in tissue is an indication that these proteins are not contaminants acquired in lymph or plasma. It may be assumed that they have some relationship to lipoprotein formation and fat transport. The proof of this will require more investigation.

Acknowledgments

We thank Misses Mary Elanne Smootz and Kathryn M. John for their capable technical assistance.

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Terminal Labeling of High Molecular Weight Ribonucleic Acid with Dimedone*

Dohn G. Glitz and David S. Sigman

ABSTRACT: The reaction of dimedone (5,5-dimethyl-1,3-cyclohexanedione) with dialdehydes resulting from periodate oxidation of the 3′-hydroxyl terminus of RNA has been studied as a terminal-labeling technique in polynucleotide sequence analysis. One molar equivalent of dimedone-2-^{14}C is incorporated per free 3′ terminus of oxidized AMP, tRNA, rRNA, or bacteriophage f2 RNA. Little or no dimedone is associated with unoxidized RNA. The labeling process does not result in appreciable nonspecific incorporation or detectable polynucleotide degradation. The terminal label is sufficiently stable at neutral pH, except in the presence of amines, to permit identification of only predicted terminally labeled oligonucleotide fragments from RNA upon nuclease digestion followed by DEAE-cellulose chromatography.

End-labeling techniques have played an important role in the development of protein chemistry. By analogy, similar techniques in RNA chemistry have been valuable in the identification of terminal mono- and oligonucleotides of RNA molecules. Such information is useful in sequence work as well as for the determination of heterogeneity in RNA preparations (RajBhandary, 1968).

A number of end-labeling techniques have been developed which exploit the periodate oxidation possible only at the 3′ terminus (also called the 5′-linked or right-hand end) of a polyribonucleotide. Generally the procedures have involved first the oxidation of RNA by periodate and then derivatization of the resulting uniquely reactive dialdehyde. The dialdehyde generated by periodate oxidation has been treated with tritiated isonicotinoylhydrazine (Hunt, 1965), tritiated 2-phenylethylammonium chloride (Wimmer and Reichmann, 1969), ^{14}C-labeled semicarbazide (Steinschneider and Fraenkel-Conrat, 1966a), and tritiated sodium borohydride (RajBhandary, 1968; DeWachter and Fiers, 1967; Glitz *et al.*, 1968).

None of the methods of derivatization has been generally employed, indicating a lack of clear superiority of any of the

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methods. The most common, borohydride reduction, suffers from a number of shortcomings, especially in the study of high molecular weight RNA. In particular, the nonspecific binding of tritium to macromolecular RNA and the probable isotope effect apparent in the lower specific activity of labeled terminal oligonucleotide are troublesome (see *e.g.*, Glitz *et al.*, 1968). The greatest advantage of the borohydride method is the stability of the alcohol produced upon reduction.

Recent attempts to use tritiated isonicotinoylhydrazine for the isolation of terminal nucleotides from R-17 bacteriophage have not proved successful (Thach and Boedtker, 1969). Apparently this particular derivative of the dialdehyde is not sufficiently stable to permit satisfactory isolation of the desired oligonucleotide (but, see also Hunt, 1970). The semicarbazone of the dialdehyde also suffers from the instability of the radioactive product (Steinschneider and Fraenkel-Conrat, 1966a). Primary amines are unlikely to be desirable end labels because the aldimines they can form with the dialdehyde are unstable with respect to the elimination of the 5′-phosphoryl group. In fact, procedures for the stepwise degradation of a polynucleotide have been developed from the technique of Whitfeld (1954) which involve the treatment of periodate-oxidized RNA with aniline, lysine, or cyclohexylamine (Steinschneider and Fraenkel-Conrat, 1966b; Khym and Uziel, 1968; Weith and Gilham, 1969). Methods which depend on the reduction of aldimines by sodium borohydride (*e.g.*, Wimmer and Reichmann, 1969), in order to be quantitative, must also minimize these elimination reactions.

One important factor in the choice of an end label for the dialdehyde of the periodate-oxidized RNA is whether the derivative is stable to the type of β-elimination reactions which can take place in aldimines. Since dimedone (5,5-dimethyl-1,3-cyclohexanedione), unlike hydrazines, semicarbazides, and primary amines, generally forms derivatives with aldehydes which are saturated at the aldehydic carbon atom, its derivatives should not be very susceptible to β-elimination reactions. In the present communication, we wish to report that dimedone-2-^{14}C reacts in a 1:1 stoichiometry with the dialdehyde produced by periodate oxidation of RNA. The derivative is sufficiently stable so that radioactive terminal oligonucleotides can be readily isolated following enzymic digestion and subsequent chromatography. In addition, radioactive dimedone produces neither artifacts such as backbone cleavage nor appreciable nonspecific or noncovalent binding to the RNAs studied.

