INTERCONVERSION OF THYMINE AND THYMIDINE IN A THYMINE REQUIRING STRAIN OF BACILLUS SUBTILIS

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SUMMARY

In a B. subtilis Thy^- strain, thymidine is rapidly converted into thymine and, at the steady state, the pool size of thymidine is very small as compared to that of thymine. Consequently when such strain is used for pulse incorporation experiments with labelled thymidine paradoxical results are obtained. A quantitative estimation of the rate of DNA synthesis can only be obtained by thymine pulses or by cumulative incorporation experiments. We also present evidence that, during a short pulse, thymidine is mainly utilized for replicative DNA synthesis.

The metabolism of thymidine and thymine in bacteria has been extensively studied mainly in Escherichia coli K12 (1). Thy^+ strains of this organism are unable to utilize the exogenously added thymine, whereas thymidine is incorporated only for a very short time since it is rapidly degraded to thymine (2-6). Successive experiments have shown that the utilization of thymine depends on the presence of a pool of deoxyribose-1-phosphate (7-8); in fact when deoxyribonucleosides are added to the medium (as donor of deoxyribosyl residues), exogenous thymine and thymidine are extensively incorporated into DNA (4-6). The relevant reaction is the following:

$$\text{deoxyribose-1-P} + \text{Thy} \xrightleftharpoons{\text{(Thymidine phosphorylase)}} \text{dThd} + \text{Pi}$$

The thymidine phosphorylase of E. coli (E. C. 2.4.2.4.) is an inducible enzyme which catalyzes the degradation of thymidine to thymine and can also catalyze the Thy → dThd conversion provided a pool of deoxyribosyl-1-P is available. Wild type E. coli strains are unable to utilize exogenous thymine since they have a very small deoxyribose-1-P pool. Thy^- strains (defective in thymidylate synthetase) incorporate exogenous thymine (as well as thymi-

dine) very efficiently since an increased catabolism of deoxyribonucleotides, provides a higher supply of deoxyribose-1-P (7-9).

The information available on thymine metabolism in Bacillus subtilis is more limited, but the situation seems to be similar to that found in E. coli. Exogenous thymine is not appreciably incorporated by Thy^+ strains, whereas thymidine incorporation into DNA lasts only a few minutes and is then reduced to zero (10); this is due to the rapid degradation of dThd to Thy, probably through the action of thymidine phosphorylase.

A Thy^- mutant which requires either thymine or thymidine (indicating that the thymidine phosphorylase is active), has been isolated in B. subtilis (11). Subsequently it was found (12, 13) that the thymine dependence in this mutant is determined by two mutations thy A and thy B which must be simultaneously present in order to confer a Thy^- phenotype and both of which probably affect the thymidylate synthetase activity.

The availability of such a mutant is extremely useful for an unambiguous quantitative determination of DNA synthesis in vivo, by the measurement of cumulative incorporation of labelled precursors; the use of such strains for pulse experiments, to measure transient rates of DNA synthesis, can on the contrary give paradoxical results if the rates of interconversion of the two precursors (Thy and dThd) are not taken into account.

We present here data that describe the possible artifacts arising in this type of experiments, and we indicate the way to avoid them.

MATERIALS AND METHODS

Media and Strains. Penassay broth: antibiotic medium 3 (Difco); enriched minimal medium: the minimal medium of Spizizen (14) enriched with 0.2% casaminoacids (Difco), 0.05% yeast extract (Difco) and thymine or thymidine as indicated in the text.

Strain PB 566/1 (thy- A, thy B) derivative of SB 566 (thy a, thy b, trp C2; J. Lederberg) was used in all experiments. Spores were prepared and germinated as already described (15).

Cumulative and pulse incorporation. The strain PB 566/1, grown overnight in Penassay broth supplemented with 50 µg/ml of thymidine or thymine, was diluted 1:100 at 35° in enriched minimal medium supplemented with thymidine (5 µg/ml, 10 µCi/ml) or thymine (10 µg/ml; 15 µCi/ml) respectively.

At various times 0.1 ml aliquots were removed and precipitated with 1 ml cold 5% TCA. A parallel culture was grown in the presence of cold dThd (or Thy) but without [^{3}H]dThd (or [^{3}H] Thy) and at various times 0.1 ml ali-

quots were removed and pulsed for 5 min with thymidine at 10 μCi/ml (or thymine at 15 μCi/ml). The pulses were stopped by the addition of 1 ml of cold 5% TCA. All the samples were filtered on Whatman GF/C filters and counted in a Packard scintillation counter.

