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Determining [^{3}H]Thymidine Incorporation into Bacterioplankton DNA: Improvement of the Method by DNase Treatment

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Determination of [^{3}H]thymidine incorporation into bacterial DNA versus other macromolecules is usually achieved by NaOH and hot trichloroacetic acid hydrolysis. This procedure was found not to be specific enough. An alternative method founded on DNase treatment is proposed. Under the new method, the fraction of thymidine incorporated into DNA ranged from 10 to 83%.

Determining the rate of bacterial DNA synthesis is conceptually a very elegant approach for estimating bacterial production in aquatic environments. Several procedures have been proposed for this purpose, including $^{32}PO_4$ (4, 9) and [^{3}H]adenine (5, 7, 9) incorporation measurements. However, the method of [^{3}H]thymidine incorporation into cold-trichloroacetic acid (TCA)-insoluble material, as first proposed by Fuhrman and Azam (3), is now the most widely used field procedure because of its experimental simplicity.

One possible drawback to this procedure is the variable nonspecific labeling of other macromolecules besides DNA during incubation of the sample in the presence of [*methyl*-^{3}H]thymidine. For correctly converting thymidine incorporation rates into production rates, the fraction of label incorporated into DNA with respect to other macromolecules must be determined. Several authors have discussed this point (3, 4, 6, 8, 14) and proposed a biochemical fractionation procedure on the basis of 0.5 N NaOH (60°C) and 5% TCA (100°C) hydrolysis. The former treatment is supposed to remove RNA, whereas the latter is assumed to hydrolyze all nucleic acids. The DNA fraction is then calculated as the difference between the NaOH-treated and the hot-TCA-insoluble fractions. A wide range of percentages was found by these authors, ranging from 70 to 95% in marine environments to 27 to 80% in freshwater systems (1, 3, 4, 6, 8, 10, 14, 15).

However, the justification for the acid and base hydrolysis procedure seems rather poor. Most authors, when not cross-citing themselves, refer either to Luria (11) or to Munro and Fleck (12). Luria quotes Davidson (2) who simply cites Schneider (16). The paper by Munro and Fleck (12), on the other hand, is a comprehensive review of pre-1965 work on determination of nucleic acids from animal and plant tissues. It appears from this review that "the use of hot acid as a method of extracting DNA. . .has not received sufficient critical examination to indicate the optimum conditions" and that "the conditions for full recovery vary from tissue to tissue." The authors also pointed out that some protein extraction occurs with hot acid.

To check the acid and base hydrolysis procedure, we subjected commercial [2-^{14}C]thymidine DNA from *Escherichia coli* (Amersham Corp.) and ^{14}C-methylated bovine serum albumin (Amersham) to 5% TCA hydrolysis at 100°C and monitored for 2 h the remaining cold-TCA-insoluble fraction. Hot-5%-TCA hydrolysis of labeled DNA proceeds at a rapid rate up to a point at which the residual amount of precipitable material remains stable (Fig. 1A). This behavior can be easily explained on the basis of the well-known mechanism of acid DNA hydrolysis (17), which first involves the rupture of β-osidic bonds between puric bases and deoxyribose, followed by breakage of the pentose-phosphate skeleton at the sites where the puric bases have been removed. The polypyrimidic sequences are kept intact and, if of sufficient length, remain precipitable.

In contrast, enzymatic treatment with DNase I leads to a complete hydrolysis of the same labeled DNA (Fig. 1A). DNase I (type III from Sigma Chemical Co.) is an endonu-

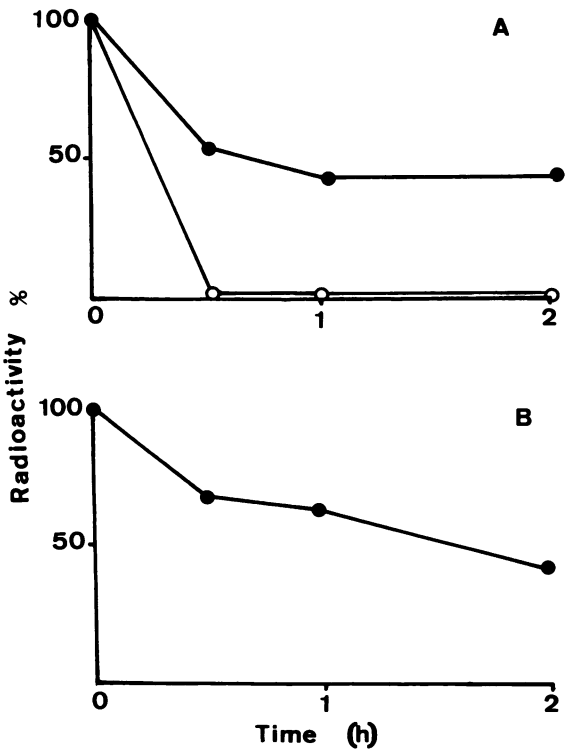

FIG. 1. (A) Hydrolysis kinetics of [2-^{14}C]thymidine DNA from *E. coli* (Amersham) in 5% TCA at 100% (●) and in the presence of 0.2 mg of DNase I per ml at 20°C (○). (B) Hydrolysis kinetics of ^{14}C-methylated bovine serum albumin (Amersham) in 5% TCA at 100°C. Results are expressed as percentage of radioactivity added.