Materials and Methods

a. Synthesis of Dimedone-2-^{14}C. Dimedone-2-^{14}C was synthesized from diethyl malonate-2-^{14}C (Amersham-Searle Co., Des Plaines, Ill.) and mesityl oxide according to a scaled-down published procedure (Shriner and Todd, 1943). Diethyl malonate-2-^{14}C (0.010 ml) was added with a syringe to 0.2 ml of a 2.5 M solution of sodium ethoxide in ethanol. The syringe was washed with 0.3 ml of ethanol and the washings added to the reaction vessel. Unlabeled diethyl malonate (0.050 ml) was then added to the reaction mixture. The solution was stirred for 10 min and then 0.050 ml of mesityl oxide was added. The reaction mixture was refluxed for 2.5 hr. Next, 54.5 mg of potassium hydroxide in 0.251 ml of water was added and the reaction mixture was refluxed for an additional 6 hr. Then 0.3 ml of 4 N HCl was added to the reaction mixture, which was stirred at room temperature for 48 hr. At the end of this period a precipitate was evident; it was collected and dried at 40° at 50 μ The infrared and ultraviolet spectra of the precipitate were characteristic of dimedone. The dimedone product had a specific activity of 1.54 mCi/mmole; paper electrophoresis and Sephadex chromatography indicated there was less than 5% of radiochemical impurities.

b. Nucleic Acids and Nucleotides. Nucleosides and nucleotides were purchased from Calbiochem, Los Angeles, Calif., or Sigma Chemical Co., St. Louis, Mo., except for generally tritiated adenosine 5′-monophosphate (AMP) which was purchased from Amersham-Searle Co. Yeast transfer RNA was also a commercial product (Calbiochem, lot no. 40278); any residual terminal amino acids were removed by incubation for 1 hr at 37° in a solution of 0.5 M Tris·HCl (tris(hydroxymethyl)aminomethane, Sigma Chemical Co.), pH 9.0. RNA from *Escherichia coli* B was prepared by three successive phenol extractions of *ca.* 40 mg/ml of log-phase cell suspensions in 0.1 M sodium acetate (pH 5.5) containing 0.6 mg/ml of sodium dodecyl sulfate and 0.1 mg/ml of bentonite (both purchased from Sigma Chemical Co). Bacteriophage f2 was grown in *E. coli*, American Type Culture Collection strain 15766, and phage and RNA were prepared as described by Glitz *et al.* (1968).

c. Periodate Oxidation and Dimedone Labeling. AMP (0.05 M) was dissolved in 0.05 M phosphate buffer (pH 7.0) and oxidized by at least 1 molar equiv of sodium periodate for 30–60 min in the dark at 0°. If more than 1 equiv of periodate was used, the reaction mixture was passed through Sephadex G-15; this prevents subsequent periodate oxidation of dimedone (Wolfrom and Bobbitt, 1956). Dimedone, in equivalent amounts or up to a 100% molar excess, was added at 0.05 M in 0.05 M phosphate buffer (pH 7). The reaction was allowed to proceed for 18–20 hr at 0–4°. Tritium-labeled AMP (2.5 μmoles) was oxidized and treated with a twofold excess of dimedone-^{14}C as above; reaction times varied from 15 to 72 hr with no apparent effect.

Transfer RNA was dissolved in 0.1 M sodium acetate buffer (pH 5.5) at a concentration of 10 mg/ml and the solution made 0.01 M in sodium metaperiodate. After 30–60 min in the dark at 0°, 2 volumes of ethanol was added, and after 10 min the suspension was centrifuged for 15 min at 12,000*g*. The supernatant was discarded and the precipitate redissolved in a minimal volume of 0.1 M potassium phosphate buffer containing 0.06 M dimedone-^{14}C of the appropriate specific activity. After 18–20 hr at 0–4° the RNA was freed of excess dimedone by repeated ethanol precipitation from 0.01 M phosphate buffer (pH 7). Usually 5–6 precipitations were needed to bring the RNA to constant specific activity.

