CAUTIONARY NOTE ON THE USE OF [METHYL-3H]THYMIDINE TO MEASURE RATES OF CHLOROPLAST DNA SYNTHESIS IN CHLAMYDOMONAS REINHARDTII

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It has been reported that exogenously supplied thymidine and thymidine analogues, e.g. 5-bromo-deoxyuridine, are specifically incorporated into chloroplast (β-component) DNA in Chlamydomonas reinhardtii (10). The chloroplast-specific incorporation is found in vegetative cells, but this specificity is lost during meiosis when both nuclear DNA and chloroplast DNA can be labeled by radioactive thymidine (1). The specificity in vegetative cells has been attributed (though not yet demonstrated) to the presence of thymidine kinase in the chloroplast and the absence of this enzyme in the nucleus and/or cytoplasm (1, 10).

Since radioactive thymidine is incorporated only into chloroplast DNA, thymidine labeling, potentially, could be used to measure the rate of chloroplast DNA synthesis in whole cells. However, we have encountered problems in measuring rates of chloroplast DNA synthesis with the use of [methyl-3H]thymidine, due to a contaminant which is efficiently incorporated by C. reinhardtii cells.

Several other reports in the literature warn about using [3H]thymidine to measure DNA synthesis in other organisms; however, these studies are concerned with the nature of the product into which bona fide thymidine is incorporated (2, 4, 6, 8).

MATERIALS AND METHODS

Chlamydomonas reinhardtii strain 137C (mt⁺) obtained from W. T. Ebersold (University of California Los Angeles, Calif.) was grown autotrophically on high salt medium (HSM) (9). Cultures were bubbled with 3% CO₂ in air, maintained at 25°C, and exposed to 4,000 lx fluorescent illumination.

Radioisotopically labeled thymidine or thymine was obtained from New England Nuclear (Boston, Mass.). [Methyl-3H]thymidine was stored at 2°C and used within 1 mo after it was received. The labeled compounds cited in these experiments are [methyl-3H]thymidine (Lot no. 787-220) 43 Ci/mmol, [6-3H]thymidine (Lot no. 744-060) 9.3 Ci/mmol, [2-14C]thymidine (Lot no. 744-024) 54 mCi/mmol, and [methyl-3H]thymine (Lot no. 824-276) 14.9 Ci/mmol.

RESULTS AND DISCUSSION

When [methyl-3H]thymidine or thymine is added to an exponentially growing (late log) culture of vegetative C. reinhardtii cells, label is incorporated at a rapid rate into acid-precipitable material for 20–40 min (Fig. 1). At the end of the period of rapid incorporation, about 0.2% of supplied thymidine is converted to acid-precipitable material.

Chromatographic analysis in one dimension was performed with Polygram Cel 300 cellulose-coated thin-layer chromatography sheets. The solvent used was water (7) or the upper phase from a mixture of ethyl acetate, water, and formic acid (60:30:10) as modified from a procedure described by Fink et al. (3). Unlabeled thymidine and thymine were used as UV-visible markers.
After rapid incorporation, a slower rate of incorporation continues, which will be discussed more fully below.

It was curious both that linear incorporation proceeded for only a short time and that only a small percentage (~0.2%) of total ³H-label was ultimately incorporated. These unusual incorporation characteristics could arise from three alternative situations: (a) radioactive thymidine is being rapidly converted to a compound that is more slowly incorporated; (b) cells might physiologically respond to the addition of thymidine and exclude its further rapid incorporation; or (c) cells might be rapidly incorporating a contaminant in the [methyl-³H]thymidine preparation.

To investigate the first possibility, that [methyl-³H]thymidine might be converted en masse to a nonutilizable compound during 30 min of incubation, we analyzed an aliquot of medium after 30-min incubation with cells by one-dimensional thin-layer chromatography. The chromatography conditions (solvent, water) clearly separated thymidine from thymine (7). The results showed that there was no difference in the chromatographic pattern of the label before and after incubation, and that 95% of the total counts per minute comigrated with a UV-visible thymidine marker. We conclude, therefore, that [methyl-³H]thymidine is not converted, en masse, to thymine or to a different chromatographically identifiable compound during incubation with cells.

To test the second possibility, that cells might rapidly develop a tendency to block further thymidine incorporation upon exposure to exogenous thymidine, we preincubated cells with unlabeled thymidine for 30 min and then added [methyl-³H]thymidine. As seen in Fig. 2, preincubated cells incorporated [methyl-³H]thymidine as efficiently and to the same extent as did cells which were not previously exposed to exogenous thymidine. Thus, cells apparently do not develop an exclusion mechanism to block further rapid incorporation of thymidine.

