017	0.159	1.65	15.66	22.80
Hot acid-soluble cell extract						
50	0.0003	0.0029	0.034	0.227	0.455	0.744
97	0.0003	0.0033	0.029	0.211	1.157	1.074
121	0.0003	0.0029	0.029	0.240	1.653	2.397

[a] The figures were calculated from the radioactive data and represent the amount of TdR in each cell extract.

The amount of ^{3}H-labeled material bound intracellularly (hot acid-soluble cell fraction) also increased roughly in proportion to the amount of TdR added, up to 4.15 μM. However, the amount bound did not increase with time after the first sample at 50 h except when the amount of TdR added was increased to 41.5–83 μM. These results are compatible with the idea that TdR was rapidly degraded by sugarcane cells and that, with low levels of added TdR, by 50 h TdR was no longer available for incorporation into cellular DNA. At higher extracellular concentrations, not all of the TdR was degraded during the experiment, and therefore TdR was available for intracellular DNA synthesis and the amount of ^{3}H bound continued to increase with time, up to 121 h. To identify the labeled material accumulated in the cold acid-soluble fraction of the cells, the remaining portion of each fraction from the 121-h samples of the three cultures originally containing 4.15, 415, and 830 μM TdR was neutralized with KOH, chilled, and the $KClO_4$ was removed by centrifugation. The supernatants were concentrated *in vacuo*, and an aliquot of each was examined by paper chromatography. More than 90% of the ^{3}H-labeled material applied to the chromatogram from each sample co-chromatographed with BAIB, and the remainder was found with T. No label was associated with TdR. Intracellular acid-soluble ^{3}H label derived from [^{3}H]methyl-labeled TdR, therefore, is not a DNA precursor, since formation of BAIB from TdR is irreversible (2). This labeled material, BAIB, can be converted to cellular components in intact plants (23). The free ^{3}H-BAIB itself is unlikely to interfere in radioautographic studies, since it would be removed during the fixation process (24), except in unusual procedures such as the cytological studies of acid-soluble ^{3}H-labeled material in unfixed tissue (22).

Cells remaining in one of the above cultures (83 μM TdR) were collected immediately after the 121 h sample, washed with water by suction on a filter paper disc and frozen. The DNA was subsequently extracted and purified by the method of Bendich and Bolton (1) with a recovery of 57% of the total DNA. Based on the specific activity of the isolated DNA, no more than 10% of the hot acid-soluble labeled material found in the cells at 121 h could be in DNA. No attempt was made to identify the non-nucleic acid ^{3}H-labeled cellular component(s), but this labeling result appears similar to that found in root tips of *Vicia faba* (23) and in *Euglena* (21), where methyl-labeled [^{3}H]TdR was used and a significant proportion of the label was found in non-DNA acid-insoluble cell material. It would seem that if methyl-labeled [^{3}H]TdR is used to label plant cell DNA, care should be taken to ensure that incorporated (cold acid insoluble) label is present only in DNA, at least in sugarcane cells.

[^{14}C]Thymidine Utilization. As shown above, most of the [^{3}H]methyl TdR was degraded, and little was used for synthesis of DNA. [2-^{14}C]TdR was used in a similar incorporation experiment at a concentration of 4.1 μM. After 91 h incubation the cells had nearly tripled in mass but only 0.2 and 1.4% of the label was in the cold and hot acid-soluble fractions, respectively. The greatly reduced amount of label in the cold acid-soluble cell fraction relative to that found previously with the [^{3}H]methyl-labeled TdR could be accounted for by the known catabolic pathway of TdR (5) which proceeds through T, dihydrothymine, β-ureidoisobutyrate and then, with loss of the ^{14}C label in the 2 position as CO_2, to unlabeled BAIB. Further breakdown of BAIB probably produces alanine (6). A methyl group label, ^{14}C or ^{3}H, in T could produce labeled alanine by this pathway, and some labeled protein would be expected to be synthesized from the alanine.

The DNA was isolated and purified from the remaining cells of the 91-h sample of the above culture, and it was calculated on the basis of its specific activity that >90% of the label found in the hot acid-soluble cell fraction could be in DNA. It appeared likely that [2-^{14}C]Tdr had been utilized only for DNA synthesis and that any labeled dihydrothymine or β-ureidoisobutyrate derived from it was not used for synthesis of other cellular constituents unlike the result obtained previously with [^{3}H]TdR. Any BAIB produced could not contribute since it would be unlabeled.