Bacteriophage f2 RNA or RNA from *E. coli* B, at a concentration of 6–20 mg/ml, was dissolved in 0.05 M sodium acetate (pH 5.5) and sodium metaperiodate added to a level of 0.01 M. After 30–60 min at 0° in the dark, the RNA was precipitated with 2 volumes of ethanol, redissolved in buffer, and again precipitated with ethanol to remove excess periodate. The pellet was then dissolved in 0.016 M dimedone (of specific activity 2.83×10^6 cpm/μmole) in 0.1 M potassium phosphate buffer (pH 7.0) to give the highest RNA concentration possible (at least 10 mg/ml). Residual ethanol was removed by blowing air over the surface of the solution for 5–10 min at 37°.

Reaction was allowed to proceed in the dark at 0–4° for 16 hr, or in a few cases up to 4 days. Excess dimedone was removed by repeated precipitation from 0.01 M phosphate buffer with 2 volumes of cold ethanol.

d. Chromatography and Electrophoresis. Paper chromatography was performed using Whatman DE-81 DEAE paper with 0.05 M sodium phosphate buffer (pH 6.2). This system separates dimedone from mononucleotide product in about 4 hr. Paper electrophoresis was carried out using Whatman No. 3MM paper and buffers of 0.025 M sodium citrate (pH 2.9 or 3.5). Electrophoresis at 300 V (5 V/cm) for 24 hr cleanly resolved dimedone from reaction mixtures containing oxidized nucleotides, and partially resolved nucleotides from their dimedone derivatives. Gel electrophoresis with 2% polyacrylamide plus 0.5% agarose gels (pH 8.3) was performed according to Peacock and Dingman (1968), except that Tris·HCl was replaced with 0.01 M NaH_2PO_4 in the gel buffer.

Column chromatography on DEAE-cellulose using buffers containing 7 M urea was performed as described by Tomlinson and Tener (1962), except that pH was in general maintained with 0.01 M potassium phosphate buffer (pH 7.5) and nucleotides eluted with a linear gradient of 0–0.4 M sodium chloride.

e. Other Methods. Radioactivity was measured using a Packard Tri-Carb Model 3003 liquid scintillation spectrometer and the scintillation fluid described by Steinschneider and Fraenkel-Conrat (1966a), or with a Packard Model 7201 radiochromatogram scanner. Ultraviolet spectra were measured in a Zeiss PMQ II or a Cary 14 spectrophotometer. Infrared spectra were measured in KBr pellets with a Perkin-Elmer 237B infrared spectrophotometer.

Enzymic digestion was carried out at 37° for 18 hr in 0.05 M phosphate buffer, pH 7.0. Pancreatic ribonuclease (EC 2.7.7.16), purchased from Worthington Biochemical Co., was used at a level of 2 μg/mg of RNA, while ribonuclease T_1 (EC 2.7.7.26, Sankyo Ltd., purchased from Calbiochem) was used at a level of 5 units (Takahashi, 1961) per mg of RNA. Sucrose density gradient centrifugation was performed using 28 ml of 5–20% sucrose gradients in 0.05 M sodium phosphate buffer (pH 6.0) and a Spinco L2-65B centrifuge and SW25 rotor. Centrifugation at 4° was for 20 hr at 23,500 rpm.

Results

A. Stoichiometry of the Reaction. The stoichiometry of the reaction of dimedone with periodate-oxidized 5′-mononucleotides, tRNA, and viral RNAs was determined and in each case it was found to be 1 dimedone per oxidized ribonucleotide. For example, the reaction of dimedone with periodate-oxidized AMP (AMP dialdehyde) was studied by paper electrophoresis or chromatography of reaction mixtures. The "titration" of a fixed amount of dimedone with varying quantities of AMP dialdehyde (Figure 1) shows all the dimedone reacted upon the addition of 1 molar equiv of AMP dialdehyde. The slope of −1 until the equivalence point is reached not only strongly indicates a 1:1 stoichiometry but also suggests the reaction of dimedone with AMP dialdehyde has a very favorable equilibrium constant. If the AMP dialdehyde–dimedone product is eluted from the above paper chromatograms and the absorption spectra of AMP dialdehyde and dimedone are assumed

