A Proton Exchange between Purines and Water and Its Application to Biochemistry*

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ABSTRACT: Purine-containing compounds will exchange one proton with $[^3H]H_2O$ when heated in $[^3H]H_2O$. Such exchange allows the *in vitro* preparation of numerous highly labeled ^3H compounds of biochemical interest (*e.g.*, purine nucleosides and nucleotides, puromycin, and nucleic acids).

The resultant ^3H compounds are relatively stable at room temperature under neutral or acidic conditions and therefore lend themselves to many biochemical applications and assays.

The C_8 proton of purines and purine nucleosides readily exchanges with the deuterium of D_2O[1] during short periods of refluxing (Schweizer *et al.*, 1964; Fox, 1965). These observations suggest that many purine-containing compounds can be labeled with tritium by means of a similar exchange with $[^3H]H_2O$. Such exchange of tritium from $[^3H]H_2O$ with the C_8 proton of purines could provide a simplified procedure for labeling biologically significant purine-containing compounds and thereby prove useful in various biochemical assays. This paper reports the properties of purine C_8 proton exchanges with $[^3H]H_2O$ and demonstrates some of the practical uses of this reaction.

Experimental Procedures

High Vacuum Line Operation and Proton-Exchange Procedure. Meaningful ^3H proton exchanges from $[^3H]H_2O$ require the use of highly labeled $[^3H]H_2O$. Accordingly, a high vacuum line procedure was developed to minimize health hazards. In a typical experiment, 1–40 mg of previously neutralized (pH 7 with NaOH) and then lyophilized material is placed in the reaction vessel (H in Figure 1) using a long-stemmed funnel. The open arm of the reaction vessel is sealed into the vacuum line apparatus at point 1 and then constricted to about 4-mm i.d. at point 2. A glass-encased iron bar (2.5 × 0.6 cm) is placed inside the line at point 3. Then a sealed reaction vessel (J) containing 1.0–1.5 g of $[^3H]H_2O$ is sealed through its break-seal side arm into the line at point 3. This seal must allow contact of the iron bar with the break seal of the vessel containing $[^3H]H_2O$. The seals are checked for leaks by evacuation of the line (1–2 μ) and examination with a high-frequency induction coil (Electro Technic Products, Chicago, Ill.). $[^3H]H_2O$ (in vessel J) is then frozen, first in a Dry-Ice bath, then in liquid N_2, and the break seal is opened by manipulating the iron bar with a magnet. The complete system is then evacuated for 15–20 min. After evacuation, the transfer line (G) is isolated by closing the stopcock above the transfer line. The liquid N_2 bath is then removed from the vessel containing the $[^3H]H_2O$ (J) and placed on the reaction vessel (H) containing the solid material. The $[^3H]H_2O$ is quantitatively transferred into the vessel containing the solid by applying a warm water bath to the other vessel (J). This is followed by flaming of the empty vessel and the transfer line above the liquid N_2 bath. The reaction vessel containing the solid plus the $[^3H]H_2O$ is then removed from the system by sealing off the reaction vessel (still under vacuum and partially submerged in liquid N_2) at point 2.

The tritium-exchange reaction is facilitated by placing the sealed reaction vessel (containing the $[^3H]H_2O$ and the dissolved solid material) in a water bath maintained at 100° for varied periods of time. The $[^3H]H_2O$ is then transferred from the sample to another reaction vessel by the water-transfer procedure described above. Finally, readily exchangeable protons (*e.g.*, as on hydroxyl groups) are removed by dissolving the tritiated solid three times in a 1000-fold excess of H_2O followed by freeze drying.

The labeling of nucleotides and oligoribonucleotides by the above procedure requires careful control of the pH so as to avoid depurinization and other forms of degradation. Accordingly, such samples are dissolved in H_2O along with 15 μmoles of sodium phosphate buffer (pH 8.0) and then lyophilized to dryness within the reaction vessel before initiating the ^3H exchange from $[^3H]H_2O$.

Assay of the Rate of ^3H Exchange from $[^3H]$Purine

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[1] Abbreviations used: D_2O, water containing 100% deuterium in place of hydrogen; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazolyl)benzene; AMP and GMP, adenosine and guanosine monophosphates.