With [6-^{14}C]TdR, BAIB resulting from the above degradative pathway would be labeled (6). Cultured carrot cells in a similar experiment were supplied with [6-^{14}C]TdR, and as expected, a large part of the label was found within the cell as [^{14}C]BAIB. In these cells there was no evidence for utilization of labeled BAIB during a subsequent period of two cell divisions (unpublished results).

To follow [2-^{14}C]TdR utilization and degradation by sugarcane cells, distribution of the label between the culture medium, CO_2, cold acid-soluble and hot acid-soluble cell fractions was determined with time. The results (Fig. 1) showed a rapid removal of label from the medium, and this was balanced by a similarly rapid appearance of label as $^{14}CO_2$, an indirect measurement of degradation of TdR to BAIB. A small intracellular pool of cold acid-soluble ^{14}C-labeled material formed during the first 30 min, decreased subsequently, and appeared to be used for DNA synthesis. The rate of DNA synthesis, as measured by incorporation of ^{14}C, decreased sharply between 1 and 2 h. Chromatography of an aliquot of the centrifuged, cell-free medium showed that only about 10% of the labeled TdR remained at 60 min, and only a trace of radioactivity was present as TdR at 120 min. The remaining label in the medium at 120 min was present as T. These results indicated that at this cell concentration (1.9 mg/ml, dry weight) about 0.165 nmol/ml of TdR was degraded in 90 min by sugarcane cells cultured at 21 C. No measurable reutilization of released $^{14}CO_2$ was found in a separate experiment.

Since the above results were obtained, we found this degradative pathway to be present in other cultured plant cells. There was little variation in the rate or extent of TdR breakdown in cultured cells of broad bean, tobacco, carrot or immature pollen cells of tobacco removed from the anther and suspended in liquid culture medium (unpublished results).

The production of $^{14}CO_2$ was used to compare the rate of degradation of [2-^{14}C]TdR and [2-^{14}C]T added to a sugarcane cell suspension at equal concentrations (8 μM). The cell concentration was increased in this experiment to 3.0 mg/ml, dry weight, and samples were taken at 5-min intervals to detect any delay of degradative activity due to induction of the degradative enzyme system. The results (Fig. 2) show an immediate breakdown of T and a delay of only 2–3 min before TdR degradation was measurable. The activation of the degradative system was rapid, and since the rate of degradation was the same for both compounds, the release of T from TdR by these cells was not limiting for production of CO_2 from TdR.

An attempt was made to localize the degradative enzyme system in the cell wall matrix of sugarcane cells by removing the walls enzymically and incubating the resulting protoplasts with 2-^{14}C-labeled TdR. The protoplasts, prepared by the method of Maretzki and Nickell (14), were washed and resuspended in Syn II media (containing 0.7 M mannitol as osmoticum) at a density equivalent to 1.9 mg/ml of the original cells. [2-^{14}C]TdR was added to one

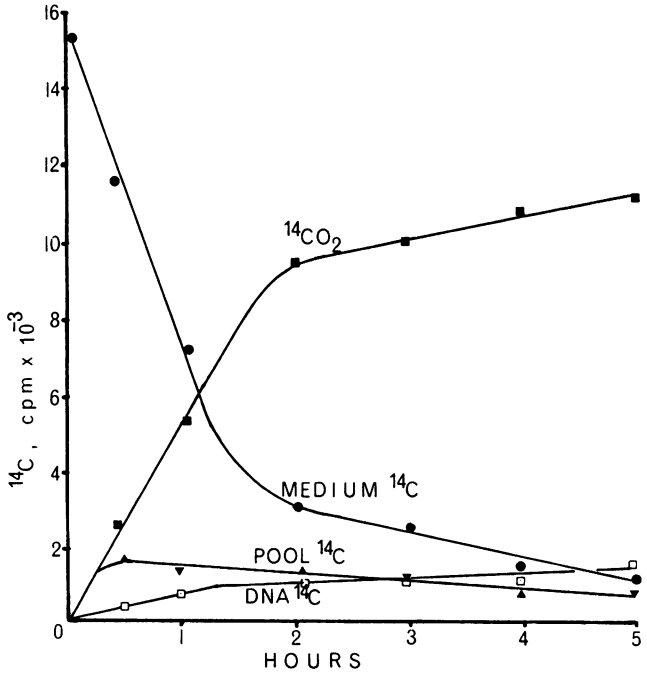

FIG. 1. Distribution of label from [2-^{14}C]TdR added to a culture of sugarcane cells with time. [^{14}C]DNA was measured as hot acid-soluble, and pool-^{14}C as cold acid soluble, radioactivity. After 2 h most of the medium ^{14}C was present only as T.

