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Hybridization of Ribosomal RNA Labeled to High Specific Radioactivity with Dimethyl Sulfate†

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ABSTRACT: RNA radioactively methylated with dimethyl sulfate has the advantage of relatively low background noise level when utilized in DNA saturation hybridization employing the membrane filter technique. In addition, the RNA can be methylated with either ^{3}H- or ^{14}C-labeled methyl groups. However, the low specific radioactivity usually obtained with dimethyl sulfate has limited the use of this labeling technique. We describe a detailed characterization of the methylation of rRNA with dimethyl sulfate giving specific radioactivities on the order of 10^5 cpm/μg. Kinetics and optimum conditions for the methylation reaction of rRNA were studied. The methylation did not cause excessive degradation of RNA in neutral aqueous solution, and the methyl derivative of RNA was stable under normal hybridization conditions. Specific radioactivity of the methylated RNA was found to be a linear function of the product of RNA concentration and specific radioactivity of the dimethyl sulfate in the reaction mixture at a constant incubation time. The methylated bases of the RNA lowered the thermal stability of the DNA–RNA hybrids by 1° in T_m per 1.6% methylated RNA bases. rRNA gene dosage values using high specific radioactive methylated RNA were determined for mouse and human liver tissues and were found to be 81 and 180 genes/haploid genome, respectively. Dissociation constants of the hybridization reaction ranged from 0.90×10^{-10} to 2.37×10^{-10} M.

A problem frequently encountered in designing DNA–RNA hybridization experiments is to obtain either DNA or RNA at sufficiently high specific radioactivities. These high specific radioactivities are particularly difficult to achieve in *in vivo* experiments using whole animals because of the low efficiency of label incorporation. A rapid, simple, and economical means of introducing sufficient amounts of radioactive label into nucleic acids would therefore be of considerable advantage. The radioactive labeling of purified nucleic acids *in vitro* has several important advantages. In addition to being able to obtain nucleic acids at a high specific radioactivity more economically, different nucleic acid species can be radioactively labeled at a uniform specific radioactivity independent of their *in vivo* nucleotide precursor pool size or synthesis rate.

Radioisotopes may be introduced into nucleic acids *in vitro* by several methods. The *in vitro* enzymatic synthesis of RNA by DNA-dependent RNA polymerase (Melli and Pemberton, 1972) and the enzymatic synthesis of DNA by RNA-dependent DNA polymerase (Kacian *et al.,* 1972) in the presence of labeled nucleotides have been used to obtain nucleic acids with high specific radioactivities. The disadvantages of these techniques are the requirements for a suitable enzyme and a highly purified template and the resultant low fidelity and nonuniformity of copying. Another method is to label nucleic acids directly by chemical modification. This has been achieved by halogenation (Commerford, 1971), photoreduction (Lee and Gordon, 1971), and alkylation (Smith *et al.,* 1968). While iodination affords a means of labeling DNA and RNA to as much as 10^7 cpm/μg (Tereba and McCarthy, 1973; Scherberg and Refetoff, 1973), the isotope is not randomly introduced into the preformed polynucleotide but reacts exclusively with cytosine (Commerford, 1971). In addition, there are difficulties encountered with the stability of the label (Holmes and Bonner, 1974; Getz *et al.,* 1972; Scherberg and Refetoff, 1973), excessively high nonspecific background binding to membrane filters (Scherberg and Refetoff, 1973; Tereba and McCarthy, 1973), and loss of the ability to renature when extensively iodinated (Commerford, 1971). The *in vitro* introduction of label into nucleic acids by photoreduction of uracil and thymine with [^{3}H]borohydride circumvents some of the problems of iodination (Lee and Gordon, 1971), but rather low specific radioactivities are obtained, and the isotope is not randomly introduced (Cerutti *et al.,* 1965).

