IN VITRO TRITIUM LABELLING OF DNA*

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Current day biochemistry encompasses many applications involving radioactively labeled nucleic acids. To date, most radioactive nucleic acids employed in such experiments have been obtained from in vivo syntheses. Various chemical treatments of intact nucleic acids, for example, proton exchange with $^3H_2$ gas (Wilzbach, 1957; Borenfreund et al., 1959), derivitization with radioactive halogen forms (Brammer, 1963; Ascoli and Kahan, 1966) and methylation with radioactive dimethyl sulfate or diazomethane (Brimacombe et al., 1967) have also yielded radioactive DNAs and RNAs. These methods, although useful, suffer from the fact that they either derivitize the native nucleic acid or require large scale commercial operations to achieve labeling.

The recently described exchange of ^3H protons from ^3H_2O into purine containing compounds (Shelton and Clark, 1967) offers a simplified method of preparation of ^3H-nucleic acids without derivitization of the original material. This paper reports the successful preparation of ^3H-DNA by ^3H proton exchange from ^3H_2O and establishes that such ^3H-DNA retains its specificity during DNA-DNA hybrid formation.

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MATERIAL AND METHODS

Isolation of DNAs. The following procedure, performed at room temperature unless stated otherwise, yields DNA optimal for hybridization assays.

Seven grams of SSC† washed, log phase E coli B cells were lysed in 150 ml of 0.15 M NaCl, 0.1 M EDTA, 1% sodium dodecylsulfate, 1% sodium triisopropylnaphthalene sulfonate by quick freezing and thawing. This suspension was made 0.5 M with $NaClO_4$, incubated for 10 minutes, deproteinized by successive treatments with $CHCl_3$-isoamyl alcohol (Marmur, 1961) until the aqueous solution yielded a clear interface. The nucleic acids were then collected by precipitation with 1 volume of ethanol and centrifugation. The resultant pellet was dissolved in 60 ml of 1/100 SSC and treated with RNAse and pronase (Gillespie and Spiegelman, 1965) before being deproteinized by 3 additional cycles of $CHCl_3$-isoamyl alcohol treatment. The DNA was then precipitated by addition of a 1/10 volume of 2 M potassium acetate and a volume of ethanol and stored (4^0) in a 1/100 SSC solution (0.6 mg/ml) until use.

A solution of 0.8 mg salmon sperm DNA (Sigma Chemical Company) per ml of 1/100 SSC was subjected to the above $CHCl_3$-isoamyl alcohol, RNAse, pronase, etc. treatments before use.

Preparation of ^3H-DNA for use in Hybridizations. A frozen 2 ml aliquot of purified DNA (0.6 mg/ml 1/100 SSC) was sealed in the ^3H_2O transfer apparatus of Shelton and Clark (1967), lyophilized to dryness and mixed with 1 ml of ^3H_2O (90 curies/mole, Nuclear Chicago Corp.) by high vacuum transfer. The sealed DNA-^3H_2O sample was then heated for 20 minutes at 100^0, to facilitate ^3H exchange, before removal of the ^3H_2O by high vacuum transfer. Readily exchangeable ^3H protons were then back exchanged from the ^3H-DNA by 3 cycles of solubilization in 2 ml aliquots of 1/100 SSC

† SSC, standard saline citrate containing 0.15 M NaCl, 0.015 M sodium citrate, pH 7; other concentrations are multiples (e.g., 6X SSC) or fractions (e.g., 1/100 SSC) of this concentration.

followed by precipitation with 1/10 volume of 2 M potassium acetate and 1 volume of ethanol. This ^{3}H-DNA was stored overnight (4°) in distilled H_2O (150 mg/ml) before fragmentation (30 min, 0°) in a Mullard ultrasonicator. The fragmented ^{3}H-DNA was then denatured by a 3 min, 25°, pH 13 treatment; diluted with 1 volume of 6X SSC and adjusted to pH 7 for use as the solution phase in the hybridization assay.

DNA--^{3}H-DNA Hybridization Assay. DNAs were denatured with alkali and bound to filters in the presence of 6X SSC according to Gillespie and Spiegelman (1965). Dry filters containing 50/mg of DNA were placed into scintillation vials and preincubated for 5 hours (65°) with 5 ml Denhardt's (1966) hybridization media before addition of varying aliquots of ^{3}H-DNA (see above). The 65° incubation was continued for 19 hours after which unhybridized ^{3}H-DNA was washed from the filters by the procedure of Warnaar and Cohen (1966). The dried (15 min, 90°) filters were then analyzed by liquid scintillation counting.