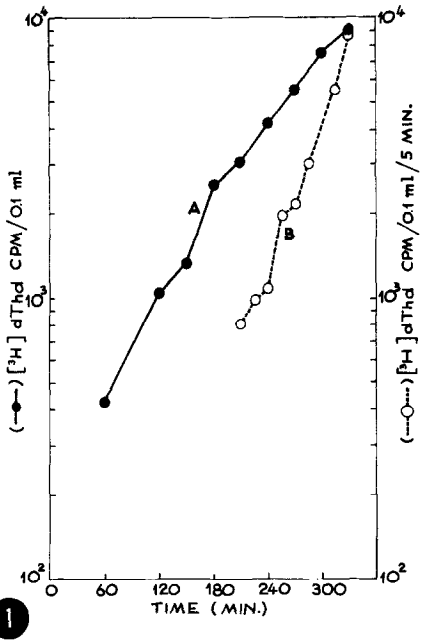
Extraction of soluble pools and chromatography. Samples of the culture (0.5 ml) labelled as described above were centrifuged and the cells resuspended in 50 μl of 0.15M NaCl. 25 μl of 4M formic acid were then added, and, after 30 min in ice, the suspension was centrifuged 15 min at 10,000 x g at 4°. 30 μl aliquots of the supernatant were applied on Whatman n°1 paper together with appropriate amounts of cold thymidine and thymine as absorbance markers. A descending chromatography was then performed using the following solvent: three parts of 1M ammonium acetate (adjusted to pH 7.5 with NH_4OH) plus seven parts of ethanol 95% (v/v). After 24 hours the paper was dried and the spots (visualized with a 254 nm UV lamp) were cut out and divided into several slices, perpendicularly to the direction of flow at 0.3 cm intervals, in order to optimize the resolution of the two spots. The paper slices were then dried and counted separately in a Packard liquid scintillation counter.

RESULTS AND DISCUSSION

The strain PB 566/1 is routinely used for the quantitative determination of DNA replication by measuring the incorporation of radioactive thymidine in the DNA on the assumption that the specific activity of thymidine in the DNA is the same as in the medium.

In the experiment shown in Fig. 1, Thy^- cells were grown in enriched minimal medium containing 5 μg/ml of thymidine: in one case (curve A: cumulative incorporation) [^{3}H] dThd was added at time zero at the specific activity of 2 μCi/μg and the samples were taken at the indicated times. In the second case (curve B: pulse incorporation) cells were grown as before but without radioactive label, and, at the indicated times, aliquots of the cultures were pulsed with [^{3}H] dThd at 10 μCi/ml for 5 min (final specific activity 2 μCi/μg). During the 5 min pulse much more radioactivity was incorporated into the DNA than expected from the cumulative data, despite the fact that the initial specific activity of [^{3}H]dThd was the same in the two cases. The discrepancy between the cumulative and the pulse incorporations increases with time and with the total cell mass so that at 330 min the amount of label incorporated was equal to that incorporated in a 5 min pulse. When the same kind of experiment was performed with [^{3}H] Thy no discrepancy was observed between the two types of incorporation (Fig. 2, curves A and B).

These results strongly indicate that, also in this strain, thymidine is



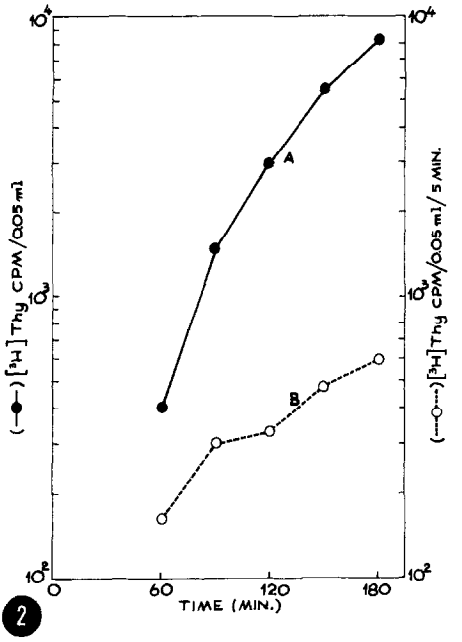


Fig. 1. Cumulative and pulse incorporation of [^{3}H] dThd during vegetative growth of PB 566/1 cells. An overnight culture at 30° in Penassay broth containing thymidine (50 µg/ml) was diluted 1:100 at 35° in enriched minimal medium containing thymidine (5 µg/ml). A) (cumulative incorporation); [^{3}H] dThd (10 µCi/ml) was added at time t = 0 min; samples were taken at the indicated times and acid precipitated to measure the radioactivity incorporated in the DNA. B) (pulse incorporation); at the indicated times aliquots of the culture were pulsed for 5 min with [^{3}H] dThd (10 µCi/ml) and processed as in A).

Fig. 2. Cumulative and pulse incorporation of [^{3}H] Thy during vegetative growth of PB 566/1 cells. An overnight culture at 30° in Penassay broth plus thymine (50 µg/ml) was diluted 1:100 at 35° in enriched minimal medium containing thymine (10 µg/ml). A) [^{3}H] Thy (15 µCi/ml) was added at time t = 30 min, samples were taken at the indicated times and acid precipitated to measure the radioactivity incorporated in the DNA. B) At the indicated times aliquots of the culture were pulsed for 5 min with [^{3}H] Thy (15 µCi/ml) and processed as in A).