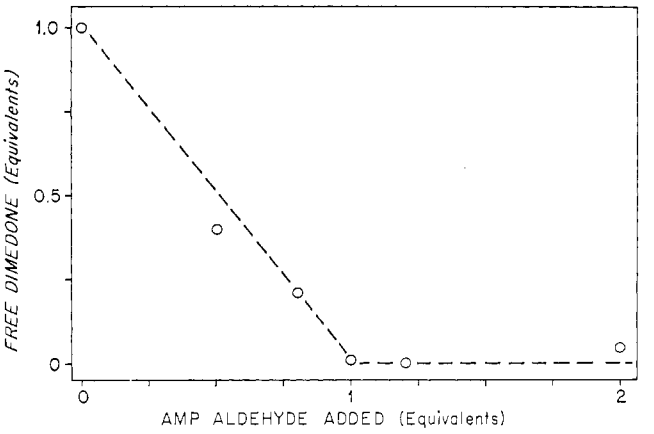


FIGURE 1: Reaction of dimedone with adenosine 5′-phosphate. Dimedone (0.5 ml, 0.05 M) was reacted with varying amounts of 0.05 M periodate-treated AMP in a total volume of 1.5 ml of 0.05 M potassium phosphate, pH 7.0, for 20 hr at 4°. Portions (25 μl) were spotted on Whatman No. 3MM paper, and electrophoresis at 300 V (5 V/cm) carried out for 24 hr using a buffer of 0.025 M sodium citrate, pH 2.9. Dimedone spots (or areas corresponding to dimedone) were cut from the paper, the compound was eluted with 4–5 ml of 0.01 M NH_3 solution, and dimedone concentration was determined spectrophotometrically.

to be additive in the region of 250–300 mμ a molar ratio of 0.7 dimedone per AMP dialdehyde can be calculated. The relatively low value of the molar ratio may indicate the assumption of additive absorption spectra is not completely valid.

Sephadex chromatography of an equimolar mixture of AMP dialdehyde and dimedone yielded a product whose absorption spectrum varied with pH in the same manner as unreacted dimedone or the dimedone derivative of acetaldehyde. By assuming that the change in the extinction coefficients in going from the un-ionized to the ionized dimedone in the AMP dialdehyde–dimedone product is the same as was observed for free dimedone or the dimedone derivative of acetaldehyde, a molar ratio of 0.68 dimedone per AMP dialdehyde was calculated. Although the value of the molar ratio obtained depends on assumptions with regard to the extinction coefficients, this experiment demonstrates that an ionizable proton still exists in the AMP dialdehyde–dimedone product.

A more direct approach in determining the stoichiometry of the dimedone product with AMP dialdehyde involved a double-labeling experiment. Tritium-labeled AMP dialdehyde containing 1.95×10^5 cpm per μmole was reacted with an excess of ^{14}C-labeled dimedone with a specific activity of 2.46×10^5 cpm per μmole. The product was isolated by DEAE paper chromatography or paper electrophoresis. ^{14}C:^{3}H ratios indicated dimedone to AMP stoichiometries of 0.82, 0.78, and 1.22 in three separate experiments. Since the AMP dialdehyde was prepared from ^{3}H randomly labeled AMP, these stoichiometries would not have been possible if significant elimination of adenine from the AMP dialdehyde–dimedone product had occurred.

As indicated above, we have also studied the stoichiometry of the reaction between dimedone and periodate-oxidized transfer, bacteriophage, and rRNA. Dimedone labeled with ^{14}C at a specific activity of 7.43×10^4 cpm per μmole

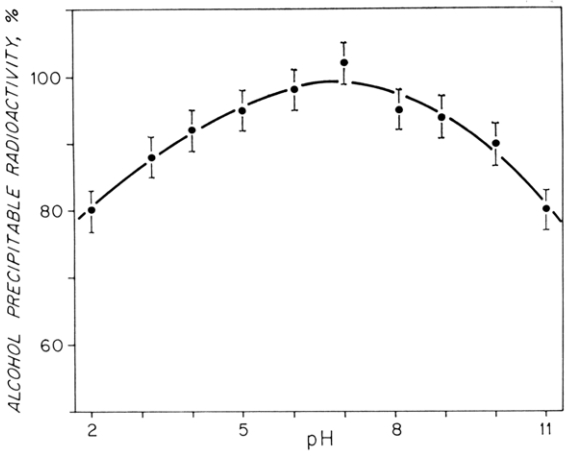


FIGURE 2: Effect of pH on the stability of dimedone–tRNA. Incubation mixtures containing 8 mg/ml of dimedone-^{14}C-labeled tRNA, in a buffer containing 0.06 M (each) of H_3PO_4, H_3BO_3, and CH_3COOH adjusted to the desired pH with KOH, were held at 0° for 24 hr and 0.05-ml portions were transferred to 1 ml of cold 70% ethanol. After 15 min the sample was centrifuged for 15 min at 8000*g* and the precipitate dissolved in 0.2 ml of H_2O. Absorbance was measured on a 1:40 dilution, radioactivity determined in a 50-μl sample, and specific activities calculated and compared to the starting material (100%).