Dimethyl sulfate has been used to introduce label into RNA (Smith *et al.,* 1968) and DNA (Akiyoshi and Yamamoto, 1970). The background noise level using the membrane filter technique for methylated RNA ranges from 0.003 to 0.01% of total input counts, whereas the level normally obtained for iodinated RNA is tenfold greater—which seriously limits sensitivity. Polynucleotides were found to be randomly labeled with no apparent degradation (Smith *et al.,* 1968; Lawley and Shah, 1972) and nucleic acids could be labeled with either ^{3}H or ^{14}C-labeled di-

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methyl sulfate. However, the specific radioactivities obtained were low (about 10^3 cpm/μg). In this paper we present details for methylating rRNA to high specific radioactivities as well as a description of physicochemical properties of the methylated rRNA, the use of methylated rRNA in hybridization experiments, and the effect of methylation on the thermal stability of the hybrid product.

Materials and Methods

Organisms and Tissues. Female mice (C57BL/6J inbred strain) were purchased from Jackson Laboratory (Bar Harbor, Maine). Mice (440 days old) were killed by cervical dislocation, and their livers were immediately removed for nucleic acid extraction. Human liver tissue (8 months old) was obtained from normal autopsy specimens. Frozen *Escherichia coli* paste (¾ log phase) was purchased from the 3-M Corporation.

Isolation of DNA and rRNA. Each mouse DNA and RNA preparation originated from the combined livers of 5–10 mice of identical age. For human, a random sample of liver tissue (10 g wet weight) was used for DNA and RNA isolation. Mouse and human DNAs were purified from isolated nuclei by a method described by Prashad and Cutler (1973 and in preparation) to eliminate possible selection artifacts of nucleotide sequences. DNA purified by this method gave yields of 93 ± 5% for mouse and human liver tissues. *E. coli* DNA was purified by the method of Marmur (1961).

Ribosomes were purified using a conventional differential centrifugation procedure. RNA was extracted from the ribosomal pellets using a low ionic strength sodium dodecyl sulfate–phenol procedure (Moldave, 1968), treated with DNase, and further purified on a Sephadex G-25 column. *E. coli* rRNA was prepared by a similar procedure. The resulting 260 nm/280 nm ratios were greater than 2.1 for all rRNA preparations used. The 260 nm/230 nm ratios were from 2.2 to 2.4. Protein and DNA contents were ≤0.5% (w/w), as determined by standard colorimetric assay methods. Possible degradation of rRNA during purification was determined by sedimentation analysis of samples routinely removed after different purification steps.

Methylation of rRNA. [^{3}H]Dimethyl sulfate (330 Ci/mol) was purchased from New England Nuclear. (Specific radioactivity tenfold greater than this can be obtained by special order.) The isotope was obtained in 5 mCi/ampoule aliquots, and the methylation reaction was carried out in this container; 1 mg (0.2 ml) of rRNA in 0.1 M sodium phosphate buffer (pH 7.6) was added directly to 5 mCi of [^{3}H]dimethyl sulfate. To terminate the reaction, 1.0 ml of sodium phosphate buffer was added directly to the ampoule, and the unreacted [^{3}H]dimethyl sulfate was separated from the RNA by chromatography with a Sephadex G-25 column (2.5 × 25 cm) equilibrated with 0.1 × SSC (SSC is 0.15 M NaCl–0.015 M sodium citrate (pH 7.0)). Methylation with nonradioactive dimethyl sulfate was carried out in an identical manner.

In Vivo rRNA Labeling. Young mice (43 days old) were used for labeling rRNA *in vivo* by intraperitoneal injections of [^{32}P]phosphate (Schwartz/Mann, carrier-free). The scheme of labeling followed that of Mohan *et al.* (1969) with 2 mCi initially being injected, followed by two injections of 1 mCi 12 hr apart. The livers were removed 24 hr after the last injection and the rRNA was isolated as described above. The specific radioactivity was approximately 6000 cpm/μg. *In vivo* tritium-labeled *E. coli* rRNA (16 S) was purchased from Miles Laboratories.

Radioactivity Measurements. The efficiency of tritium counting was approximately 53%. For specific activity calculations, RNA concentration was determined by absorbance at 260 nm using the relation of 1 O.D. unit being equivalent to 44 μg.