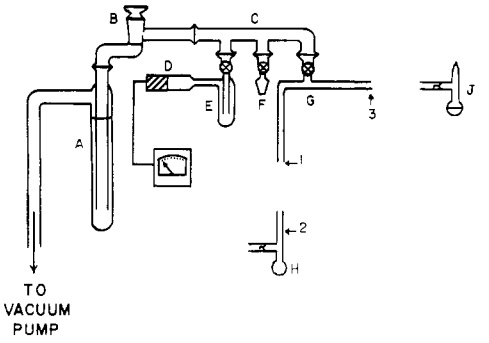

FIGURE 1: High vacuum transfer line set up. (A) Trap cooled by liquid N_2; (B) main stopcock; (C) side arm equipped with stopcock controlled outlets; (D) vacuum detector connected to vacuum meter (Fredericks Co., Bethayres, Pa.); (E) trap cooled by liquid N_2; (F) external opening for use during formation of glass connections; (G) transfer arm; (H) reaction vessel containing solid sample; and (J) reaction vessel containing $[^3H]H_2O$.

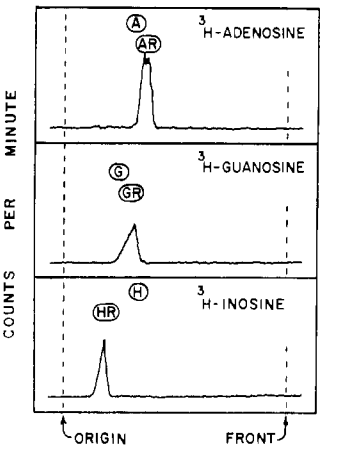

FIGURE 2: Isotopic homogeneity of [^{3}H]nucleosides after paper chromatographic separation. Aliquots of individual [^{3}H]nucleosides prepared by 5.5 hr of exchange with $[^3H]H_2O$ were applied to Whatman No. 1 paper (approximately 10^5 dpm/spot) along with standards of adenosine (AR), adenine (A), guanosine (GR), guanine (G), inosine (HR), and hypoxanthine (H). The [^{3}H]-adenosine, [^{3}H]guanosine, and appropriate standards were developed by ascending chromatography with isopropyl alcohol–HCl–H_2O (65 ml:16.7 ml:H_2O to 100 ml). The [^{3}H]inosine and appropriate standards were developed by ascending chromatography with 1-butanol saturated with water. Each chromatogram was then scanned for counts with a radiochromatogram scanner.

Nucleosides at 100° (pH 7).[2] The exchange of ^{3}H from [^{3}H]purine nucleoside to H_2O is measured by first dissolving 7–8 μmoles of [^{3}H]adenosine or [^{3}H]guanosine (both prepared by 5.5 hr of 100° exchange with $[^3H]H_2O$ at pH 7) in 75 ml of H_2O. A series of 2-ml aliquots of this solution are then sealed in vials and placed in a 100° water bath. At timed intervals, duplicate vials are removed and stored at −15° until opened for assay. Each sample (1 ml) is freeze dried and then washed free of readily exchangeable protons by redissolving it in 2 ml of H_2O followed by further lyophilization to dryness. The washed samples are finally dissolved in 1.0 ml of H_2O and 0.5-ml aliquots are removed and counted by liquid scintillation counting.

Assay of the Rate of ^{3}H Exchange from [^{3}H]Purine Nucleosides at 30° at Varied pH Values. Preparations of [^{3}H]adenosine and [^{3}H]guanosine (both prepared by 5.5 hr of 100° exchange with $[^3H]H_2O$ at pH 7) are adjusted to 2×10^{-5} M [^{3}H]nucleoside–0.02% NaN_3 in appropriate buffer (0.01 M glycine-HCl, pH 2.0; 0.01 M Tris-Cl, pH 7.5; and 0.01 M sodium phosphate buffer, pH 11.0). The solutions are maintained at 30°. Daily, duplicate 2-ml samples are removed, lyophilized to dryness, and stored at −15° before final solubilization in 2.0 ml of H_2O, removal of a 0.5-ml aliquot, and counting by liquid scintillation counting.

Radioisotope Counting Procedures. Aqueous 0.5-ml aliquots containing ^{3}H materials are counted by liquid scintillation counting after mixing with 6 ml of absolute ethanol and 8 ml of 0.8% PPO and 0.01% POPOP in toluene.