In examining the third possibility, that cells incorporate a minor contaminant in the [methyl-³H]thymidine preparations, we considered that cells might deplete such a contaminant from the medium during short incubation, and this depleted medium should not support further incorporation. That situation was the case as shown in Fig. 3. [Methyl-³H]thymidine was added to a cell culture and incubated for 50 min. The culture was cleared

![Figure 2](image1.png)  
**Figure 2** Time-course of incorporation of 1 μCi/ml [methyl-³H]thymidine after 30-min preincubation with 5.6 ng/ml unlabeled thymidine (O—O). Time-course of incorporation without preincubation (●—●), where 5.6 ng/ml unlabeled thymidine was added simultaneously with labeled thymidine. Final thymidine concentration (labeled and unlabeled thymidine) in each experiment was 11.2 ng/ml. Measurement of incorporation of label is the same as in Fig. 1.

![Figure 3](image2.png)  
**Figure 3** Time-course of incorporation of 2 μCi/ml [methyl-³H]thymidine (●—●). After 50 min of incubation the medium was cleared of cells by centrifugation, and then clarified medium was used to resuspend new cells at the same cell density as in the original incubation (10⁶ cells/ml). Time-course of incorporation during second incubation (O—O).
of cells by centrifugation, and clarified medium was used to resuspend a pellet of fresh cells. A time-course of samples taken from the second incubation showed no rapid incorporation of the \(^3\)H-label. Hence, all the \(^3\)H-label that could be rapidly incorporated was utilized in the first incubation.

To substantiate further the point that rapid incorporation of \(^3\)H-label results from a minor contaminant, the \([\text{methyl-}^3\text{H}]\)thymidine preparation was chromatographed and the incorporation of the \(^3\)H-labeled material eluting from various places on the chromatogram was tested (Fig. 4). The material which was most extensively incorporated in a 30-min incubation with cells did not comigrate on the chromatogram with \([\text{methyl-}^3\text{H}]\)thymidine. This material migrated somewhat more rapidly than thymidine on the chromatograms. This \(^3\)H-labeled compound(s) has not yet been identified.

Since it appeared that a minor contaminant is being efficiently incorporated into acid-precipitable material, we asked whether this contaminant is incorporated into DNA. To answer this question, cells were permitted to incorporate label from a \([\text{methyl-}^3\text{H}]\)thymidine preparation for 30 min. With a modified Schmidt-Thannhauser extraction procedure (5), it was found that 76% of the incorporated radioactivity was recovered as acid-hydrolyzable, base-nonhydrolyzable material. Therefore, most of the \(^3\)H-label incorporated during the first 30 min of incubation enters DNA.

Swinton and Hanawalt have reported that radioactive label from \([\text{methyl-}^3\text{H}]\)thymidine preparations (presumably found in both thymidine and the contaminant) exclusively enters chloroplast DNA and is recoverable in chloroplast DNA hydrolysates as dTMP (10).

We determined whether this rapidly incorporated contaminant was also found in ring-labeled \(^3\)H-thymidine preparations, such as \([6-^3\text{H}]\)thymidine. Fig. 5 shows that it is not. Little \(^3\)H-label is incorporated in a 90-min time-course with either \(10^6\) or \(10^4\) cells/ml. The culture with \(10^6\) cells/ml was permitted to grow further in the presence of \([6-^3\text{H}]\)thymidine, and it continued to incorporate label at a low rate commensurate with growth of the cells. The same observation was made with
[14C]thymidine. We conclude that the rapidly incorporated contaminant was found only in [methyl-3H]thymine or thymidine preparations. Therefore, it is recommended that ring-labeled thymidine preparations be used to specifically measure the rate of synthesis of chloroplast DNA.

SUMMARY
Commercial [methyl-3H]thymidine preparations tested here contain about a 0.2% contaminant which is rapidly incorporated into Chlamydomonas DNA. This contaminant obscures the measurement of the rate of chloroplast DNA synthesis when methyl-labeled preparations are used. Such contaminants are not present in ring-labeled (either 3H or 14C) thymidine preparations. In ring-labeled thymidine preparations, a slower incorporation rate commensurate with cell density is observed. These slower, long-term incorporation kinetics would be expected for the utilization of bona fide thymidine into chloroplast DNA.

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