Hybridization. Mouse and human DNAs were alkaline denatured and fixed to Schleicher and Schuell Type B-6 nitrocellulose membrane filters (100 μg/24 mm filter) according to a procedure described by Birnstiel *et al.* (1972). Filters were dried overnight *in vacuo* at 25°, heated *in vacuo* at 80° for an additional 4 hr and treated with Denhardt's preincubation media (Denhardt, 1966) for 1 hr at room temperature. Mouse and human DNA–rRNA hybridization was carried out in a 5 × SSC, 50% formamide solution for 8 hr at 50° (McConaughy *et al.*, 1969). In addition to a DNA-containing filter, each reaction vial contained a blank filter to determine nonspecific background binding. After hybridization, filters were extensively washed with 2 × SSC and treated with pancreatic RNase according to Birnstiel *et al.* (1972) before drying and counting. DNA loss from the filters during the hybridization was determined by either acid hydrolysis of DNA from the filters after counting or running ten filters in parallel mock hybridization. All hybridization values are corrected for DNA loss and nonspecific background counts. Each hybridization value is the average of three filters. *E. coli* DNA–rRNA hybridization was carried out according to the method of Gillespie and Spiegelman (1965).

Thermal Stability of DNA–rRNA Hybrids. Filters were incubated in toluene to remove RNase activity and then washed extensively with 2 × SSC. Five to ten filters were pierced by one straight pin to facilitate handling. The filters were then transferred through a series of vials, each containing 7.0 ml of 0.5 × SSC at a temperature ranging from 25 to 100°—increasing in 5° intervals. The stack of filters was incubated for 10 min in each vial before transfer, and the amount of radioactivity eluting off in each vial was determined by measuring Cl_3CCOOH insoluble radioactivity.

Results

Methylation of rRNA. The kinetics of the methylation reaction are shown in Figure 1. The reaction is essentially complete at 100 min. The kinetics of labeling *E. coli* rRNA with [^{3}H]dimethyl sulfate are very similar to those obtained with total *E. coli* RNA (Smith *et al.*, 1968). Purification of the methylated rRNA from the reaction mixture was accomplished by chromatography on a Sephadex G-25 column. The methylated rRNA elutes with the void volume as a single symmetrical peak. The remaining radioactivity is retarded and subsequently elutes at approximately two void volumes—providing satisfactory separation of the methylated rRNA. In spite of the high concentration of RNA, most of the dimethyl sulfate radioactivity does not react with the RNA and spontaneously degrades to nonreactive radioactive components.

The effects of the ratio of RNA to dimethyl sulfate concentration (w/w) and the pH of the reaction mixture on the resultant specific radioactivity, degradation, and per cent recovery were determined by using nonradioactive dimethyl sulfate. It has previously been shown that methylation of nucleic acids (Lawley and Shah, 1972) and homopolymers (Brimacombe *et al.*, 1965) with dimethyl sulfate in a neutral aqueous solution does not cause any detectable degra-

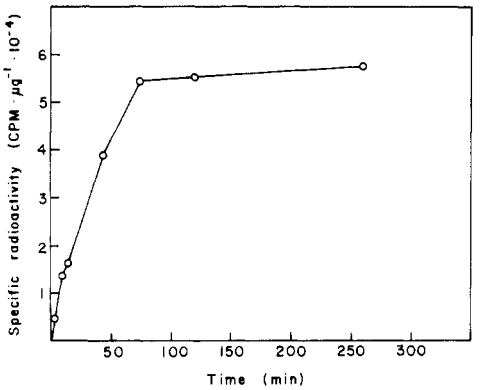

FIGURE 1: Kinetics of labeling *E. coli* rRNA with [^{3}H]dimethyl sulfate. The reaction conditions were 10 mCi of [^{3}H]dimethyl sulfate (3.8 mg) and 2 mg of rRNA in a total volume of 0.4 ml of 0.1 M sodium phosphate buffer (pH 7.6) at 25°. At various times 40 μl (200 μg of rRNA) of the reaction mixture was removed and chromatographed on a Sephadex G-25 column. Those fractions containing [^{3}H]rRNA were pooled and the rRNA was concentrated by ethanol precipitation for specific radioactivity determinations.

