New fluorescent hydrazide reagents for the oxidized 3'-terminus of RNA

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ABSTRACT

The synthesis and properties of four new fluorescent reagents capable of forming moderately stable links to the 3' oxidized end of RNA are reported. All are hydrazide derivatives: pyrene butyric acid hydrazide, proflavine monosemicarbazide, proflavine monosuccinic acid hydrazide, and anthracene-9-carboxaldehyde carbohydrazide. In addition, procedures are given for coupling the bifunctional reagent carbohydrazide to the 3' end of RNA. These carbohydrazide adducts can easily be coupled in turn to a wide variety of fluorescent reagents having specificity for aliphatic amino groups, including isothiocyanates and sulfonyl halides. Thus a route exists for the preparation of an enormous variety of 3' fluorescent labeled RNAs. The carbohydrazide adducts are also useful for other synthetic procedures such as preparation of covalent tRNA dimers.

INTRODUCTION

One of the main obstacles to fluorescence studies on nucleic acids has been the lack of specific stable dye conjugates. It has been known since 1960 that aldehyde reagents can be coupled under mild conditions to the periodate-oxidized 3' terminus of tRNA.¹ This fact has been exploited by various workers, who coupled fluorescent amines^{2,3} or hydrazines⁴ to 3' oxidized tRNA. The dyes used in the studies possessed a common characteristic -- all were acridines containing various functional groups. Because of the common chemical basis of these dyes, their spectral characteristics were quite similar. Furthermore, the aromatic amines used were found to form tRNA adducts which were quite unstable.

The purpose of this study was to develop new fluorescent reagents for use with periodate-oxidized RNA, and to expand the chemistry of the RNA 3' terminus so that existing fluorescent groups could be attached there. In addition, we sought to design a crosslinking reagent

which would attach to 3' oxidized RNA via a stable covalent bond, and then be capable of further reaction.

MATERIALS AND METHODS

1-Pyrenebutyric acid hydrazide (PBH): 1.0 g (3.47 mmoles) of 1-pyrene-butyric acid (purchased from Eastman Chemical Co.), was dissolved in 25 ml absolute ethanol. 50 mg of p-toluenesulfonic acid were added, and the solution was refluxed for 12 hr. TLC (acetone) on silica gel showed complete disappearance of the starting acid, $R_f=0.1$, and formation of a new product with $R_f=0.8$. The solution was concentrated to a few ml by evaporation. Ether and water were added, and the aqueous layer of the resulting two-phase mixture was washed with ether. The organic layers were then combined, washed in succession with saturated aqueous NaHCO_3 solution, and saturated NaCl and finally dried over anhydrous Na_2SO_4 . Rotoevaporation of the ether yielded 0.95 gm (87%) of nearly pure ester. An analytical sample, obtained by sublimation, had m.p. 49-50°. Its IR (KBr pellet) showed strong absorption at 5.79 (ester $\text{C}=\text{O}$). The NMR (CDCl_3) displayed signals at δ 8.1 (m, 9H, aromatic), 4.2 (quartet, 2H, $-\text{OCH}_2-$), 3.3 (triplet, 2H, $-\text{CH}_2-\text{CO}-$); 2.3 (m, 4H, aliphatic) and 1.2 (triplet, 3H, methyl).

900 mg (2.85 mmole) of 1-pyrenebutyric acid ethyl ester were suspended in 6 ml of 33% aqueous hydrazine. 50 ml of ethanol were added and the solution was refluxed with stirring for 2 hr, during which time some solid material deposited. TLC (acetone) on silica gel showed complete disappearance of the starting ester, $R_f=0.8$, and formation of a new product, $R_f=0.5$. The solution was cooled, and water was added to complete the precipitation of the hydrazide. The solid was filtered, washed with water, and recrystallized from ethanol/water. 820 mg (90%) of fine yellow needles were obtained. An infrared spectrum of the hydrazide revealed absorptions at 6.10 and 6.55 μ ($-\text{C}(\text{O})-\text{N}$). The NMR (CDCl_3) showed singals at δ 8.0 (m, 9H, aromatic), 3.9 (broad singlet, 3H, N-H), 3.4 (m, 2H, CH_2CO), 2.2 (m, 2H, aliphatic). Analysis: Found (calculated), C, 79.28 (79.44), H, 5.86 (6.00); N, 9.25 (9.27).

