Reprinted from THE JOURNAL OF PROTOZOOLOGY.
J. PROTOZOOL., 21(2), 379–382 (1974)
Made in United States of America

Labeling of *Crithidia fasciculata* DNA with [^{3}H]Thymidine

AGDA M. SIMPSON and LARRY SIMPSON
Department of Biology, University of California, Los Angeles, California 90024

SYNOPSIS. Attempts at continuous labeling of *Crithidia fasciculata* DNA with [^{3}H]thymidine led to a pulse-chase situation due to a cell-mediated conversion of thymidine to thymine in the medium. The uptake of thymine was slow compared to that of thymidine. Neither the addition of deoxyadenosine nor the sequential addition of several aliquots of [^{3}H]thymidine had an effect on the pattern of labeling.

Index Key Words: *Crithidia fasciculata*; thymidine; DNA; thymidine phosphorylase.

THE presence of the enzyme, thymidine phosphorylase, has been shown to be the cause of the relatively low extent of labeling of DNA by [^{3}H]thymidine in several different cell types, e.g. *E. coli* (2, 5, 8-11), certain murine and human tumor cells (13, 14) and normal mammalian liver (3, 4). This enzyme catalyzes a phosphorolytic cleavage of pyrimidine deoxyribonucleosides and is involved in the salvage pathway of thymine metabolism in these cells (7). The uptake of thymine is relatively slow compared to that of thymidine and thus the incorporation of label into DNA is effectively inhibited by the cleavage of [^{3}H]thymidine to [^{3}H]thymine.

In the course of a study of DNA replication in the parasite protozoan, *Crithidia fasciculata*, we discovered an analogous situation. This report describes several preliminary experiments which indicate that continuous labeling of *C. fasciculata* DNA is hindered by the apparent presence of a thymidine phosphorylase-like enzyme activity.

MATERIALS AND METHODS

Culture of the cells.—The cells represent a clone of a *Crithidia fasciculata* culture originally obtained from Dr. Stuart Krassner. This clonal cell line has been growing in our laboratory for 3 years in Brain-Heart Infusion Medium (Difco Laboratories) with 10 μg/ml hemin added after autoclaving. The cells were grown at 27 ± 0.5 C either in 3.5 liter quantities in a fermentator (Fermentation Industries, Allentown, Pa.) or in 150 ml to one liter quantities in Pyrex bottles rotating at 6 rpm. Antifoam B (Dow Corning) was added for defoaming as required. The experimental cultures were grown in either modified Trager's defined Medium C for *Leishmania tarentolae* (12) or in Kidder & Dutta's defined medium for *Crithidia* (6). Cells were grown for at least 3 rapid subcultures in the appropriate defined medium prior to use in labeling experiments. The modification of Trager's Medium C consisted of the replacement of the purine-pyrimidine mixture by 20 μg/ml adenine.

Labeling of the cells.—[Methyl-^{3}H]thymidine (18 C/mM) and [methyl-^{3}H]thymine (14 C/mM) were purchased from Schwarz/Mann Co. Experimental cultures were grown in bottles with rotation at 27 C. Incorporation of label was measured by spotting 100 μl of the cell culture onto Whatman 3 MM discs, drying and processing the discs through 5% (w/v) trichloroacetic acid, 70% (v/v) ethanol, 95% ethanol and ether. The discs were counted in toluene-Omnifluor (New England Nuclear Co.) in a Nuclear Chicago Scintillation Counter.

Cell counts were performed by mixing samples of culture 1:1 with 3% (v/v) formalin and counting 200-500 cells in a hemacytometer.

Paper chromatography of [^{3}H]thymidine and [^{3}H]thymine.—Samples, which consisted of neutralized aliquots of the culture medium after removal of cells by centrifugation and precipitation with 0.5 N $HClO_4$, were spotted on Whatman #1 paper, and unlabeled thymine and thymidine were added as OD markers. The strips were equalibrated for 16 hr in the presence of the lower phase of the mixture ethyl acetate-water-formic acid (60:35:5); then descending chromatography was performed using the upper phase of this mixture. The spots were eluted overnight in 0.1 N HCl and then Aquasol (New England Nuclear) was added and the samples counted in a Nuclear Chicago Scintillation Counter.