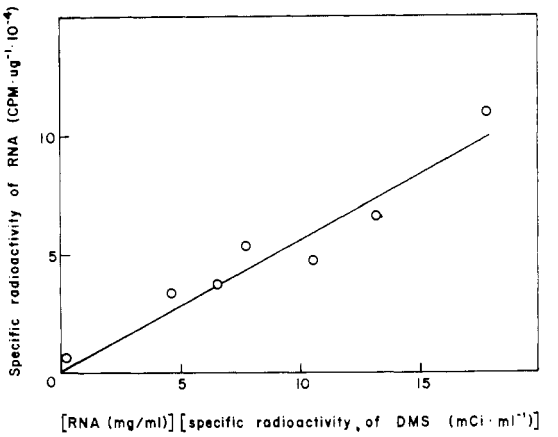

FIGURE 2: Specific radioactivity of RNA as a function of RNA concentration and specific radioactivity of dimethyl sulfate in the reaction mixture.

dation even when 45% of the bases are methylated. Under acidic conditions (pH 4.5–5.0) the specificity of the reaction changes, and the phosphodiester bond is hydrolyzed (Brimacombe *et al.*, 1965). As the reaction proceeds, H_2SO_4 is generated as a by-product, and the pH changes with time. Stability of pH was found to be a problem if the ratio of rRNA (at a concentration of 5 mg/ml in 0.1 M sodium phosphate buffer (pH 7.6)) to dimethyl sulfate becomes less than unity. The pH of the reaction mixture can be controlled by varying the molarity of the buffer and the initial pH, or the pH can be monitored and maintained at neutrality by periodic addition of alkali. We found that, at a rRNA/dimethyl sulfate ratio of 0.5 in 0.2 M sodium phosphate buffer (pH 7.8), the pH was approximately 7.0 at the end of 20 hr.

Methylated rRNA was analyzed for degradation during the labeling period by sedimentation analysis on sucrose density gradients. *E. coli* rRNAs, not methylated and methylated to 4740 and 57,200 cpm/μg, were compared. The sedimentation rates for both of the methylated *E. coli* rRNAs were faster compared to the nonmethylated rRNA, indicating some degradation had occurred. The most prominent new peak was at approximately 7 S for both labeled rRNAs, although there was still some 18S and 28S RNA evident. Specific radioactivity of the RNA (cpm/OD) was essentially constant throughout the gradient for both labeled samples. The sedimentation rates of the labeled rRNA samples were essentially the same, indicating that the observed degradation was a result of the methylation procedure and not a consequence of the methylation reaction. Because rRNA molecules of different sizes (3.5 S to 28 S) have been found to hybridize with equal fidelity (Birnstiel *et al.*, 1972), the degree of degradation encountered in the above procedure is considered to be negligible.

A direct correlation between the extent of methylation of RNA and the concentration of dimethyl sulfate in the reaction has been reported (Smith *et al.*, 1968). We have found that the extent of methylation also depends upon the RNA concentration. The specific radioactivity of methylated rRNA was found to be directly proportional to the product of the rRNA concentration and the specific radioactivity of the dimethyl sulfate (Figure 2). A typical value obtained is 6.6×10^4 cpm/μg, when 1.9 mg of [^{3}H]dimethyl sulfate (5 mCi) was reacted with 1.0 mg of rRNA in a total volume of 0.2 ml of 0.2 M sodium phosphate buffer (pH 7.8).