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TABLE 1. Partition of [*methyl*-^{3}H]thymidine into macromolecules

Sample	% Partition of [*methyl*-^{3}H]thymidine into macromolecule[a]		
	DNA	RNA	Protein
Meuse River	38	28	41
	36	15	43
Belgian coastal zone	55	20	25

[a] Results expressed as percentage of total incorporation into cold-TCA-insoluble material.

clease which hydrolyzes the bond between phosphoric acid and hydroxyl 3′.

On the other hand, hot-acid treatment of labeled proteins results in a significant degradation of this material (Fig. 1B).

These observations at least cast some doubt on the above-mentioned results concerning thymidine partitioning into macromolecules. Only Witzel and Graf (18) proposed a different approach for determining specific DNA labeling, on the basis of nucleic acid separation by adsorption onto a hydroxyapatite column and subsequent elution with phosphate buffers of increasing molarity. The method, however, is tedious and impractical for large series of samples.

This prompted us to develop an alternative procedure for specifically determining tritiated thymidine incorporation into DNA versus other macromolecules. It involves cell disruption by sonication in the presence of Triton X-100 (13), followed by DNase digestion of the sample. The detailed experimental protocol is as follows.

After incorporation of tritiated thymidine, the sample (10 ml) is sonicated (5 min with a Braun Labsonic 2000 at 90 W) (using the needle probe) in an ice bath in the presence of 0.02% Triton X-100. After sonication, a 5-ml subsample is added to an equal volume of cold 10% TCA, allowed to stand for 10 min, and filtered through a 0.2-μm-pore-size Sartorius cellulose acetate filter. To the other 5-ml subsample, 1 mg of DNase I is added (Type III; Sigma); after 30 min of incubation at 20°C, 5 ml of cold 10% TCA is added, and the subsample is filtered as described above. Radioactivity associated with the two filters is determined by liquid scintillation spectrometry by using a Packard Instrument Co., Inc., Tri-Carb scintillation counter. The radioactivity in DNA is calculated as the difference between the radioactivity on the two filters. The same procedure can be used for determining RNA or protein labeling by simply replacing DNase with RNase (Boehringer Mannheim Biochemicals) or proteinase K (Boehringer Mannheim), which is specially used for the isolation of RNA and DNA.

In a few samples, all three treatments were applied in parallel. The results show that close to 100% of the radioactivity incorporated into cold-TCA-insoluble material is recovered in the DNA, RNA, and protein fractions (Table 1). This demonstrates the quantitativeness of the method used.

The DNase treatment was applied after [^{3}H]thymidine incorporation in a wide variety of natural water samples, collected from either freshwater or seawater environments. The results are summarized in Table 2. The percentage of incorporation into DNA ranges from 21 to 83% of total incorporation.

A much lower percentage of thymidine incorporation into DNA was recorded with Meuse river water samples, which had been allowed to incubate for several days to exhaust their organic substrates (although large amounts of mineral nitrogen remained in solution). In these starved bacterial populations, less than 10% of total thymidine incorporation occurs in DNA. It is likely that under conditions of organic carbon limitation, thymidine may serve more readily as a carbon source than as a DNA precursor. This extreme example shows how misleading results of thymidine incorporation into total cold-TCA-insoluble fraction can be when the fraction incorporated into DNA is not determined. We suggest that thymidine incorporation measurements be made in terms of incorporation into DNA instead of total TCA-insoluble fraction, unless a constant proportionality between those two fractions has been demonstrated in the environments and the conditions under study. The DNase procedure described here, because of its simplicity, can be routinely used for that purpose.

TABLE 2. Thymidine incorporation into bacterioplankton DNA, expressed as percentage of total incorporation in cold-TCA-insoluble material

Environment	Site	No. of determinations	% of total thymidine incorporated into DNA	
			Avg	Range
Mediterranean Sea (Spanish North coast)	Surface water	28	50	21–83
	Deep water (700 m)	2	54	43–65
North Sea (Belgian coast)	Surface water	3	75	66–82
River (Spain)	Ebro	17	45	26–79
	Llobregat	2	38	21–54
River (Belgium)	Meuse	4	35	30–38

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