Anthracene-9-carboxaldehyde carbohydrazone (ACH): 1.0 g (4.9 mmole) of 9-anthraldehyde (Aldrich), dissolved in 50 ml of ethanol, was heated to reflux. 3.0 g (33 mmole) of carbohydrazide, dissolved in 10 ml of water, were added. A straw-colored product quickly precipitated. TLC (ethyl acetate) on silica gel after 1 hr indicated

complete disappearance of the starting aldehyde. Additional water was added to the cooled solution, which was filtered after an hour at 0°C. The yellow-orange product was washed thoroughly with excess water to remove any remaining carbohydrazide. A yield of 1.25 g (93%) was obtained.

The product was obtained in analytical purity by recrystallization from ethanol. Analysis: Found (calculated): C, 69.05 (69.05), H, 5.41 (5.07).

Proflavine monosemicarbazide (PSC): 1-2 g of proflavine (Aldrich) were suspended in 1500 ml of distilled H₂O, heated to boiling, and kept at 100° for 20 min with stirring. The hot solution was filtered under vacuum and concentrated to 1200 ml by heating under vacuum. It was allowed to cool slowly, during which time long, brown needles began to crystallize. After standing at 4°C overnight, crystals were filtered and dried. The recrystallized material showed a very clean NMR spectrum (DMSO d₆) with signals, at δ 8.0 (S, 1H), 7.3 and 6.55 (AB quartet, 2H) and 6.5 (S, 2H).

209 mg of recrystallized proflavine (1.0 mmole) were dissolved in 30 ml of boiling THF. The solution was cooled, and 54 mg (0.5 mmole) of ethyl chloroformate in 2 ml of THF were added. After several minutes, a precipitate formed in the stirred solution. One hour later, the precipitate was filtered, and both the supernatant and precipitate were analyzed by TLC (acetone). The precipitate, after treatment with Na₂CO₃ solution, was found to consist of approximately 10% monocarbamate and 90% proflavine. The supernatant contained roughly equimolar amounts of proflavine ($R_f=0.0$) and proflavine ethyl carbamate ($R_f=0.4$) plus a small amount of proflavine dicarbamate ($R_f=0.7$). The precipitate was redissolved in THF and retreated with ethyl chloroformate, and the solution filtered. The combined filtrates were rotoevaporated onto Celite, and the Celite was placed on a silica gel column. Elution with 1:1 hexane/acetone separated the products into pure mono and diurethane. A yield of roughly 55 mg (20% based on proflavine) of purified monourethane, in the form of shiny yellow flakes, was obtained. NMR signals (DMSO d₆) were observed in the regions δ 8.5-7.0 (7H, aromatic), 6.0 (S, 1H, H-N-C(O)), 4.2 (quartet, 2H, O-CH₂-), and 1.3 (triplet, 3H, CH₃).

To a solution of 300 mg of proflavine ethyl carbamate (1.07 mmole) dissolved in 10 ml of absolute ethanol, were added 3 ml of hydrazine

hydrate. The solution was refluxed for 50 hr, after which time TLC (acetone) showed nearly complete disappearance of the starting material. Two new spots, one with $R_f=0.1$, and the other with $R_f=0.0$, had formed. The faster moving spot was assumed to be the desired semicarbazide, and the slower one to be proflavine. The solution was rotoevaporated, and the residue dissolved in THF. After washing twice with aqueous NaCl, and drying over anhydrous $MgSO_4$, the THF solution was evaporated onto Celite. A silica gel column was run, using acetone as eluant, to purify the product as its acetone adduct. Approximately 50 mg (17%) of yellow solid were obtained.

Proflavine monosuccinic acid hydrazide (PSH): 209 mg (1 mmole) of recrystallized proflavine were dissolved in 100 ml of boiling THF. The solution was cooled to room temperature, and 75 mg (0.5 mmole) of β -carbomethoxypropionyl chloride (prepared as described by Cason⁵) in 10 ml of THF were added dropwise. A precipitate formed immediately, and was filtered after 30 min at room temperature. TLC (acetone) on silica gel showed that the filtrate contained a new product, $R_f=0.29$, in addition to a small amount of faster moving material, and a trace of proflavine. The solution was rotoevaporated onto Celite, and a silica gel column was run. 1:1 acetone/hexane was used to elute a product believed to be di-succinylated proflavine. Then 2:1 acetone/hexane eluted the purified monosuccinylated proflavine as an orange crystalline solid. Its NMR spectrum ($DMSO-d_6$) showed aromatic protons identical to those of monoacetylproflavine, indicating that reaction had indeed occurred to acylate one of the primary amino groups. In addition, signals were observed at δ 3.6 (s, 3, $O-CH_3$), and 2.65 (s, 4, $-CH_2-CH_2-$).