Isolation of nuclear and kinetoplast DNA.—Kinetoplast DNA (K-DNA) was isolated from early stationary phase cells (~2 × 10^8 cells/ml). The cell pellet was washed once in 0.15 M NaCl, 0.02 M glucose, 0.02 M phosphate buffer, pH 7.9. The final pellet was resuspended in 0.5 M EDTA—0.15 M NaCl (pH 8.0) to a 5% (w/v) final concentration. Sodium Dodecyl Sarkosinate (Sarkosyl NI 97, Geigy Industrial Chemicals, Ardsley, N. Y.) was added to 1% (w/v) final concentration. Pronase B grade or pronase CB B grade, Calbiochem, predigested for 30 min at 37 C was added to a concentration of either 2 mg/ml or 0.5 mg/ml respectively. The solution was incubated at 56 C for 3-5 hr. The lysate was then passed through a #18 needle at 25 lb/in^2 and then centrifuged at 20,000 rpm for 20 min in the SW 39 rotor at 4 C. The supernatant fluid was decanted and saline-sodium citrate (0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) was added to resuspend the pellet, and the solution was centrifuged as described previously. This final pellet was resuspended in saline-sodium citrate and subjected to RNase A + T_1 (20 μg/ml and 20 U/ml respectively) treatment for 30 min at 37 C. Pronase was added to 100 μg/ml and the solution incubated again at 37 C for 30 min. Deproteinization was performed with chloroform-iso-amyl alcohol (24:1 v/v), and the DNA solution was dialyzed extensively against saline-sodium citrate.

This DNA was then subjected to ethidium bromide CsCl equilibrium centrifugation (40 hr, 40,000 rpm, 20 C, 6.5 ml, #50 rotor, n_D 25C = 1.3876). The lower band consisting of covalently closed circular K-DNA networks and the upper band consisting of nuclear DNA (N-DNA) were recovered, and the dye was removed by extraction with iso-amyl alcohol and dialysis. The specific activities of the N-DNA and K-DNA were measured by reading the A_{260nm} and by spotting samples onto 3 MM discs for counting as described above.

RESULTS

Attempts at Continuous Labeling of DNA

Cells were grown in Medium C containing 10 μc/ml of [^{3}H]thymidine and different concentrations of unlabeled thymidine. Growth curves were followed and the specific activities of acid-precipitable [^{3}H] label were measured at various

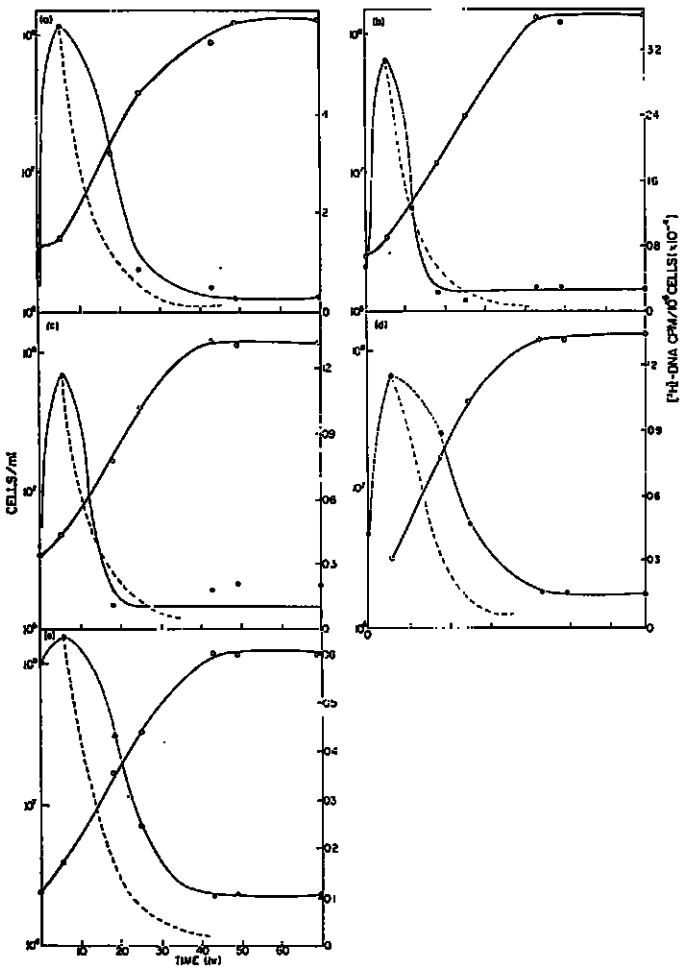

Fig. 1. Incorporation of [^{3}H]thymidine into acid-insoluble material by *C. fasciculata* cells in a long term labeling situation in Medium C. [^{3}H]Thymidine (10 μc/ml, 18 C/mM) was added at time 0 and the specific activities (cpm/10^6 cells) of the incorporated material followed during growth. The concentration of unlabeled thymidine present in each culture was as follows: (a) 0; (b) 2 μg/ml; (c) 5 μg/ml; (d) 10 μg/ml; (e) 50 μg/ml. (●——●), acid insoluble cpm/10^6 cells ($\times 10^{-4}$); O——O), number of cells per ml; (- - - - - - - - -), theoretical simple dilution curve assuming that no further synthesis occurs after the indicated point.