Thermal stability of radioactive methyl groups on RNA was determined by incubating samples of the low (4750 cpm/μg) and high (57,200 cpm/μg) specific radioactive *E. coli* rRNAs at 65° in 2 × SSC for various times. No significant differences between the two rRNA samples were observed, as determined by the relative amount of Cl_3CCOOH precipitable counts obtained over a 10-hr period at 65°. After 10 hr at 65°, some Cl_3CCOOH soluble counts were detected. However, this degradation followed a kinetic pattern characteristic of the thermal excision of the RNA phosphodiester bond (Eigner *et al.*, 1961). rRNA methylated to high specific radioactivities has remained unchanged with respect to hybridization results after being frozen for over a year.

Hybridization. The hybridization properties of methylated rRNA labeled to high specific radioactivity were examined using the membrane filter technique. rRNA gene dosage for human and mouse was determined by the RNA excess technique. The results of these experiments are shown in Figure 3. Nonspecific background binding to blank filters ranged from 0.003 to 0.011% of the input counts, which permitted high concentrations of RNA to be used to accurately determine the point of saturation. *E. coli* DNA filters hybridized with methylated mammalian liver rRNA had the same percentage of counts bound as blank filters. Mouse and human rRNAs, however, were found to cross-hybridize to approximately 85%.

The hybridization reaction using high specific radioactive methylated rRNA was observed to follow the normal kinetics expected in a solution with the DNA being fixed to a membrane (Birnstiel *et al.*, 1972). The reaction rate with mouse liver DNA (100 μg/filter) had a $t_{1/2}$ of 10 min at 10 μg/ml of rRNA. RNase sensitivity of methylated *E. coli* rRNA was determined by incubation with RNase A (10 μg/ml at 37° for 30 min in 2 × SSC). Approximately 95% of the counts were acid soluble with this treatment.

The saturation curves of Figure 3 for mouse and human liver tissues were analyzed using the Scatchard plot (Marsh and McCarthy, 1973), as shown in Figure 4, to determine rRNA gene dosage and the dissociation constant of the reaction. The calculated number of rRNA genes per haploid genome and the reaction dissociation constant (negative reciprocal slope) are given in Table I. The number of rRNA genes was calculated using a genomic molecular

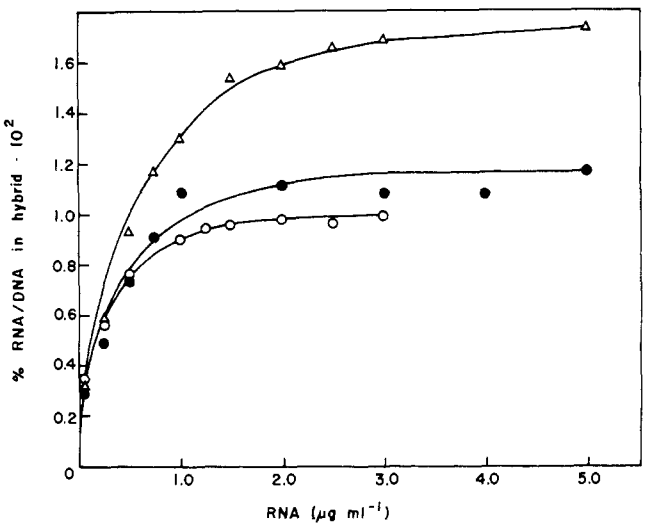

FIGURE 3: DNA saturation hybridization to homologous rRNA for mouse and human liver tissues. Filters with a constant amount of DNA were incubated with increasing concentrations of rRNA. (○) Mouse liver methylated [^{3}H]rRNA, 12,200 cpm/μg; (●) ^{32}P *in vivo* labeled mouse liver rRNA 6000 cpm/μg; and (△) human liver methylated [^{3}H]rRNA, 33,200 cpm/μg. Each point is the average of triplicate determinations.