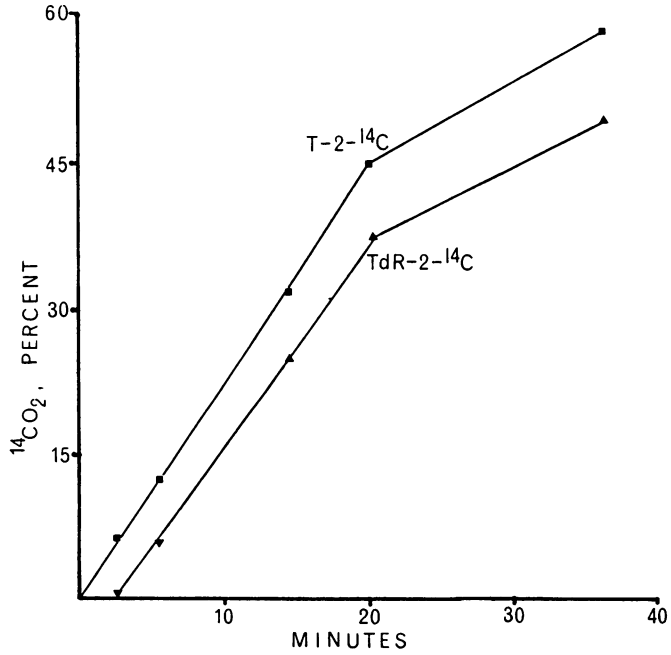

FIG. 2. $^{14}CO_2$ evolution from labeled T or TdR by sugarcane cells. Equal concentrations (0.015 nM and 500,000 cpm/ml) of T and TdR were added to separate aliquots of the same culture and the amount of degradation ($^{14}CO_2$ production) measured with time.

aliquot and [2-^{14}C]T to a second, each at a concentration of 4 μM. There was no detectable formation of $^{14}CO_2$ in either culture after incubation with gentle shaking for 60 min at 26 C. A control of untreated whole cells at an equivalent density was incubated with labeled TdR at the same concentration for 60 min, and 75% of the label was released as $^{14}CO_2$. The protoplasts were removed from both test suspensions by centrifugation at 200*g* for 5 min and an aliquot of each supernatant chromatographed. All ^{14}C was present as TdR or T in protoplast suspensions containing added labeled TdR and T, respectively. Active degradative enzymes were not associated with the protoplasts and had been apparently removed with the cell walls or inactivated by the enzymes used to digest the cell walls. Under these conditions, sugarcane cell protoplasts are metabolically active (14). In bacteria, six enzymes, all involved in degradative pathways, are released during cell wall removal in the formation of spheroplasts, which are equivalent to protoplasts of plant cells (15). However, neither the presence of phosphorylase nor of any other enzymes in the TdR degrative pathway could be demonstrated in our experiments using the culture medium supernatant containing digested sugarcane cell walls after protoplast preparation.

As shown in Table II, addition of a large excess of unlabeled T does not prevent degradation of TdR by cultured cane cells. To determine whether incorporation of TdR was influenced by T, equivalent amounts of [^{14}C]T and [^{14}C]TdR (8 μM) were added to separate aliquots of a cell culture, and to a third aliquot the same amount of [^{14}C]TdR was added with a large excess of unlabeled T (0.5 mM). The cells were collected after 2 h growth and the radioactivity in the hot acid-soluble cell fractions determined. With labeled T, 0.5%, with labeled TdR, 17%, and with labeled TdR plus unlabeled T, 16.6%, of the initial radioactivity was found in the hot acid-soluble cell fraction. The absence of appreciable [^{14}C]T incorporation might be due to a lack of deoxyribosyl donors similar to the results found in experiments with *Escherichia coli* (12). Using germinating rape seeds Evans and Axelrod (5) showed rapid degradation of labeled T and also found little incorporation of label into nucleic acid.