^{3}H materials present on paper after paper chromatographic or paper electrophoretic separations are scanned with a Packard Model 7200 radiochromatogram scanner or counted on the paper by liquid scintillation counting in 0.4% PPO and 0.01% POPOP in toluene.

Materials. $[^3H]H_2O$ (12–18 c/mole) was obtained from New England Nuclear Corp., Boston, Mass. Nucleosides and nucleotides (5′-phosphonucleosides) were obtained from Sigma Chemical Corp., St. Louis, Mo., and P-L Biochemicals, Milwaukee, Wis., respectively. Dinucleoside monophosphates were obtained from Gallard-Schlesinger Co., New York, N. Y. Puromycin dihydrochloride was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Cellulose MN Polygram 300 thin layer sheets were purchased from Brinkmann Instruments, Waterbury, N. Y.

Results

^{3}H Exchanges from $[^3H]H_2O$ into Nucleosides. Purine nucleosides can be labeled readily with ^{3}H as a result of heating with $[^3H]H_2O$ (pH 7) and subsequent lyophilization (see Experimental Procedures). The conditions specified cause little or no degradation of these nucleosides for [^{3}H]adenosine, [^{3}H]guanosine, and [^{3}H]inosine produced in this manner are isotopically homogeneous (*i.e.*, free of [^{3}H]purine when examined in three different paper chromatographic systems). Figure 2 shows examples of such assays.

[2] All instances of ^{3}H materials (*e.g.*, [^{3}H]adenosine) refer to compounds labeled with ^{3}H by exchange with $[^3H]H_2O$ as described in this paper.

TABLE I: ^3H Observed in Nucleosides After 5.5 hr of Exchange with $[^3H]H_2O$.[a]

Compound	μmoles Added at Start	μmoles Recovd	Dpm/μmoles	No. of Protons Exchanged/ Compd
		Expt I[b]		
Adenosine	3.48	2.92	1.00×10^7	0.99
Guanosine	1.63	1.37	0.983×10^7	0.97
Cytidine	1.92	1.61	$<2 \times 10^5$	
Uridine	1.97	1.65	$<2 \times 10^5$	
		Expt II[c]		
Cytidine	85.2		2.7×10^4	0.003
Uridine	84		2.8×10^4	0.003

[a] Mixtures containing known quantities of nucleosides as determined by A_{260} (expt I) or weights (expt II) were heated (100°) for 5.5 hr in $[^3H]H_2O$ (sp act. 9.10 c/mole as determined by direct counting). [b] In expt I, the ^3H nucleosides recovered after losses during transfer and lyophilizations were determined from the A_{260} of the combined sample (assuming per cent losses to be equal). Individual ^3H nucleosides were counted with standards on paper after separation by ascending paper chromatography (1% concentrated NH_4OH in 1-butanol saturated with H_2O) or paper electrophoresis (0.05 M ammonium formate, pH 3.2, 3 hr, 5°, 16 v/cm). [c] In expt II, 0.56 μmole of the combined [^3H]uridine and [^3H]cytidine (determined by A_{271}) was separated on cellulose MN Polygram 300 thin layer sheets by ascending chromatography in isopropyl alcohol–concentrated HCl–H_2O (65 ml:16.7 ml:H_2O to 100 ml); eluted quantitatively with 1 N HCl and then assayed for ^3H by liquid scintillation counting. The number of protons exchanged per compound represents the ratio, specific activity of the ^3H compound:specific activity of ^3H in $[^3H]H_2O$.

The ^3H-exchange reaction from $[^3H]H_2O$ is limited to the purine nucleosides. This is seen in the experiment of Table I wherein mixtures of nucleosides are subjected to the ^3H-exchange conditions at pH 7. Only the purine nucleosides retain appreciable ^3H label after removal of the readily exchangeable protons.

Rates of Exchange of ^3H from $[^3H]H_2O$ into Purine Nucleosides at pH 7. Experimental convenience dictates that the rate of exchange of protons between purine-containing compounds and water at 100° and pH 7 is best measured by examination of the rate of back-exchange (*i.e.*, from [^3H]purine-containing compounds into H_2O). Such analysis reveals different first-order reaction rates for the ^3H exchange between [^3H]-adenosine and [^3H]guanosine and H_2O (Figure 3). These assays also indicate that equilibrium of ^3H protons between $[^3H]H_2O$ and [^3H]adenosine or [^3H]-guanosine is greater than 98% complete after 5 hr. This is substantiated by the observation (Table I) that the extent of ^3H label within various purine nucleosides after 5.5 hr of exchange treatment at 100° (pH 7) is proportional to the quantity of purine nucleoside originally present.