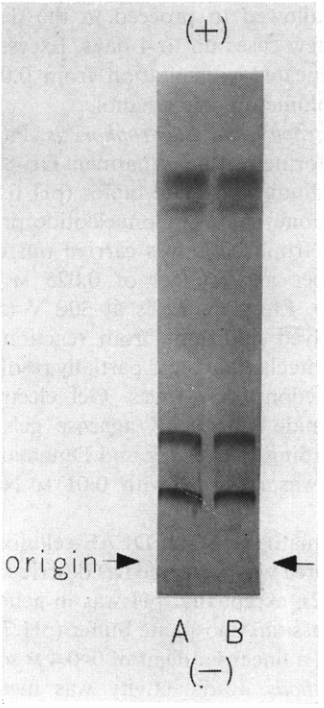


FIGURE 3: Gel electrophoresis of terminally labeled RNA. The gel contained 2% polyacrylamide plus 0.5% agarose. Electrophoresis at pH 8.3 was carried out at 4° for 90 min at 200 V. The gel was stained in 0.2% methylene blue in pH 4.5 sodium acetate (0.4 M). Samples (about 20 μg); (A) control *E. coli* RNA, (B) dimedone-^{14}C-labeled *E. coli* RNA.

was incubated with untreated and with periodate-oxidized tRNA, and excess dimedone was removed by repeated ethanol precipitation of the product. Radioactivity was incorporated into oxidized RNA at a level of 117.6 cpm per A_{260} mμ. Assuming an average molecular weight for the RNA to be 25,000, and using an extinction coefficient of 250 for a 1% solution of RNA, this incorporation of ^{14}C corresponds to 7.25×10^4 cpm per μmole of RNA, a ratio of 0.97 dimedone per RNA molecule. Identical experiments with unoxidized RNA resulted in ^{14}C incorporation at less than 2% of the level found with periodate-oxidized RNA.

High molecular weight RNA from bacteriophage f2 was oxidized and reacted with dimedone ^{14}C with a specific activity 2.83×10^6 cpm per μmole, and then precipitated with ethanol to remove excess dimedone, and centrifuged in a sucrose density gradient. Peak tubes averaged about 90 cpm per optical density unit, or 2250 cpm per mg of RNA. Using a molecular weight estimate of 1.1×10^6 for this RNA (Erikson, 1968), the incorporation is equivalent to 0.80 dimedone per 3′ terminus. A similar experiment with high molecular weight f2 RNA which had not been oxidized showed levels of radioactivity undetectable above background after centrifugation.

RNA extracted from *E. coli* B was oxidized, labeled with dimedone of specific activity 2.83×10^6 cpm per μmole, and fractionated by sucrose density gradient centrifugation. The 23S and 16S rRNA isolated from the gradients showed specific activities of 116 and 165 cpm per optical density unit, equivalent to one dimedone per 9.8×10^5 and one dimedone per 6.8×10^5 molecular weight, respectively.

B. Stability of the Terminally Labeled Product. tRNA was oxidized and treated with dimedone-^{14}C with a specific activity of 7.43×10^4 cpm per μmole and the stability at 0° of the product (as defined by ice-cold 70% ethanol-precipitable radioactivity) examined. The effect of pH is illustrated in Figure 2. Increasing the temperature to 37° resulted in appreciable degradation of labeled or unlabeled RNA (presumably due to a minor nuclease contamination of the commercial tRNA preparation), but the specific activity of the precipitated material was only slightly less than the result shown in Figure 2 except at pH values above 8. At neutral pH, frozen, labeled RNA appears completely stable for at least 1 month. Less complete experiments showed 0.1 M NH_4Cl or Tris·HCl labilized the derivative. Some (20%) of the ^{14}C label was lost in 24 hr at 0° and pH 7.0 in the presence of either of the above amines.

C. Stability of RNA to Dimedone Treatment. Terminally labeled RNA of *E. coli* was fractionated by gel electrophoresis and, as shown in Figure 3, could not be distinguished from untreated RNA and showed no signs of degradation due to the labeling process. The same RNA, subjected to sucrose density gradient centrifugation, gave a normal sedimentation profile and showed the level of incorporation of dimedone-^{14}C outlined in section A.

