Fluorescence Reaction of Ribonucleosides and Ribonucleotides with 1,2-Bis(4-methoxyphenyl)ethylenediamine

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Ribonucleosides and ribonucleotides produce fluorescence (excitation maxima, *ca.* 340 nm; emission maxima, *ca.* 470 nm) when heated in an acidic solution with *meso*-1,2-diarylethylenediamines which have phenyl groups substituted with electron-donating groups at the 4-positions, after periodate oxidation. Of the 1,2-diarylethylenediamines tested, *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine was the most sensitive and permitted the fluorometric determination of ribonucleosides and ribonucleotides at concentrations as low as 60—500 pmol/ml. The compound also produces fluorescence with reducing sugars.

Keywords 1,2-diarylethylenediamine; *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine; fluorogenic reagent; ribonucleoside; ribonucleotide; periodate oxidation

Although non-specific measurement of ultraviolet absorption has frequently been used for the determination of nucleic acid bases and their nucleosides and nucleotides, several fluorogenic and chromogenic reagents selective for these compounds have also been reported: chloroacetaldehyde,[1,2] glyoxal hydrate trimer[3] and phenylglyoxal[4] react with adenyl and cytosyl, adenyl, and guanyl groups of the compounds, respectively, to form fluorescent derivatives; molybdic acid that has been employed as a chromogenic reagent for phosphoric acid,[5,6] has been applied to the color reaction of the acid derived from nucleotides; 3,5-diaminobenzoic acid[7-9] yields a fluorescent compound and diphenylamine[10] and orcinol[11] give coloration, when they react with 2-deoxyribose, 2-deoxyribose and ribose liberated by acid hydrolysis of nucleosides and nucleotides.

1,2-Diarylethylenediamines (DAEs) have been developed as fluorogenic reagents for both catecholamines[12] and reducing carbohydrates.[13] It has recently been found that some DAEs also provide fluorescent compounds with ribonucleosides and ribonucleotides when heated in an acidic solution after periodate oxidation. This paper describes the fluorescence reactivities of DAEs towards ribonucleosides and ribonucleotides and the optimum conditions for their fluorometric determination with *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine (p-MOED), chosen as the most suitable reagent among the tested DAEs. Guanosine, adenosine-5′-triphosphate (ATP), cytidine-5′-triphosphate (CTP) and guanosine-5′-monophosphate (GMP) were used as model compounds to establish suitable reaction conditions.

Experimental

Reagents and Solutions The following 28 DAEs were synthesized as described previously[12]: 1,2-diphenylethylenediamine, 1,2-bis(2-hydroxyphenyl)ethylenediamine, p-MOED, 1,2-bis(2- and 3-methoxyphenyl)-ethylenediamines, 1,2-bis(2- and 4-ethoxyphenyl)ethylenediamines, 1,2-bis(2-, 3- and 4-methylphenyl)ethylenediamines, 1,2-bis(4-ethylphenyl)-ethylenediamine, 1,2-bis(3,4-dimethoxyphenyl)ethylenediamine, 1,2-bis-(3,4-methylenedioxyphenyl)ethylenediamine, 1,2-bis(4-fluorophenyl)-ethylenediamine, 1,2-bis(2-, 3- and 4-chlorophenyl)ethylenediamines, 1,2-bis(2,6- and 3,4-dichlorophenyl)ethylenediamines, 1,2-bis(4-cyanophenyl)-ethylenediamine, 1,2-bis(4-biphenylyl)ethylenediamine, 1,2-bis(1- and 2-naphthyl)ethylenediamines, 1,2-bis(2-furyl)ethylenediamine and 1,2-bis-(3-pyridyl)ethylenediamine, all in the *meso* form; and 1,2-diphenylethylenediamine, 1,2-bis(4-methoxyphenyl)ethylenediamine and 1,2-bis(4-methylphenyl)ethylenediamine, all in the DL form. Each DAE solution (10 mM) was prepared in methanol. Deionized, distilled water was used. All other chemicals were of reagent grade. The standard solutions of nucleosides and nucleotides were prepared in water except that guanosine was dissolved in 0.1 M hydrochloric acid (10 μmol/ml) and diluted to various concentrations with water.

Apparatus Uncorrected fluorescence spectra and intensities were measured with a Hitachi MPF-4 spectrofluorometer using semimicro quartz cells (10-mm length parallel to the excitation beam, 3-mm width parallel to the emission beam, 1 ml); spectral bandwidths of 10 nm were used in both the excitation and emission monochromators.