Analyses of the extent of labeling of adenosine and guanosine after 5.5 hr of heating at 100° (pH 7) in an excess of $[^3H]H_2O$ of a known specific activity are in agreement with the concept that only one proton of the purine-containing compounds is exchanging with the $[^3H]H_2O$. Previous work of others (Schweizer *et al.*, 1964; Fox, 1965) would dictate that this proton is on the 8 position of the purine moiety.

Effect of pH upon the Rate of Exchange of ^3H from [^3H]Nucleosides into H_2O. An analysis of the rate of exchange of ^3H from [^3H]adenosine and [^3H]guanosine into H_2O under sterile conditions at 30°, as a function of pH, provides further information about this proton exchange of purines. As seen in Figure 4, the back-exchange of ^3H from [^3H]purine nucleosides is minimal at neutral or acid pH values while the loss of ^3H from [^3H]guanosine is greatly accelerated at

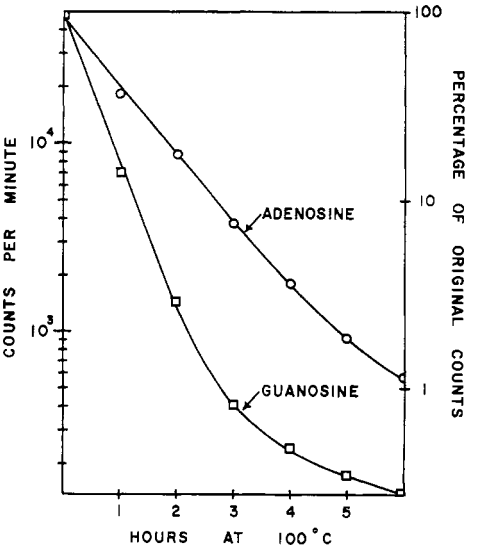

FIGURE 3: Rates of exchange of ^3H from [^3H]adenosine and [^3H]guanosine into H_2O at 100°.

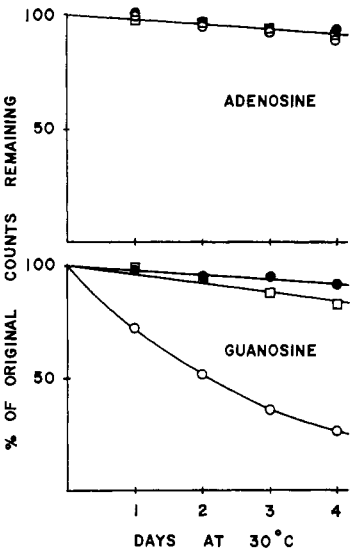

FIGURE 4: Rates of exchange of ^3H from [^3H]adenosine and [^3H]guanosine into H_2O at 30°. Assayed as described in Experimental Procedures. Incubations (30°) are in 0.01 M glycine-HCl, pH 2.0 (●—●); 0.01 M Tris-Cl, pH 7.5 (□—□); and 0.01 M sodium phosphate, pH 11.0 (○—○).

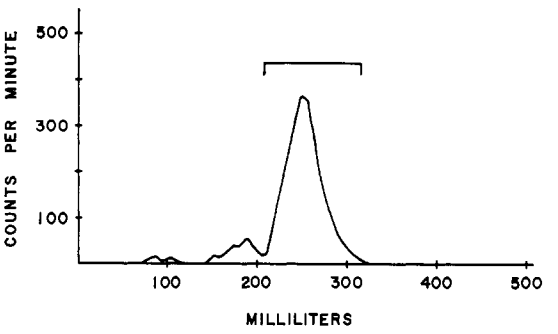

FIGURE 5: Gel filtration analysis of crude [^3H]puromycin. Crude [^3H]puromycin (23.65×10^{-9} mole) (determined by A_{260}) containing 37.8×10^4 dpm was added to a 26 × 2.5 cm column of polyacrylamide gel P-2 (Bio-Rad Laboratories, Richmond, Calif.) preequilibrated with 0.01 M $(NH_4)_2CO_3$–0.05% sodium dodecyl sulfate. The column was eluted (25°) with equilibration fluid (0.2 ml/min) while collecting 2.5-ml fractions. Aliquots (0.5 ml) of every third fraction were assayed for ^3H by liquid scintillation counting. The bracketed zone indicates the region in which commercial puromycin elutes as a peak of A_{260}-absorbing material paralleling the major peak containing the ^3H label.