D. Isolation of Terminal Oligonucleotides. RNA isolated from bacteriophage f2 was terminally labeled with dimedone-^{14}C of specific activity 2.83×10^6 cpm per μmole, and the excess reagent removed by repeated precipitation with ethanol. The RNA was then digested with ribonuclease T_1 and chromatographed on DEAE-cellulose. If phosphate buffers were used for chromatography, the recovery of labeled terminal nucleotide (see Figure 4) was 0.60–0.82 μmole per μmole of RNA digested. The terminal oligonucleotide, identified by ^{14}C incorporation, was found to move with the peak containing primarily heptanucleotides, as was found

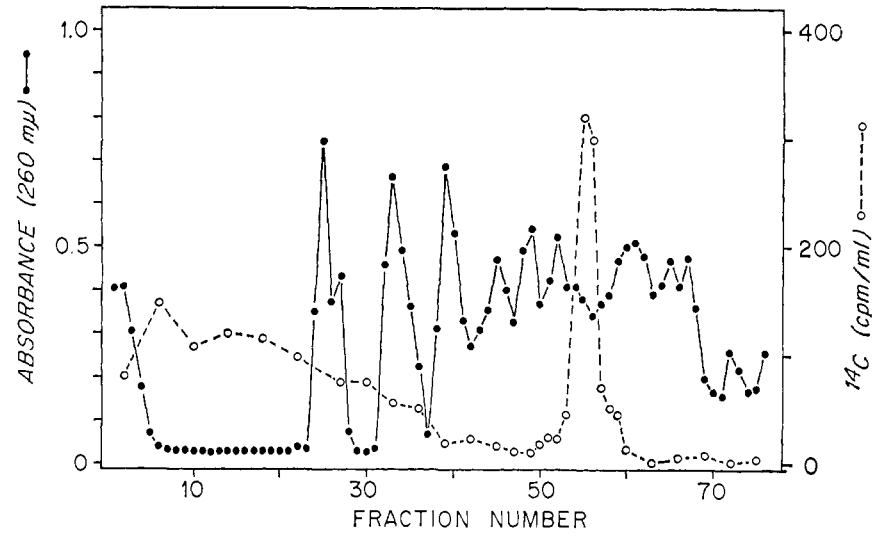


FIGURE 4: DEAE-cellulose chromatography of an RNase T_1 digest of terminally labeled f2 RNA. Dimedone-labeled bacteriophage f2 RNA (6 mg) was digested with 30 units of RNase T_1 and the digest applied to a column of DEAE-cellulose (1 × 12 cm) long. Nucleotides were eluted with 700 ml of buffer containing 7 M urea and 0.01 M potassium phosphate, pH 7.5, and a linear gradient of 0–0.4 M NaCl. Fractions (10 ml) collected at a flow rate of 0.4 ml/min.

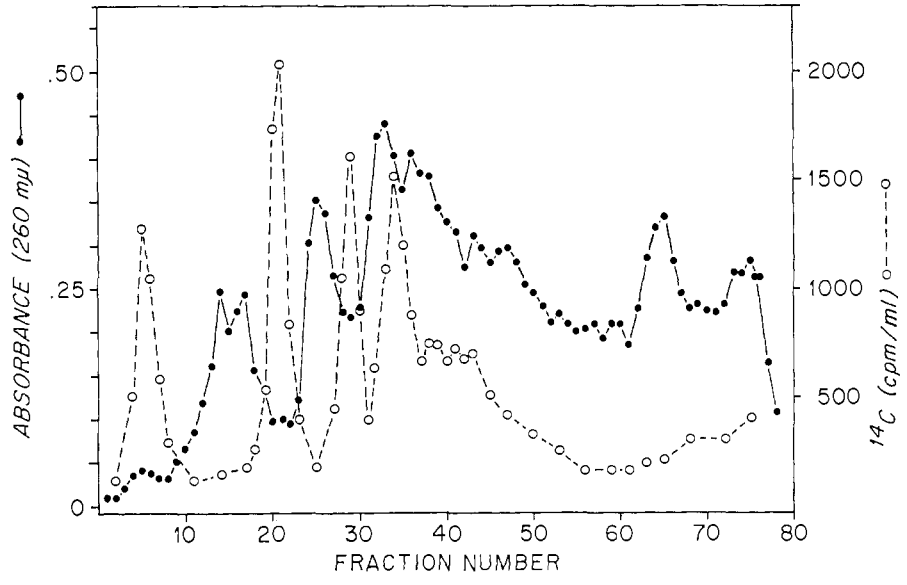


FIGURE 5: DEAE-cellulose chromatography of an RNase T_1 digest of terminally labeled tRNA. Dimedone-labeled tRNA (10 mg) was digested and fractionated as described in Figure 4.

in similar experiments using borohydride reduction (Glitz *et al.*, 1968). If buffers containing 0.01 M Tris·HCl were employed the yield of labeled terminal oligonucleotide was much lower, and often ^{14}C could not be detected in the samples in which the terminal oligomer should have been eluted from the column. Variable quantities of radioactivity were eluted from the column before the major radioactive peak. This label appeared to be nonspecific and was reduced (but never fully eliminated) by more extensive reprecipitation of the labeled RNA as was carried out in the case of tRNA illustrated in Figure 5.