100 mg (0.3 mmole) of the succinylated proflavine derivative above were dissolved in 20 ml of ethanol. 1 ml of hydrazine hydrate was added, and the solution was refluxed at 60° for 1 hr. TLC (methanol) indicated complete disappearance of the ester and formation of a new, slower moving product. THF and aqueous NaCl were added. The organic layer was separated and washed three times with salt solution, dried over Na_2SO_4 , and rotoevaporated. The residue was redissolved in a small amount of methanol. Benzene was added and the solution was rotoevaporated again. The product was used without further purification.

Attachment of Labels to the 3' terminus of RNA: The selective

oxidation of the 3' terminal ribose ring of RNA is described by Zamecnik *et al.*¹ Excess IO_4^- was precipitated at 0° at KIO_4 and removed. The oxidized tRNA was twice precipitated by the addition of 0.1 volume of 2M NaCl and two volumes of ethanol to remove any remaining KIO_4 .

A typical direct labeling procedure: 10 mg of 3'-oxidized tRNA were dissolved in 1 ml of .05M NaOAc, pH 5.6, and 1.0 mg of PBH in 0.7 ml of spectral grade DMSO (Eastman) was added. (For each reagent, the minimum amount of DMSO needed to solubilize the dye during the reaction was used.) The cloudy solution was incubated for 2 hr at 37° after which time 2 volumes of ethanol were added to precipitate the tRNA. After centrifugation, the tRNA was redissolved in 3 ml of .01M Tris (Sigma Chemical Co.), 0.1M NaCl, .03M EDTA, pH 7.5. Centrifugation at 12,000x g for 10 min, to remove any undissolved dye, was followed by reprecipitation of the tRNA with ethanol. After 6 ethanol precipitations the supernatant was colorless and nonfluorescent. An unoxidized sample of tRNA contained less than 0.3% as much dye/tRNA as did the oxidized material. For the modified proflavine dyes, which intercalate strongly into nucleic acids, as many as 15 ethanol precipitations were needed to remove all non-covalently attached dye.

A typical carbohydrazide procedure: To 5 mg of periodate-oxidized tRNA, dissolved in 1 ml of .05M acetate buffer, pH 5.6, were added 4.5 mg of carbohydrazide (Aldrich Chemical Co.). The reaction was allowed to proceed for 2 hr. at 37°, followed by ethanol precipitation of the tRNA. Complete removal of unreacted carbohydrazide was achieved by 3 additional ethanol precipitations, or passage of the tRNA through a Sephadex G-100 column. To 5 mg of carbohydrazide-tRNA in 1.0 ml of Fluorescein isothiocyanate (FITC) in 0.2 ml DMSO. After incubation at 37° for 2 hr, the tRNA was precipitated by the addition of 2.5 ml of ethanol. Free dye was removed by 5 additional ethanol precipitations. After this treatment, control samples of oxidized tRNA which had not been exposed to carbohydrazide showed no evidence of reaction with FITC. The bishydrazides succinic acid dihydrazide and oxalyl dihydrazide were reacted with periodate-oxidized tRNA in the same way as was carbohydrazide.

Preparation of tRNA dimers: 15 mg of carbohydrazide-tRNA were incubated with 15 mg of periodate-oxidized tRNA in 2 ml of .05M acetate buffer, pH 5.3 for 4 hr. at 37°. The tRNA was then ethanol precipitated

CD measurements of monomeric and dimeric tRNA were run in a 1 cm cell, using a solution of 0.8 A₂₆₀ of tRNA in .01 M sodium phosphate, 0.1 M MgCl₂, pH 7.0 buffer.

Preparation and Labeling of Ribosomal RNA: Phenol extracted, ethanol precipitated E. coli A-19 ribosomal RNA was a gift from Dr. Kuei Huang.