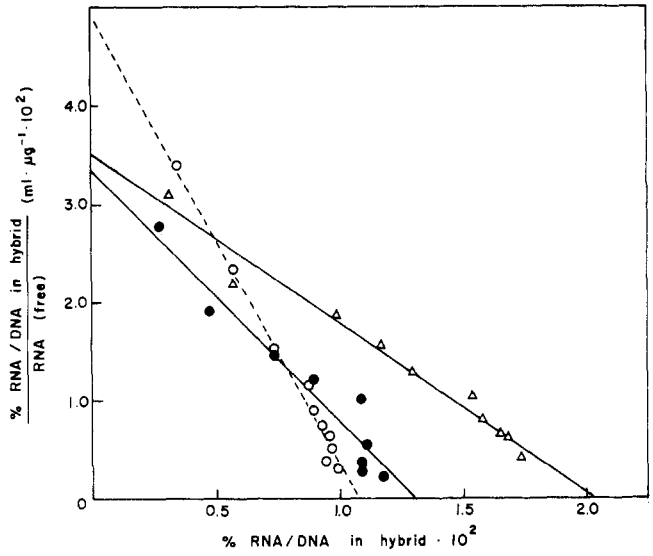

FIGURE 4: Scatchard plots of the DNA saturation hybridization curves in Figure 3. Mouse liver DNA with ^{32}P *in vivo* labeled rRNA (●) or ^{3}H methylated rRNA (○); (△) human liver DNA. The lines were determined by linear regression and analysis of the data. The constants determined by this analysis are given in Table I.

weight of 2.2×10^{12} for human (Bachmann, 1972) and 1.8×10^{12} for mouse (Laird, 1971). The molecular weights for the combined mass of the rRNA subunits used in calculations were 2.4×10^6 for mouse and human rRNA (Birnstiel *et al.*, 1971).

The human rRNA gene redundancy value obtained with methylated rRNA is in good agreement with other estimates using *in vivo* labeled rRNA (Bross and Krone, 1972; Scherberg and Refetoff, 1973). We are not aware of a published value for rRNA gene dosage for mouse, but the *in vivo* ^{32}P-labeled rRNA gave very similar results compared to methylated rRNA from mice of the same age. Since repeat experiments gave results within 10% of the mean value, the difference of 15 genes given here between the *in vivo* and *in vitro* labeled mouse liver rRNA is not considered significant.

The dissociation constants (K_d) of the DNA–rRNA hybrid for mouse liver are very close to other reported values (Quincey and Wilson, 1969; Bishop, 1970). The K_d for human is slightly high but well within the range of other values (Kennel, 1971). The difference in K_d for the *in vivo* ^{32}P-labeled and *in vitro* ^{3}H-methylated mouse liver rRNA may reflect differences in background binding, different relative amounts of 18S and 28S rRNA in the preparations, and/or differences in contaminating RNA species.

Table I: rRNA Gene Dosage and DNA–rRNA Dissociation Constants for Different Organisms.[a]

Tissue	% Hybridization at Saturation	No. of rRNA Genes/ Haploid Genome	Dissociation Constant (K_d) × 10^{10} M
Mouse liver			
[^{3}H]rRNA	0.011	81	0.90
[^{32}P]rRNA	0.013	96	1.59
Human liver	0.020	180	2.37

[a] Constants used in calculations are given in text.

Thermal Stability of Hybrid Molecules. The major alkylation product of the dimethyl sulfate–RNA reaction is 7-methylquanine; the principle minor products are 3-methylcytosine and 1-methyladenine, with uracil being modified to a very minor degree at the N-3 position (Lawley and Shah, 1972; Brimacombe *et al.*, 1965). The major methylation products are the same as those found *in vivo* (Bernhard, 1967). The effect of these altered bases on DNA–RNA thermal stability is not known, but presumably they induce base-pair mismatch by preventing normal Watson–Crick hydrogen bonding.