RESULTS

DNA can be readily labeled with ^{3}H by heat (100°) facilitated exchange of ^{3}H from ^3H_2O. For example, 20 min of heating E. coli DNA, in the presence of ^3H_2O (90 curies ^{3}H/mole), followed by removal of readily exchangeable ^{3}H protons, yields ^{3}H-DNA containing 3880 cpm/μg. This extent of labeling is only 62% of that expected from the purine content of the DNA (Belorzersky and Spirin, 1960), the known rates of proton exchange for purine compounds (Shelton and Clark, 1967) and the specific activity of the ^3H_2O used. This reduced rate of exchange probably reflects the altered electronic state of the purines in polymeric DNA.

The biochemical functionality of such ^{3}H-DNA can be conveniently assayed by means of Gillespie and Spiegelman's (1965) membrane filter assay of hybrid formation. This assay involves binding alkali denatured, single stranded DNA to a filter and subsequent challenging of this DNA with a solution of single stranded, fragmented ^{3}H-DNA. If the soluble ^{3}H-DNA

contains sufficient structural homology with the filter bound DNA, filter bound DNA--^{3}H-DNA hybrids are formed.

As seen in Figure 1, DNA--^{3}H-DNA hybrid formation does occur when filter bound E. coli DNA is challenged with an annealing solution of E. coli ^{3}H-DNA prepared by ^{3}H exchange from ^3H_2O. Further, such E. coli ^{3}H-DNA has not lost its biological specificity for the heterologous control of filter bound salmon sperm DNA and soluble E. coli ^{3}H-DNA does not yield appreciable hybrid formation (Figure 1).

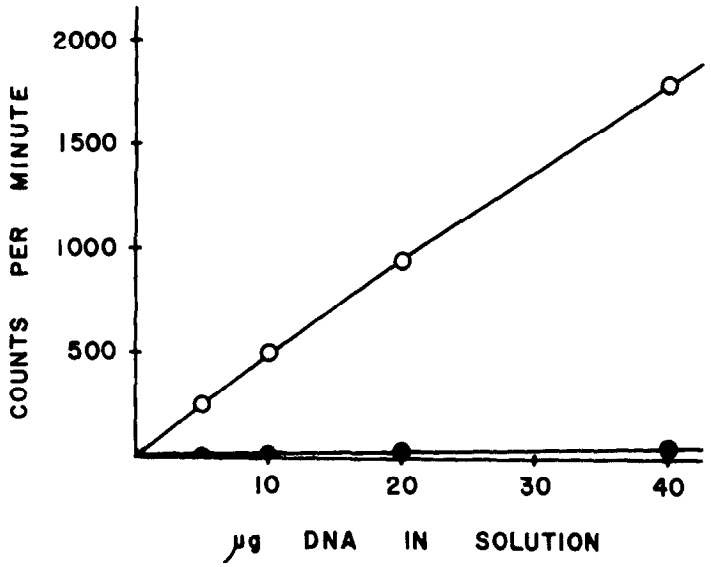

Figure 1. Specificity of DNA--^{3}H-DNA hybrid formation. Filters contained either E coli DNA (○—○) or salmon sperm DNA (●—●). Solution phase of all assays contained varying amounts of E coli ^{3}H-DNA as determined by A_{260}.

DISCUSSION

These data establish that DNA can be labeled by treatment with ^3H_2O at 100°. This labeling most probably represents exchange of the C-8 protons of the purines with ^{3}H protons of ^3H_2O (Shelton and Clark, 1967) but may also reflect some exchange of protons on thymine as observed in DNA labeled by proton exchange with $^3H_2$ (Borenfreund et al., 1959). The resultant ^{3}H-DNA retains its hybridization specificity after 20 minutes

of pH 7 heating at 100° (Figure 1). This suggests that one may employ longer heating times in the presence of ^3H_2O and obtain useful ^{3}H-DNAs of higher specific activity.

The data also establish that ^{3}H-DNA prepared by 100° exchange of protons with ^3H_2O does not lose excessive ^{3}H label due to back exchange with the media during the 65° hybrid forming step. Analysis of the back exchange in single stranded ^{3}H-DNA during the 19 hr, 65° hybrid forming period reveals that 55% of the ^{3}H label is lost from the ^{3}H-DNA. This loss necessitates the relatively high amount of filter bound DNA used in our assay. Use of the recently developed low temperature hybridization procedure (Bonner, et al., 1967) will probably both limit the extent of back exchange with the media and allow use of lower levels of filter bound DNA.

Knowledge of the extent of back exchange under our conditions allows one to calculate that approximately 3% of the input ^{3}H-DNA hybridizes with filter bound DNA. This value compares favorably with results of Denhardt (1966) using in vivo labeled DNA and suggests that our in vitro labeling procedure does not markedly harm the hybridization potential of DNAs.

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