270 A₂₆₀ of ribosomal RNA was dissolved in 3 ml of 0.1 M sodium acetate buffer, pH 5.6, and oxidized by addition of 1.0 mg of NaIO₄. The solution was made 0.02% in SDS to inhibit any ribonuclease activity, and allowed to stand in the dark at room temperature for 30 min. It was then dialyzed against the acetate/SDS buffer to remove NaIO₄. 0.65 mg of anthracene-9-carbohydrazone, dissolved in 1.5 ml of DMSO, were added to the RNA solution, and the combined solution was incubated for 1.7 hr. at 37° C. Reaction was stopped by the addition of 3 volumes of ethanol to precipitate the RNA.

Determination of Fluorescence Quantum Yields: Quantum yields (ϕ) were determined by comparison of uncorrected emission spectra with the emission of either fluorescein in 0.1 M NaOH or quinine sulfate in 0.1 M H₂SO₄. The quantum yields used for these reference compounds were 0.96 and 0.70 respectively.^{6,7} To determine the unknown ϕ , both the reference compound and the dye in question were excited at a particular wavelength. ϕ_{dye} was then calculated, using a computer program which compares the integrated emission spectra, per absorbance unit, for the unknown and reference dyes. This program was written by Mr. Robert Fairclough.

RESULTS

A series of fluorescent compounds has been linked to the periodate oxidized 3' terminus of tRNA. These dyes fall into two categories: 1) newly synthesized fluorescent aldehyde reagents which react directly with the 3' terminus of RNA; 2) amine reagents originally developed for use with proteins, or fluorescent aldehydes, which have been attached to RNA for the first time. The latter group of reagents were linked to the RNA via a bridging carbohydrazone molecule.

Synthesis of Fluorescent Aldehyde Reagents: This group of reagents all possess the hydrazide function, and can be represented by the general structure R-CO-NH-NH₂. Four such dyes have been synthesized; two of these contain in the R group a proflavine moiety, one an anthracene, and one a pyrene chromo-

phore.

The syntheses of proflavine monosemicarbazide (PSC) and proflavine monosuccinic acid hydrazide (PSH), are illustrated in Figure 1. Both syntheses begin with monoacylation of 2,6-diamino-acidine, or proflavine. Although none of the more volatile organic solvents were found to be particularly suitable for dissolving proflavine, tetrahydrofuran (THF) proved to be best, and was used as a general solvent for this type of compound. Acylation was carried out by addition of the appropriate acid chloride to a solution of proflavine in THF, which also contained triethylamine to scavenge HCl.

Monoacylation could be accomplished quite easily, with the formation of only minor amounts of diacetylated material. Such selectivity has also been observed in the reaction between stoichiometric amounts of proflavine and acetic anhydride, which forms monoacetyl proflavine in nearly quantitative yield.⁸ The deactivation of the second proflavine amino group after reaction of the first is apparently quite substantial, since the high reactivity of the acyl halide should otherwise lead to random distribution of products.

The monoacetylated proflavine derivatives were reacted with excess hydrazine, to give the final products in moderate yield. Some proflavine was regenerated in the reaction, due to cleavage of the amide bond by hydrazine.

Anthracene-9-carbohydrazone (ACH) was produced by the condensation of anthracene-9-carboxaldehyde with carbohydrazone (Figure 2). The use of a 10-fold excess of carbohydrazone was desirable, to avoid further reaction of ACH with another molecule of the starting aldehyde. Since ACH seemed to precipitate from solution soon after it was formed, this precaution made it extremely unlikely that such coupling would occur. In fact, no trace of any coupled by-product was observed.

As shown in Figure 2, the synthesis of 1-pyrenebutyric acid hydrazide was accomplished in two steps, using standard techniques for the preparation of acid hydrazides. Both reactions proceeded in very high yield, and the pure hydrazide was easily crystallized from an ethanol/water mixture.

Attachment of Fluorescent Aldehyde Reagents: Figure 3 illustrates the technique, developed by Zamecnik *et al.* (1960),

SYNTHESES OF DYES FOR THE 3' END OF RNA

Fig.1. Reaction sequences used for the syntheses of the aldehyde reagents proflavine monosemicarbazide (PSC) and proflavine monosuccinic acid hydrazide (PSH)

SYNTHESES OF DYES FOR THE 3' END OF RNA

Fig.2. Reaction sequences employed in the syntheses of the hydrophobic aldehyde reagents anthracene-9-carbohydrazone (ACH) and pyrenebutyric acid hydrazone (PBH).