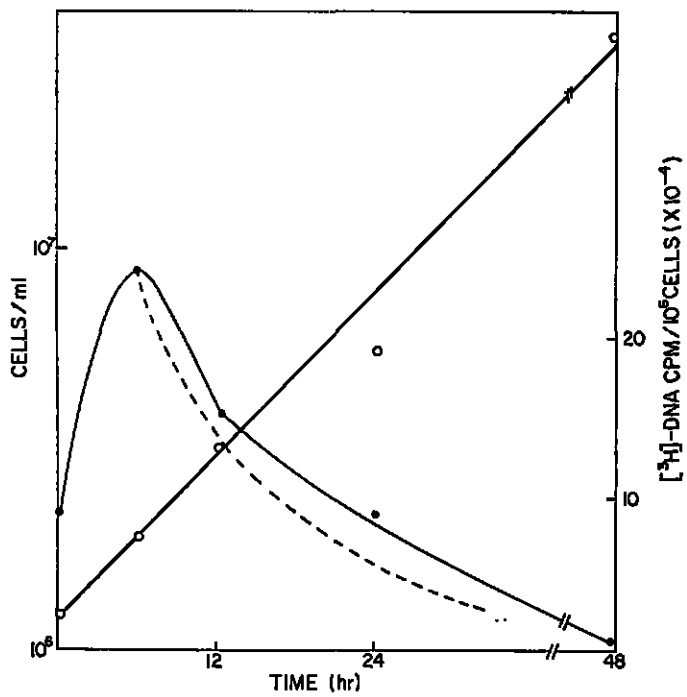

Fig. 2. Incorporation of [^{3}H]thymidine into acid-insoluble material by *C. fasciculata* cells in a long term labeling experiment in Kidder & Dutta's medium. [^{3}H]Thymidine (10 μc/ml, 18 C/mM) was added at time 0 and the specific activities (cpm/10^6 cells) of the incorporated material followed during growth. See Fig. 1 for description of symbols.

times during the growth curves. We have previously demonstrated that [^{3}H]thymidine is incorporated solely into the DNA (nuclear and kinetoplastic) of *C. fasciculata* and is found entirely in the thymine moiety in total cell DNA hydrolysates (unpublished results). As shown in Fig. 1 the specific activity of the total cell DNA reached a maximum before one cell division and then decreased approximately by simple dilution, indicated by the dashed line. This occurred even in the presence of 50 μg/ml of unlabeled thymidine (Fig. 1c). Thus attempts at continuous labeling of DNA led to an apparent pulse-chase situation.

Identical results were obtained in Kidder and Dutta's defined medium for *Crithidia* as shown in Fig. 2. However, in the case of this culture medium, which is known to be optimal for the growth of *C. fasciculata*, the maximum specific activity attained was 4.2 × that attained in Medium C.

These experiments did not distinguish between incorporation into N- K-DNA, the latter of which is known to represent ~25% of the total cell DNA (Simpson & Simpson, unpublished data). Therefore, it is possible that the specific activity of K-DNA increased to a plateau value, whereas the N-DNA specific activity showed an early peak and a decrease. That this was not the case was clear both from the fact that the final total cell DNA specific activity was much less than 25% of the maximum value attained (Fig. 1), and that the specific activities of purified N- and K-DNAs from cells grown with 10 μc/ml of [^{3}H]thymidine for 3 days were equal within experimental error (Table 1).

Presence of Thymidine Phosphorylase Activity During Growth of Cells

An explanation for the failure of continuous labeling of cells with [^{3}H]thymidine was the apparent presence of thymidine phosphorylase activity. Cells were grown in Kidder & Dutta's medium with 20 μc/ml of [^{3}H]thymidine with no additional unlabeled thymidine. The conversion of [^{3}H]thymidine to [^{3}H]-

TABLE 1. *Specific activities of purified N- and K-DNA after growth of cells for 3 days in [^{3}H]thymidine.*

DNA	Specific Activity† (cpm/μg DNA)	Ratio of Specific Activities‡ K/N
nuclear	30,394	
kinetoplast	42,897	1.09

* 10 μc/ml, in the medium of Kidder & Dutta (6).
† Concentration measured by A_{260nm} assuming 1 OD = 41.5 μg/ml.
‡ Ratio corrected for 21.5% thymine in nuclear DNA *vs* 28% thymine in kinetoplast DNA (unpublished data).