Transfer RNA, labeled with dimedone-^{14}C of specific activity 7.43 × 10^4 cpm per μmole, was similarly digested and chromatographed on DEAE-cellulose to give the pattern of Figure 5. The heterogeneous distribution of radioactivity would be expected from mixed species of tRNA. (If the CpCpA termini of the tRNA molecules were not fully intact, some labeled nucleoside and dinucleoside monophosphate would be released by RNase T_1 digestion and would be eluted in the first radioactive peak of Figure 5.)

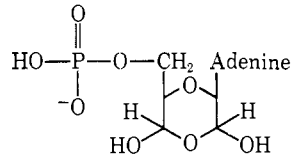
Degradation of terminally labeled tRNA with pancreatic ribonuclease should release ^{14}C-labeled nucleosides. Fractionation of such a digest by paper electrophoresis and strip counting to detect ^{14}C showed all of the radioactivity detectable to be associated with materials moving with the dimedone derivatives of adenosine and cytidine, with less than 10%

of the radioactivity applied to the paper (5800 cpm) not recovered from the area in which the dimedone derivatives of nucleosides moved.

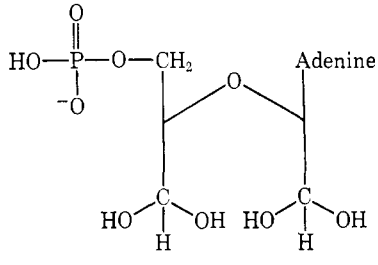
Discussion

The reaction of dimedone with periodate-oxidized RNA possesses most of the properties necessary for an effective end-labeling technique. All our evidence is consistent with the facile formation of a product composed of one dimedone per periodate-oxidizable terminus of RNA or mononucleotide. The ^{14}C label incorporated as dimedone into oxidized RNA is sufficiently stable to allow practical use as a terminal label. Dimedone remains associated with oxidized RNA or oligonucleotide through a variety of fractionations, and permits ready identification of the terminus-containing components. One noteworthy drawback is the instability of the product in the presence of amines, since it prohibits Tris- or ammonia-containing buffers for chromatography and electrophoresis.

From the data presently available we are not able to propose a unique structure for the nucleotide dialdehyde–dimedone product. Some suggestions of it however can be obtained from consideration of the probable structure of AMP dialdehyde. The infrared spectrum of AMP dialdehyde fails to demonstrate the presence of any carbonyl absorption characteristic of free aldehyde. Therefore, the AMP dialdehyde must exist in some type of hydrated form and the cyclic dioxane-like structure Ia would seem to be preferred over Ib. Related cyclic structures involving periodate-oxidized nucleosides and sugars have been suggested by a number of authors (Khym and Cohn, 1960; Khym, 1963; Hunt, 1965; Barry and Mitchell, 1953).

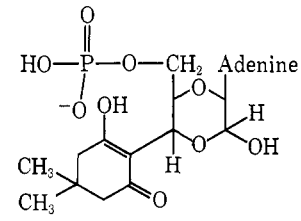


Ia

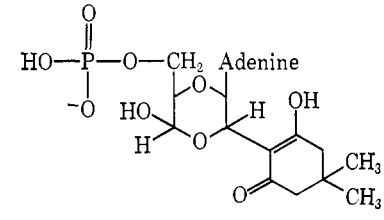


Ib

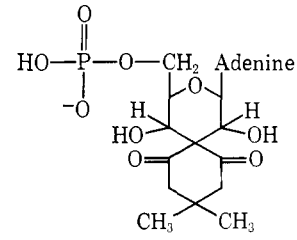
Assuming the cyclic structure Ia for AMP dialdehyde, the most probable structure for the AMP dialdehyde–dimedone product would appear to be IIa or IIb. A cyclic structure IIc in which the C-2 carbon of dimedone would be bound to the two aldehydic carbons (C-2 and C-3 of the initial ribose) would appear to be ruled out by the ultraviolet absorption spectrum of the derivative. This absorption spectrum has a pH dependence similar to that of either dimedone or the dimedone derivative of acetaldehyde. Therefore an enolizable proton with a pK_a of about 6 still



IIa



IIb



IIc

exists in the AMP dialdehyde–dimedone product. Despite our inability to propose an exact structure for the AMP dialdehyde–dimedone derivative at present, we have no evidence from numerous paper chromatograms and electrophoreses of the formation of more than one product.