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massively converted into thymine and that, at the steady state, the thymidine pool is very small as compared to the thymine pool. This conversion would have no effect on the cumulative incorporation which would in any case measure the increase of the amount of DNA. On the contrary, the amount of [^{3}H] dThd incorporated during a short pulse, would be strongly affected by the ratio between the thymine and thymidine pools in the cell.

Our results are therefore probably due to a progressive increase of the

TABLE I

Thymine and thymidine pool size in B. subtilis (566/1)

Samples	% of the total[a]	
	Thy	dThd
[^{3}H] Thy Batch	99	1
[^{3}H] dThd Batch	12	88
Growth in dThd (I)[b]	75	25
Growth in dThd (II)[b]	85	15
Growth in Thy[c]	91	9

a) The relative amounts of dThd and Thy were measured by dividing the chromatography spots as described in Materials and Methods and by calculating the area under the resulting peaks.
b) (I) sample taken at 210 min (Fig. 1); (II) sample taken at 330 min (Fig. 1).
c) Sample taken at 180 min of growth (Fig. 2).

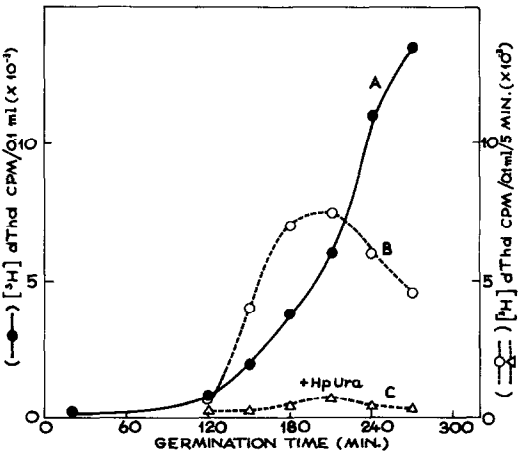

Fig. 3. Cumulative and pulse incorporation of [^{3}H] dThd during germination of PB 566/1 spores. Spores were prepared as described in Materials and Methods and germinated at 35° in enriched minimal medium containing 5 µg/ml of thymidine. A) [^{3}H] dThd was added at time t = 0 min at the specific activity of 2 µCi/µg, samples were taken at the indicated times and acid precipitated to measure the radioactivity incorporated in the DNA. B) At the indicated times aliquots of the culture were pulsed for 5 min with [^{3}H] dThd (10 µCi/ml) and processed as in A). C) Same as in B) but in the presence of 0.2 mM HpUra.

specific activity of [^{3}H] dThd in the pulses resulting from a decrease of the pool size of thymidine during the growth.

This hypothesis has been tested directly by measuring the pool size of thymine and thymidine during the exponential growth. The base and the nucleoside were extracted and separated as described in Materials and Methods. The results shown in Table I demonstrate that, during the exponential growth in the presence of exogenous thymidine, the intracellular pool of thymidine decreased to the advantage of the thymine pool which progressively increased. When cells were grown in the presence of thymine, only a small fraction of it (6-10%) was converted into thymidine. The conversion of thymine into thymidine is probably limited by the amount of endogenous deoxyribose-1-P available.

No differences were observed between the Thy and dThd pools inside and outside the cells (data not shown).

The effect described above was observed both in exponentially growing cells and in germinating spores. In this last case, as Fig. 3 shows, the effect is particularly dramatic probably because the experiment was started with a high cell concentration ($\simeq 10^8$ spores/ml) and because DNA synthesis starts rather late thus allowing time for the dThd → Thy conversion.

In this experiment it was also observed that the [^{3}H] dThd incorporation during the 5 min pulse is totally inhibited by 6-(p-hydroxyphenylazo)-uracil (HpUra) a specific inhibitor of DNA polymerase III (16), thus indicating that the [^{3}H] dThd incorporation during the pulse is due to replicative DNA synthesis. The fact that [^{3}H] dThd pulses are sensitive to HpUra (see Fig. 3) seems to rule out the possibility of thymidine being mainly used for repair synthesis as opposed to thymine being preferentially used for replicative DNA synthesis (17). This possibility is further weakened by the finding that thymine is partially converted into thymidine (see Table I); it is thus likely that thymidine is in fact the precursor of dTTP.

One important conclusion which emerges from this work is that although thymidine and thymine work equally well for the continuous labelling of DNA, thymidine pulses can not be used to quantitatively estimate the rate of DNA synthesis in a Thy$^-$ *B. subtilis* strain. Therefore for this kind of experiments thymine pulses should always be used.

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