MODIFICATION OF 3' TERMINUS OF RNA

Fig.3. (Top) Illustration of the conditions required for oxidation of the 3' terminus of RNA, and subsequent reaction with NH_2 -containing compounds. (Bottom) Addition of carbohydrazide to the oxidized 3' terminus. B = any of the RNA bases.

for coupling aldehyde reagents to the 3' terminus of RNA. The diagram depicts the theoretical formation of hydrazones at both aldehyde groups, a situation which has not been achieved in practice with the compounds under consideration. Table I lists the actual dye/tRNA ratios which have been achieved. It can be seen that in no case was a ratio of greater than 1:1 dye/tRNA above the unoxidized control obtained. Because of the uncertainty in the extinction coefficient of bound dye, this number may actually represent a 1:1 ratio of dye/tRNA. The hydrazine adducts in Figure 3 are written as ring-opened bis derivatives. However the 1:1 yield and stability of the products suggests that they may actually be monoadducts, followed by ring closure to yield morpholine derivatives. This kind of product has been suggested previously by others.⁹

Attachment of Fluorescent Reagents via the Carbohydrazide

Method: In an effort to link dyes which do not contain a hydrazide function to the 3' terminus of RNA, we explored the possibility of first attaching a dihydrazide molecule at this site. The lower diagram of Figure 3 illustrates the general reaction, using the smallest dihydrazide available -- carbohydrazide. Again, a 2:1 reaction with the terminal ribose is depicted for simplicity, although in practice this structure has not been demonstrated. In performing the reaction, a large excess of the dihydrazide is used, to minimize coupling of the aldehyde functions.

As can be seen, the desired product contains unreacted hydrazide groups. The availability of these groups for further reaction was measured as shown in Figure 4. The tRNA-carbohydrazide adduct was treated with anthracene-9-carboxaldehyde, a dye which does not react with unmodified or oxidized tRNA. In order for linkage between the anthracene group and the tRNA to occur, a carbohydrazide bridge must form. It was found that 0.64 dye molecule were covalently bound per carbohydrazide-treated tRNA. The product of both reactions is that shown in Figure 4. In one reaction carbohydrazide is joined first to the dye, while in the other it is attached first to the tRNA.

Two other dihydrazides were similarly tested for their ability to link anthracene-9-carboxaldehyde to tRNA. Both succinic acid dihydrazide and oxalyl dihydrazide stimulated the attachment of the dye, although to a considerably lesser extent

TABLE I Dyes Attached Directly to the Oxidized 3' Terminus of tRNA^a

Dye	Dye/tRNA ^b		λ_{abs} max	λ_{em} max	ϵ^c	ϕ
	3' Oxidized	Control				
Anthracene-9-Carbohydrazone (ACH)	0.63	0.002	393nm	456nm	8×10^3	.017
Proflavine Monosemicarbazide (PSC)	1.20	0.070	445	516	$.4.6 \times 10^3$	.021
Pyrene Butyric Acid Hydrazide (PBH)	0.62	0.004	348	397	36.5×10^3	.023
Proflavine Monosuccinic Acid Hydrazide (PSH)	1.20	0.30	457	528	13.1×10^3	.012

^a Unfractionated yeast tRNA.

^b Estimated assuming no hypochromicity of the bound dye. Recent work by Paul Koenig has shown that PBH exhibits significant hypochromicity when attached to tRNA. For this reason, the dye/tRNA ratio reported for this compound is low by 40% or more.

^c ϵ values for the free dyes are listed. Other properties are those of the tRNA-bound dyes.

<u>Bis-Hydrazide</u>	<u>Dye/tRNA Ratio</u>
$\begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{NNHCNHNH}_2 \end{array}$	0.64
$\begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N NHC} \sim \text{CNHNH}_2 \\ \parallel \\ \text{O} \end{array}$	0.31
$\begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{NNHC} - \text{C} - \text{NHNH}_2 \\ \parallel \\ \text{O} \end{array}$	0.09
NO OXIDATION	0.04
NO BIS-HYDRAZIDE	0.00

Fig.4. Use of various dihydrazides to couple ACH to tRNA. The reaction is illustrated for carbohydrazide. Under identical conditions, the other dihydrazides give the dye/tRNA ratios listed.

than did carbonylhydrazide. For this reason, the latter reagent was used exclusively to link other chromophores to tRNA.