Most important to the intended use of the labeling technique is that dimedone appears to be incorporated quite specifically at the 3′ terminus of RNA with no detectable degradation of the macromolecule in the process. The latter point is suggested by sucrose density gradient centrifugation, and especially by gel electrophoresis of labeled RNA. We have no evidence which suggests any detectable degradation of RNA during careful oxidation, dimedone labeling, and washing. The specificity of the incorporation at the terminus is shown by the data of Figures 4 and 5. The distribution of radioactivity is essentially what would be predicted from the sequence information available on the nucleic acids examined. In addition, the label is associated with the expected nucleosides upon enzymic degradation of tRNA. Whereas oxidized RNA incorporates one dimedone per RNA terminus in our experiments, unoxidized RNA shows little or no tendency to bind the label.

Nonspecific or noncovalent labeling of RNA with this technique appears to be rather small, and so it should be of value in end-group determinations with macromolecular RNA. In this respect, dimedone shows a clear advantage over borohydride reduction. The borohydride method yields more than the expected level of radioactivity in macromolecular RNA, and this nonspecific label remains associated with the RNA until it is extensively depolymerized (see, *e.g.*, DeWachter and Fiers, 1967; Glitz *et al.*, 1968).

We thus conclude that terminal labeling of RNA with dimedone provides a technique which is quantitative and

specific for 3′ termini, and produces a derivative which is sufficiently stable to permit further manipulation and characterization of terminal fragments.

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5,6-Dideoxy-5-oxoerythronolide B, a Shunt Metabolite of Erythromycin Biosynthesis*

Jerry R. Martin and Richard S. Egan

ABSTRACT: A $C_{21}H_{36}O_6$ dihydroxydioxolactone accumulated in fermentation broth by a blocked mutant of *Streptomyces erythreus* was isolated. Chemical and physical evidence indicated the structure of the lactone to be 5,6-dideoxy-5-oxoerythronolide B. The new lactone was not converted into erythromycin when added to the fermentation medium of both erythromycin-producing and -blocked-nonproducing strains of *S. erythreus*. However, the lactone is probably formed *via* the erythromycin pathway but is an aberrant metabolite of the pathway.

Erythromycins A–C, antibiotic glycosides elaborated by *Streptomyces erythreus*, are characterized by a branched-chain, macrocyclic polyhydroxyoxolactone aglycone moiety, erythronolide (Gerzon *et al.*, 1956; Wiley *et al.*, 1957). Despite relatively extensive work on the biogenesis of erythronolide, most of the process remains obscure. Early work (Vaněk *et al.*, 1961; Grisebach *et al.*, 1960; Corcoran *et al.*, 1960) indicated that propionate was somehow involved in the biosynthesis and later (Friedman *et al.*, 1964; Kaneda and Corcoran, 1961; Wawszkiewicz and Lynen, 1964; Grisebach *et al.*, 1962) the important role of 2-methylmalonate was recognized. Friedman *et al.* (1964) concluded from a wide variety of data that erythronolide biosynthesis most likely involves a "primer" of propionyl-CoA[1] and the condensation of six molecules of 2-methylmalonyl-CoA probably forming a 21-carbon polyoxolactone (I) (Scheme I). Reduction of appropriate oxo functions could result in the formation of 6-deoxyerythronolide B (II), a progenitor of erythromycin biosynthesis recently isolated by Martin and Rosenbrook (1967).

In an attempt to elucidate the biosynthesis of erythromycin we are examining the metabolites of a number of blocked mutants of *S. erythreus*. In paper III of this series (Martin and Perun, 1968), we reported the structure of 5-deoxy-5-oxoerythronolide B, a shunt metabolite of erythromycin biogenesis, from the fermentation broth of a blocked mutant (*S. erythreus*, Abbott 4EB40). Preliminary evidence indicated

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[1] The abbreviations used are: CoA, coenzyme A; TMSi, tetramethylsilane; ASIS, aromatic solvent-induced shifts.