Thermal stability of the DNA–RNA hybrid is dependent upon the number and type of base pairs and the precision of base pairing (Walker, 1969), and should therefore be sensitive to alterations in base pairing that might be caused by high levels of methylation of RNA. The thermal stability of *E. coli* DNA–rRNA hybrids with rRNA methylated to different degrees was determined by thermal elution from membrane filters. This method measures irreversible strand separations, T_{mi} (Laird *et al.*, 1969). Figure 5 shows the melting curves for *in vivo* labeled [^{3}H]rRNA (16 S) and for rRNA methylated to a low and a high specific radioactivity. The *in vivo* labeled rRNA exhibited a T_{mi} of 82° in 0.5 × SSC; this is consistent with T_{mi} values of both 23S and 16S rRNA and a G + C content of 54% (Midgley, 1962; Moore and McCarthy, 1967). The lowering of the T_{mi} value, relative to the *in vivo* labeled rRNA, for the methylated rRNAs indicates the presence of base-pair mismatching. The shapes of the thermal stability profiles for all three hybrids were similar over the interval of 25–75% dissociation, indicating that the lower T_{mi}'s were due to random distribution of base-pair mismatching. The depression of the T_{mi} could be a consequence of a contaminant found only in radioactive dimethyl sulfate. The *in vivo* labeled rRNA was therefore treated with cold dimethyl sulfate, using the same procedure of methylation as with radioactive dimethyl sulfate. The same degree of lowering of T_{mi} was obtained with the nonradioactive methylated rRNA.

The decrease in T_{mi} of the DNA–RNA hybrids relative to the unmethylated DNA–RNA hybrids is plotted as a

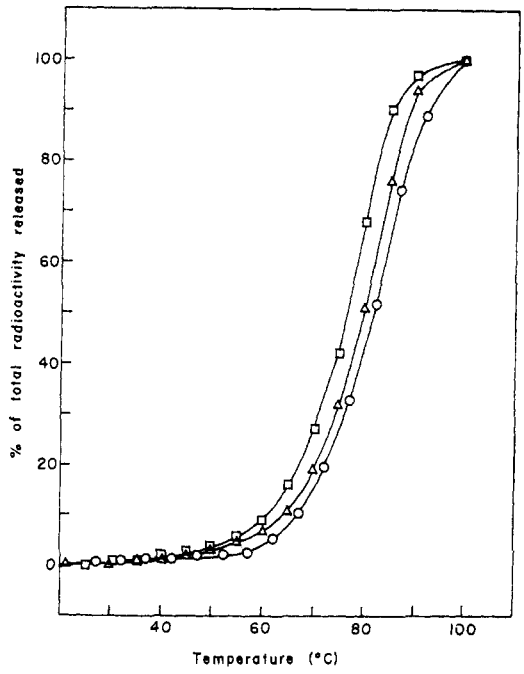

FIGURE 5: Thermal dissociation profiles of *E. coli* DNA-rRNA hybrids in 0.5 × SSC. *In vivo* labeled 16S [^{3}H]rRNA (○); rRNA methylated for 15 min, 15,200 cpm/μg (△); and rRNA methylated for 260 min, 57,200 cpm/μg (□). The radioactivity eluted from the filters was determined as outlined under Materials and Methods.

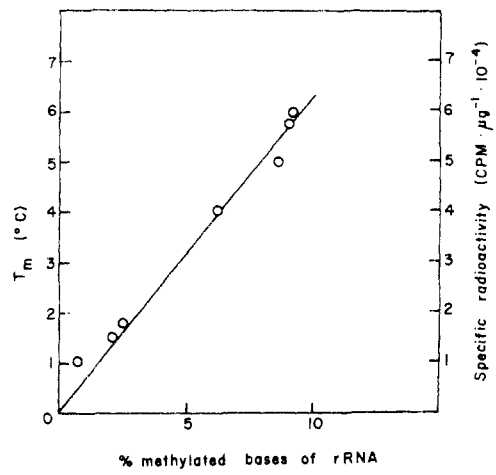

FIGURE 6: The relationship between DNA-RNA hybrid thermal stability and the per cent methylated bases of *E. coli* rRNA. The *E. coli* rRNAs methylated to different extents were those derived from the kinetic study (Figure 1). They were hybridized to a common DNA preparation for 5 hr in 2 × SSC at 67°. The T_m of each hybrid was determined under identical conditions at an ionic strength of 0.5 × SSC. The line represents a least-squares fit of the data.

function of per cent methylation in Figure 6. The slope derived from a least-squares fit gave a 1° decrease in T_{mi} per 1.6% radioactive methylated bases. Previous work on the relationship between decrease in thermal stability duplexes and the fraction of mispairing or altered base pairs has been presented by Laird *et al.* (1969) and Kohne (1970). Our value of change in T_{mi} compares well with their value of 1° decrease in T_{mi} per 1.5% altered base pairs.