The free hydrazide groups of the tRNA-carbonylhydrazide adduct will react with a wide variety of fluorescent reagents in common use for modification of protein amino groups. Table II lists four dyes which have been linked to tRNA by carbonylhydrazide. Yields in the order of 0.65 dye/tRNA have been obtained, with a high specificity for oxidized vs. unoxidized tRNA. The stability of all of these tRNA derivatives is far greater than that of previously reported fluorescent conjugates. For example, the kinetics of dye release from PBH-tRNA are apparent first order. Even at 60° in .01M Tris, pH 7.5, the half life of hydrolysis is still 95 min. At lower temperatures the conjugates are very much more stable.

Physical properties of Bound dyes: Also listed in Tables I and II are absorption and emission maxima of the tRNA-bound dyes, plus their measured quantum yields. A wide spectral range is covered by the chromophores represented. With the exception of fluorescein isothiocyanate (FITC), the bound dyes were found to possess surprisingly low quantum yields. In at least some of the cases, this phenomenon was apparently caused by tertiary interactions between the dye and a part of the tRNA molecule. Several independent lines of evidence point to this conclusion. For one, pancreatic RNAase digestion of tRNA containing 3'-linked PSH (PSH-tRNA) caused a 3-fold increase in the PSH fluorescence. The time required for completion of the fluorescence change was dependent upon RNAase concentration.

A second finding supports the notion of quenching by tertiary interactions. Yang discovered that PSH linked to an aldehyde on the dihydro U arm of tRNA has a quantum yield roughly ten times higher than that observed in this study for 3'-linked PSH.¹¹ Since covalent bonding to the chromophore is identical, noncovalent interactions must be responsible for this phenomenon. Finally, a study of PBH-tRNA and PBH linked to homopolymers revealed that under some conditions, but not all, the fluorescence of the bound dye is strongly quenched.

tRNA Dimers: Since we had demonstrated the ability to link virtually any aldehyde to the periodate-oxidized 3' terminus of tRNA, it was of interest to determine whether two 3' oxidized tRNA molecules could be joined by this method. We first measured

TABLE II Dyes Attached Via the Carbohydrazide Method to the Oxidized 3' Terminus of tRNA^a

Dye	3' Oxidized	Dye/tRNA		$\lambda_{\text{max}}^{\text{abs}}$	$\lambda_{\text{max}}^{\text{em}}$	ϵ^b	
		Control					ϕ
Dansyl Chloride	c	c		~340nm	500nm	-	-
Anthracene-9-Carboxaldehyde	0.64 ^d	0.00		393	465	8×10^3	.017
NBD Chloride ^g	e	e		397	non-fluorescent		
Fluorescein-Isothiocyanate (FITC)	0.65 ^d	0.00		496	518	43×10^3 ^f	.30

^a unfractionated yeast tRNA.

^b ϵ values for the free dyes are listed. Other properties are those of tRNA-bound dyes.

^c Not accurately determinable, since the tRNA absorption band overlaps the weak dansel absorption.

^d Assuming the same extinction of free and bound dye.

^e The ϵ value of bound NBD choride, which differs considerably from that of free dye, was not measured. Therefore, no dye/tRNA ratio could be calculated. A ratio of $A_{397}/A_{260} = 6.9 \times 10^{-3}$ was found.

^f Determined by Tengerdy and Chang¹⁰.

^g 7-chloro-4-nitrobenz-2-oxa-1,3-diazole.

the chromatographic behavior of tRNA's with various 3' modifications of Sephadex G-100. The pattern we observed of unfractionated yeast tRNA, by monitoring A_{260} eluted from the column, was essentially identical to that reported for tRNA^{ala}.¹² Unfractionated tRNA contained a small amount of high molecular weight material, a well-resolved dimer peak, and the large monomer peak. 3' oxidized tRNA shows no aggregated material, and very little of the dimeric species. The oxidized tRNA was treated with excess carbohydrazide, and rechromatographed on a Sephadex G-100 column. An elution pattern superimposable with that of the original periodate-oxidized tRNA was obtained. Clearly, no coupling of tRNA molecules, or other gross change in size, had occurred during this process.