alkaline pH. Extreme alkaline conditions (0.35 N NaOH, 18 hr, 37°) will cause extensive loss of ^3H from both [^3H]adenosine and [^3H]guanosine.

The data of Figure 4 also point out a potential use of this exchange procedure. The labeling of purine-containing compounds by ^3H exchange from [^3H]H_2O is only of practical value if the resultant ^3H materials retain their ^3H label for satisfactory periods of time under commonly used laboratory conditions. The observed 30°, aqueous solution, neutral and acid pH half-lives for these compounds of at least 2 weeks suggest that such [^3H]purine-containing compounds are readily applicable in many common biochemical assays or procedures. [^3H]Purine-containing compounds that have been freed of H_2O by lyophilization appear to decompose at a rate close to the normal half-life of ^3H.

^3H Exchange between [^3H]H_2O and Various Purine-Containing Compounds. In theory, this ^3H exchange from [^3H]H_2O can be used to label any purine-containing compound if that compound is stable under the pH conditions and elevated temperature employed during the exchange treatment. Accordingly, the labeling of various purine-containing compounds by means of ^3H exchange from [^3H]H_2O was undertaken. Treatment of AMP, GMP, and dinucleoside monophosphates (Ado-3′-P-5′-Ado, Ado-3′-P-5′-Guo, and Guo-3′-P-5′-Ado) with [^3H]H_2O at pH 8 and 100° for 5.5 hr results in the corresponding ^3H materials wherein greater than 95% of the ^3H resides in the expected ^3H nucleotide or ^3H dinucleoside monophosphate as assayed by paper electrophoretic separation (Lohrmann *et al.*, 1966) and subsequent radiochromatogram scanning.

The purine-containing antibiotic, puromycin (6-dimethylamino-9-[3-deoxy-3-(*p*-methoxy-L-phenylalanylamino)-β-D-ribofuranosyl]purine) requires longer periods of 100° heating (pH 7) with [^3H]H_2O in order to exchange one proton of puromycin with a ^3H of [^3H]H_2O. When puromycin is heated (100°) for 12 hr in [^3H]H_2O at pH 7, one obtains crude [^3H]puromycin containing greater than 90% of the theoretical one ^3H per purine. Such puromycin contains some minor impurities as shown by gel filtration analysis on polyacrylamide gel P-2 (Figure 5). When the purity of this crude [^3H]puromycin is determined by *in vitro* comparison of the inhibitory properties of the crude [^3H]-puromycin and pure puromycin toward poly U directed polyphenylalanine synthesis (Nishizuka and Lipmann, 1966), assays at various inhibitor concentrations show the [^3H]puromycin to be 70% as effective as untritiated puromycin.

Discussion

The mechanism of the proton exchange between the C_8 proton of purine-containing compounds and protons of water is not established. Our data would suggest an alkali-mediated first-order reaction mechanism. Other possible mechanisms include mechanisms that utilize the carbanion–carbene intermediate first proposed by Fox (1965). The correct mechanism of this purine C_8 proton exchange, when determined, may well explain the earlier observation that adenine can exchange

approximately one proton with D_2O or $[^3H]H_2O$ in the presence of a reduced platinum catalyst (Eidenoff and Knoll, 1953).

The purine-labeling procedure presented here suggests several practical applications. First the procedure provides a quick and simple method for the preparation of biochemically useful [^{3}H]purine-containing compounds. The ^{3}H-labeling procedure involves the eventual exchange of one proton of purine-containing compounds with one proton of $[^3H]H_2O$. It is therefore theoretically possible to prepare [^{3}H]purine-containing compounds with a specific activity equal to that of ^{3}H in the available $[^3H]H_2O$ supply. Several suppliers now offer $[^3H]H_2O$ in the range of 200 c/mole. Therefore, using such $[^3H]H_2O$, one can theoretically obtain [^{3}H]-purine-containing compounds containing up to 100 c of ^{3}H/mole.