If [^{3}H]TdR is used with an excess of unlabeled T, there would be only a trace of [^{3}H]BAIB produced. Any possibility of mislabeling due to utilization of this degradation product would be avoided, especially in radioautography experiments (23) and where unusual labeling patterns have been found when [^{3}H]TdR was used alone (21).

Table II. *Metabolism of [^{14}C]TdR by Sugarcane Cells: Effect of Added Compounds*

Test Compound[a]	^{14}C Distribution at 90 Min, % of Initial [^{14}C]TdR as:[b]		^{14}C in Culture Medium at 90 Min, % as:	
	CO_2	Hot Acid-Soluble Cell Fraction	[^{14}C]-TdR	[^{14}C]T
0.5 mM				
None	52	7	90	10
Deoxyadenosine	49	1.5	100	0
5-Fluorodeoxyuridine	2	1	98	2
6-Azauridine	17	2	100	0
5-Bromodeoxyuridine	1	0.5	95	5
Deoxycytidine	4	1	100	0
Cytidine	2.5	1	95	5
Uridine	4	0.7	100	0
6-Aminouracil	46	5	70	30
5-Fluorouracil	17	8.2	57	43
5-Diazouracil	6	15	60	40
Thymine	0.5	7.2	45	55
6-Azathymine	15	13	65	35

[a] Compounds added 5 min before [^{14}C]TdR and distribution of ^{14}C measured 90 min later.

[b] [^{14}C]TdR added was 16.5 μM.

Inhibition of Thymidine Degradation. Production of $^{14}CO_2$ from [2-^{14}C]TdR added to sugarcane cells was used to measure the effects of added compounds on TdR degradation. A block at any step in the catabolic pathway before the formation of BAIB would prevent CO_2 formation. Distribution of the ^{14}C-label between TdR and T in the medium was also determined, by paper chromatography, as a measure of the first stage of degradation. Incorporation of label into the hot acid-soluble cell fraction was also measured to ensure that DNA biosynthesis had not been inhibited. To ensure complete equilibration, the test compound was added to each cell suspension 5 min before the addition of [^{14}C]TdR.

Results show that seven of the tested compounds did markedly reduce CO_2 formation (Table II, col. 2), but two of these, T and 5-diazouracil, did not prevent the formation of T from TdR (Table II, columns 4 and 5), and were therefore of no use. It was apparent that 5-diazouracil did increase markedly the amount of label incorporated into the hot acid-soluble cell material possibly by conversion to 5-ara-uridinemonoP, known to block endogenous T-monoP formation in bacteria (2). The enhancement of TdR incorporation by 6-azathymine is not understood, but presumably occurs by inhibition of TMP synthesis by some unknown interference similar to that inhibiting TMP synthetase when FUdR is used (24). However, 6-azathymine does not prevent TdR degradation (Table II, columns 4 and 5) and is also of little use.

Of the remaining four compounds, only two showed reduced TdR degradation, FUdR and 5-bromodeoxyuridine. The latter is incorporated into plant cell DNA (17), where it interferes with the function of DNA and also sensitizes the cell to the effect of light. Howland and Yette showed (10) that FUdR efficiently reduces degradation of labeled TdR when fed to wild carrot cells, but in sugarcane cells 2% degradation in 90 min would amount to a significant effect over one generation time of about 50 h. When used with cultured cells derived from a domestic carrot (unpublished results) FUdR reduced TdR degradation to a low level and increased its incorporation into DNA. These results are similar to those found with wild carrot cells (10).

The other compounds tested (Table II) did not reduce degradation sufficiently. It is possible that suitable combinations of these at optimum concentrations may provide the needed protection from degradation. In short term labeling a more promising approach may be selection of mutant cultured plant cells lacking TdR degradative enzymes, perhaps by using aminopterin together with trimethoprim to select cells requiring TdR for growth, as has been done successfully with some bacteria (25).

Acknowledgments—S. M. Lesley acknowledges with sincere thanks his deep appreciation of the advice, facilities and hospitality offered by the staff of Hawaiian Sugar Planters Association during a one-year sabbatical at the Experiment Station.

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