After removal of unreacted carbohydrazide, the tRNA-carbohydrazide adduct was reacted with an equivalent amount of periodate-oxidized tRNA. Figure 5 (top) shows the elution pattern obtained from one such reaction. The major component of the mixture is now eluted at the same rate as the original tRNA dimers. The new covalent dimers are of course different in structure than the original "natural" dimers; however, they are apparently similar enough in overall shape and size to give closely resembling elution patterns. Figure 5 (bottom) shows a rechromatography of pooled dimer fractions from the sample. It can be seen that nearly pure dimer is obtained by a single passage through the large (90x4 cm) Sephadex column. After this work was completed Abermann and Yoshikami also reported the synthesis of covalent tRNA dimers by a similar technique using larger bishydrazides.¹³

The CD spectrum of the tRNA dimers was measured, in order to determine whether these molecules differed significantly from monomeric tRNA in terms of secondary and tertiary structure. It was found that the two species were virtually indistinguishable by CD. The trough at 295 nm, indicative of double stranded regions, was clearly present in both cases.¹⁴ No significant shift in the positive band at 265 nm was observed. These results suggest that the 3' end of tRNA is not strongly coupled to major regions of secondary and tertiary structure. This is consistent with the crystal structure of tRNA^{Phe}.¹⁵

Labeling of Ribosomal RNA with ACH: Ribosomal RNA, prepared

Fig.5. (Top) Elution pattern on Sephadex G-100 showing the appearance of tRNA dimers after reaction of tRNA-carbohydrazide with 3' oxidized tRNA. (90x4 cm column).

(Bottom) Nearly pure tRNA dimer isolated from the Sephadex G-100 column, as above, and rechromatographed on the same column. D = dimer; M = monomer. ³Eluant is 0.15 M KCl, 0.01 M sodium cacodylate; 5×10^{-3} M MgCl₂, 5×10^{-4} M EDTA, pH 7.0.

Fig.6. Centrifugation of 100 A₂₆₀ of unlabeled (A) and ACH labeled (B) ribosomal RNA through 5 ml gradients of 5-20% sucrose in .01M Tris, pH 7.5, .1M NaCl, .03M EDTA buffer (TNE buffer). The gradients were run for four hours at 190,000 x g, and then dripped into separate tubes. Aliquots of 16S RNA, isolated from large (37 ml) sucrose gradients, were centrifuged through the same (5 ml) medium for 4.5 hours at 190,000 x g, to give the profiles (C) and (D).

from 70S E. coli ribosomes by phenol extraction of the protein, was treated first with sodium periodate and then ACH. Centrifugation of the RNA through a small sucrose gradient resulted in the profile drawn in Figure 6B. Comparison of this profile with that seen in Figure 6A reveals that the labeling procedure caused no apparent damage to the RNA. Since a single break in one of the large RNA molecules could substantially change its sedimentation rate, the similarity in the two profiles rules out the possibility that an appreciable amount of such breakage occurred.

Analysis of the central band of each gradient, corresponding to 16S rRNA, was made on small gradients. Figures 6C and 6D, the profiles of the gradients, demonstrate that the labeled 16S rRNA is intact, and has been freed from other rRNA by the large gradient. A labeling ratio of approximately 0.8 dye/16S rRNA molecule was measured. This system is of interest because of the possibility of reconstituting fluorescently labeled 16S rRNA by the procedure of Traub and Nomure to produce 30S ribosomal subunits containing a fluorescent probe at a specific site.¹⁶

DISCUSSION

The general usefulness of carbonyldiimidazole as a coupling agent for attaching fluorescent chromophores to the 3' end of RNA's has been demonstrated. This should permit a wide variety of new fluorescent derivatives of tRNA to be made. The reaction conditions with hydrazide reagents are sufficiently mild that few side reactions occur. Even 16S rRNA can be labeled without apparent breakage of the polynucleotide chain. Carbonyldiimidazole should be a useful reagent for the preparation of other nucleic acid analogs. For example, 4-fluoro-3-nitro-phenylazide¹⁷ has been reacted with carbonyldiimidazole to yield an aldehyde reagent that has potential as a photoaffinity reagent¹⁸. Many similar chemically active derivatives should be accessible through the use of carbonyldiimidazole.

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