Discussion

There now exist several *in vitro* methods to radioactively label nucleic acids, each having characteristic advantages and disadvantages. We find the introduction of radioisotopes into rRNA by methylation with [^{3}H]dimethyl sulfate to be quick, reliable, and simple. Our findings should also be applicable to the use of [^{14}C]dimethyl sulfate and to the methylation of DNA (Akiyoshi and Yamamoto, 1970). The maximum specific radioactivity of labeled polynucleotides that can be obtained appears to be limited by the maximum concentration (solubility) of the nucleic acid and the specific radioactivity of dimethyl sulfate available. The specific radioactivities of RNA obtained with RNA concentrations of 5–10 mg/ml and normally available specific activities of dimethyl sulfate of 300–2000 Ci/mol are approximately 10^5 cpm/μg, which is sufficiently high for most hybridization experiments. Other advantages of methylated RNA for use in hybridization are the high stability of the radioactive derivative and the tenfold lower nonspecific background noise level found when working with the membrane filter technique—as compared to using iodated RNA.

Our experiments confirm previous reports (Smith *et al.*, 1968; Lawley and Shah, 1972) indicating the methylation reaction does not cause significant degradation in aqueous solutions. However, we have found that the thermal stability of DNA-RNA hybrids using methylated RNA is decreased to a detectable level when RNA is methylated to more than 1% of its bases. This corresponds to a specific activity of about 15,000–105,000 cpm/μg, and at this level of methylation the specificity or efficiency of hybridization is not expected to be serious.

DNA and many RNA species are normally methylated enzymatically *in vivo* (Srinivasan and Borek, 1964), and dimethyl sulfate is known to be a weak carcinogen (Druckrey *et al.*, 1970). Therefore, the effect of base methylation, resulting in a lower duplex thermal stability, could prove to be of biological significance. Presumably, methylation can induce miscoding by preventing normal hydrogen bonding (Lawley and Shah, 1972). Thus, methylated bases in DNA may increase the probability for the transition mutations by anomalous base pairing during DNA replication or for translation errors if mRNA becomes mismethylated. On the other hand, methylation of DNA may prove to be of importance in destabilizing certain specific nucleotide sequences or in determining the average tertiary configuration of methylated DNA or RNA molecules.

Dimethyl sulfate appears unstable in aqueous solutions near neutral pH, and it is suggested that this instability governs the observed labeling kinetics of RNA. A kinetic model is presented, based on (1) the RNA concentration at time zero (R_0) being in excess and not rate limiting and (2) the concentration of dimethyl sulfate (M) being rate limiting because of its natural instability in the reacting solution. Evidence supporting this latter conclusion is the relatively large amount of nonreactive radioactive label remaining after the RNA has reached the maximum specific activity.

The rate of methylation of RNA is given as

$$\frac{dR_M}{dt} = k_1 R_0 M$$

where R_m is the amount of RNA methylated, k_1 is the reaction rate constant, R_0, the concentration of unmethylated RNA at time zero, and M is the concentration of reactive dimethyl sulfate at time t. And

$$M = M_0 e^{-k_2 t}$$

where M_0 is the concentration of dimethyl sulfate at time zero and k_2 is the spontaneous degradation constant of dimethyl sulfate under these aqueous reaction conditions. On substitution and integration we obtain

$$R_M = \frac{k_1}{k_2} R_0 M_0 [1 - e^{-k_2 t}]$$

which agrees with our data indicating the linear relationship between R_m and the product R_0M_0.

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