Second, this purine-labeling procedure is potentially useful as a diagnostic tool for the detection of minute quantities of purine-containing compounds. The theoretical limit of the ^{3}H labeling is the specific activity of the ^{3}H in $[^3H]H_2O$. Thus, if one uses 200 c/mole of $[^3H]H_2O$, one can label as little as 10 $\mu\mu$moles of purine-containing compound with 2.2×10^3 dpm in the 10 $\mu\mu$moles of sample. Purine-containing compound (10 $\mu\mu$moles) is equal to approximately 1% of the current limit of detection of purines by conventional ultraviolet spectroscopy. Thus, this labeling procedure, coupled with subsequent chromatography, greatly increases the limit of detection and analysis of purine-containing compounds.

A third potential feature of this ^{3}H-labeling procedure is application of the method to detect the relative ratios of the specific purines present in minute quantities of oligonucleotides. The ^{3}H labeling of nucleosides is essentially purine specific and results, at equilibrium, in a distribution of ^{3}H among the purine nucleosides that is equal to the concentrations of purines present (Table I). Therefore, total labeling (*e.g.*, exchange from $[^3H]H_2O$ for 5.5 hr at 100°, pH 7) of the nucleosides or nucleotides derived from a given oligonucleotide by enzymatic or alkaline degradation, followed by chromatographic separation of the nucleosides or nucleotides should yield [^{3}H]purine nucleosides or nucleotides containing ^{3}H label to an extent equal to the concentrations of purines originally present.

Such an assay would prove extremely useful when applied to oligoribonucleotides obtained by exhaustive treatment with ribonuclease T_1. Such oligoribonucleotides will usually contain guanosine only in the 3′-terminal residue (Sato-Asano, 1959). The quantity of ^{3}H present in the various purine nucleosides or nucleotides, after degradation and subsequent labeling with ^{3}H by exchange from $[^3H]H_2O$, should be equal to, or some multiple of, the ^{3}H present in the [^{3}H]guanosine or [^{3}H]guanosine 3′-phosphate. The absolute quantity of other purines originally present could be obtained by dividing the ^{3}H disintegrations per minute present in the nucleoside or nucleotide in question by the disintegrations per minute present in the [^{3}H]guanosine or [^{3}H]guanosine 3′-phosphate.

The capability of this ^{3}H-labeling procedure to detect minute quantities of purine nucleosides coupled with its potential use to determine the relative ratio of purine nucleosides present in an oligonucleotide may prove particularly useful in nucleotide sequence work on hard to obtain homogeneous m- and sRNAs.

Lastly, this purine-labeling procedure may prove useful in nucleic acid hybridization work. Cellular biology and chemical taxonomy studies employing nucleic acid hybridization techniques, particularly those employing DNAs and RNAs of large animals, have long been limited by the difficulties involved in the *in vivo* preparation of DNAs and RNAs sufficiently labeled with radioisotope. The procedure presented here allows the *in vitro* labeling of any nucleic acid and therefore offers a route around this difficulty.

This purine-labeling procedure will probably prove particularly applicable to nucleic acid hybridization studies through its ability to yield *in vitro* labeled [^{3}H]-DNAs. The higher rate of C_8 proton exchange from purine-containing compounds in alkali (Figure 4) and the known stability of DNAs in alkali suggests that highly labeled and minimally degraded [^{3}H]DNA can be prepared by ^{3}H exchange from alkaline $[^3H]H_2O$. The rapid proton exchange exhibited by guanosine in alkali suggests that extensively labeled [^{3}H]DNA may even be prepared in alkaline $[^3H]H_2O$ at lower temperatures as well as shorter times. Subsequent hybridization of such highly labeled [^{3}H]DNAs under conditions of neutral pH and lower temperatures should yield readily detectable [^{3}H]DNA:RNA and [^{3}H]DNA:DNA hybrids for the level of back-exchange (*i.e.*, ^{3}H from [^{3}H]DNA into H_2O) will be reduced at the short times, lower pH values, and lower temperatures commonly used for hybridization of nucleic acids.

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