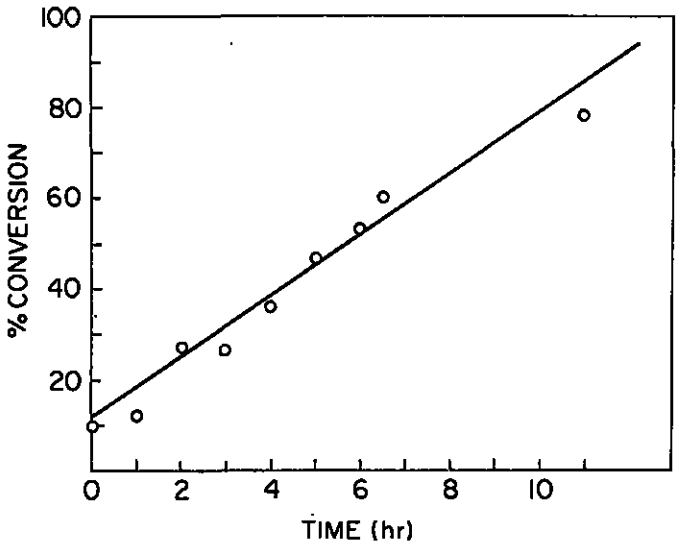

Fig. 3. Conversion of [^{3}H]thymidine (20 μc/ml, 18 C/mM) to [^{3}H]thymine by *C. fasciculata* cells growing in Medium C. No unlabeled thymidine was added. Similar results were obtained using 5 μg/ml unlabeled thymidine with 20 μc/ml [^{3}H]-thymidine. Aliquots of the culture were removed and centrifuged at 2000 *g* for 10 min. The supernate solution was made 0.5 N in perchloric acid and again centrifuged for 15 min at 5000 *g*. The resulting supernate solution was neutralized with KOH and the precipitate of potassium perchlorate removed by centrifugation. Descending paper chromatography was performed on the final solution as described in Methods. The percent conversion of thymidine to thymine was calculated using the total cpm recovered from the thymine and thymidine spots at each time point.

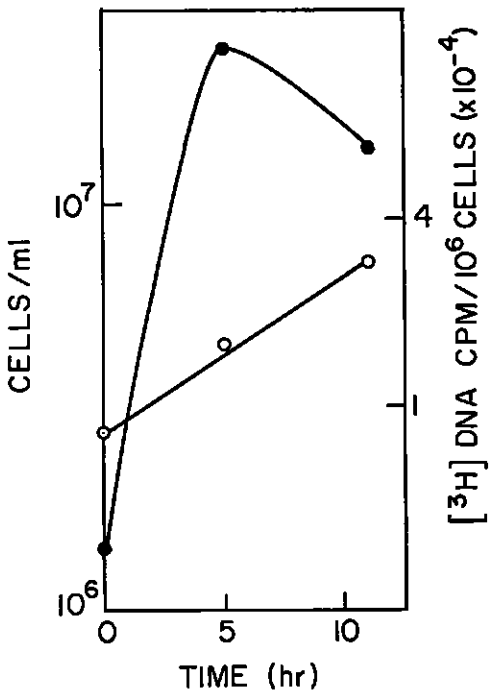

Fig. 4. Effect of the sequential addition of several aliquots of [^{3}H]thymidine on the labeling pattern of *C. fasciculata* in Medium C. [^{3}H]Thymidine (5 μc/ml, 18 C/mM) was added at time 0, 5 hr and 12 hr during growth. The cell counts and specific activities of incorporated material were measured at each time. See Fig. 1 for description of symbols.

thymine in the medium was followed by descending paper chromatography. As shown in Fig. 3, the conversion proceeded linearly with time at least for 10 hr, by which time 78% of the exogenous thymidine originally present had been converted to thymine. This phenomenon would lead to the observed decreased incorporation of label provided thymine were not taken up to the same extent as thymidine. This was demonstrated to be the case by growing cells in Medium C in the presence of 3.3 μc/ml of [^{3}H]thymine with 5 μg/ml of unlabeled thymine. After 3 days growth, the cells had attained a specific activity of 2.8×10^3 cpm/10^6 cells, which is equivalent to the basal levels attained by cells grown in [^{3}H]thymidine after 60 hr growth.

Attempts to Increase Uptake of Thymidine

Deoxyadenosine (250 μg/ml), which is a known inhibitor of thymidine phosphorylase activity in *E. coli* (1), had no effect on the kinetics of labeling of DNA with [^{3}H]thymidine in *C. fasciculata*.

Sequential addition of several aliquots of [^{3}H]thymidine during the growth period also had no effect on the pattern of labeling. In this experiment cells were given 5 μc/ml [^{3}H]thymidine at 0, 5 and 15 hr in the growth curve. As shown in Fig. 4, the specific activity reached a maximum at 5 hr and then decreased at 12 hr.

DISCUSSION

We have demonstrated that attempts at continuous labeling of *C. fasciculata* DNA by [^{3}H]thymidine in either of 2 defined media led to an apparent pulse-chase situation explainable on the basis of a cell-mediated conversion of thymidine to thymine in the medium. Attempts to increase the amount of [^{3}H]thymidine taken up, either by the addition of deoxyadenosine or by supplying additional aliquots of [^{3}H]thymidine at 5 hr intervals, failed. A recognition of this labeling pattern is of obvious importance in any study of the replication of *C. fasciculata* DNA. Furthermore, this phenomenon, if it is of general occurrence among the parasitic hemoflagellates, may be significant in terms of limiting the chemotherapeutic value of thymidine analogues.

In our experiments, we did not distinguish between incorporation into N- or K-DNA, but the equality of the specific activities of purified N- and K-DNA from cells labeled with [^{3}H]thymidine for 3 days implied that the labeling of the K-DNA also was following a pulse-chase pattern.

Similar results have been obtained independently by Gutteridge & Al Chalabi (5).

REFERENCES

1. Boyce, R. P. & Setlow, R. B. 1962. A simple method of increasing the incorporation of thymidine into the deoxyribonucleic acid of *Escherichia coli*. *Biochim. Biophys. Acta* **61**, 618-20.

2. Crawford, L. V. 1958. Thymine metabolism in strains of *Escherichia coli*. *Biochim. Biophys. Acta* **30**, 428-9.

3. Friedkin, M. & Roberts, D. 1954. The enzymatic synthesis of nucleosides. I. Thymidine phosphorylase in mammalian tissue. *J. Biol. Chem.* **207**, 245-56.

4. ——— & ——— 1954. The enzymatic synthesis of nucleosides. II. Thymidine and related pyrimidine nucleosides. *J. Biol. Chem.* **207**, 257-66.

5. Gutteridge, W. & Al Chalabi, K. 1973. Thymidine phosphorylase activity in *Crithidia fasciculata*, *in* de Puytorac, P. & Grain, J., eds., *Progress in Protozoology, Proc. 4th Int. Cong. Protozool.*, Sept., 1973, Université de Clermont, Clermont-Ferrand, 164.

6. Kidder, G. W. & Dutta, B. N. 1958. The growth and nutrition of *Crithidia fasciculata*. *J. Gen. Microbiol.* **18**, 621-35.

7. Kit, S. 1970. Nucleotides and nucleic acids, *in* Greenberg, D. M., ed., *Metabolic Pathways*, Academic Press, New York, **4**, 69-275.

8. Manson, L. A. & Lampen, J. O. 1950. Metabolism of deoxyribosides in *Escherichia coli*. *Fed. Proc.* **9**, 397.

9. Rachmeler, M., Gerhart, J. & Rosner, J. 1961. Limited thymidine uptake in *Escherichia coli* due to an inducible thymidine phosphorylase. *Biochim. Biophys. Acta* **49**, 222-5.

10. Razzell, W. E. & Khorana, H. G. 1958. Purification and properties of a pyrimidine deoxyriboside phosphorylase from *Escherichia coli*. *Biochim. Biophys. Acta* **28**, 562-6.

11. ——— & Casshyap, P. 1964. Substrate specificity and induction of thymidine phosphorylase in *Escherichia coli*. *J. Biol. Chem.* **239**, 1789-93.

12. Trager, W. 1957. Nutrition of a hemoflagellate (*Leishmania tarentolae*) having an interchangeable requirement for choline or pyridoxal. *J. Protozool.* **4**, 269-76.

13. Zimmerman, M. & Seidenberg, J. 1964. Deoxyribosyl transfer. I. Thymidine phosphorylase and nucleoside deoxyribosyltransferase in normal and malignant tissues. *J. Biol. Chem.* **239**, 2618-21.

14. ——— & ——— 1964. Deoxyribosyl transfer. II. Nucleoside: pyrimidine deoxyribosyltransferase activity of three partially purified thymidine phosphorylases. *J. Biol. Chem.* **239**, 2622-7.