Procedure for Screening of DAEs Using Guanosine Aliquots of 0.5 ml of 50 mM HCl and 0.25 ml of 0.5 mM $NaIO_4$ were added to 1.0 ml of 10 nmol/ml guanosine solution in a screw-capped 3.5-ml reaction vial, and the mixture was allowed to stand at room temperature (approximately 23—27 °C) for 5 min. Then 0.25 ml of 0.5 mM Na_2SO_3 (to decompose the excess oxidant) and 0.5 ml of a DAE solution were added. The vial was tightly closed and heated at 140 °C for 10 min, and then cooled in ice-water. The reagent blank was prepared in the same way except that 1.0 ml of the guanosine solution was replaced with 1.0 ml of water. The fluorescence intensities of the test and blank were measured at the respective excitation and emission maxima (see Table I).

Procedure for the Fluorometric Determination of Ribonucleosides and Ribonucleotides with p-MOED Aliquots of 0.5 ml of 50 mM HCl and 0.25 ml of 1.0 mM $NaIO_4$ were successively added to 1.0 ml of aqueous sample solution in a screw-capped 3.5-ml reaction vial, and the mixture was allowed to stand at room temperature (approximately 23—27 °C) for 5—10 min. Then 0.25 ml of 1.0 mM Na_2SO_3 and 0.5 ml of 10 mM p-MOED solution were added, and the mixture was heated at 140 °C for 10 min. To prepare the reagent blank, the same procedure was carried out except that 1.0 ml of the sample solution was replaced with 1.0 ml of water. The fluorescence intensities were measured at an emission wavelength of 470 nm with irradiation at 340 nm.

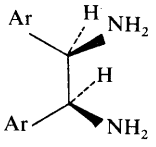
Results and Discussion

Screening of DAEs Of the DAEs examined, seven DAEs afforded fluorescence with guanosine (Table I). *meso*-DAEs which have phenyl groups substituted with electron-donating groups at the 4-positions seemed to yield strong fluorescence with longer excitation and emission wavelengths. p-MOED gave the most intense fluorescence and was selected for further investigation.

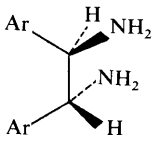
The fluorescence excitation and emission maxima for the reaction mixture of p-MOED with guanosine were at 340 and 470 nm, respectively (Fig. 1). On irradiation at 340 nm, the intensity of the reagent blank was 1.0% of that given by 10 nmol/ml guanosine.

Reaction Conditions Ribonucleosides and ribonucleotides, after periodate oxidation, reacted with p-MOED to produce fluorescence; other oxidizing agents including potassium hexacyanoferrate(III), iodine and hydrogen peroxide were ineffective. Since periodate can oxidatively cleave vicinal diol group into two carbonyl groups, and

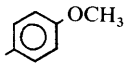
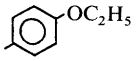
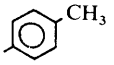
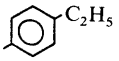
TABLE I. Excitation and Emission Maxima of the Fluorescences Produced of the Reaction by Guanosine with Various DAEs, and Their Relative Fluorescence Intensities[a]



meso form



DL form

DAE	Ar	Excitation maximum (nm)	Emission maximum (nm)	RFI[b]	
				Test	Blank
meso form					
p-MOED		340	470	100	1
1,2-Bis(4-ethoxyphenyl)ethylenediamine		340	470	81	17
1,2-Bis(4-methylphenyl)ethylenediamine		330	430	39	5
1,2-Bis(4-ethylphenyl)ethylenediamine		320	420	12	19
1,2-Bis(3,4-methylenedioxyphenyl)ethylenediamine		330	420	39	17
1,2-Diphenylethylenediamine		310	400	8	21
DL form					
1,2-Diphenylethylenediamine		310	405	10	20

a) Portions (1.0 ml) of 10 nmol/ml guanosine solution were treated according to the procedure for screening. *b*) Relative fluorescence intensity. The intensity obtained with p-MOED was taken as 100.

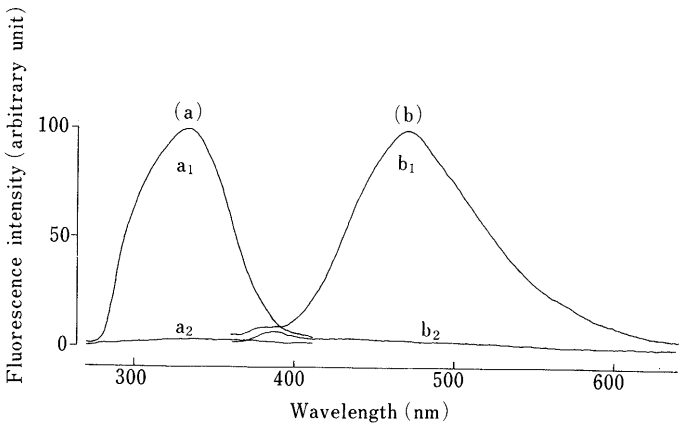


Fig. 1. Fluorescence Excitation and Emission Spectra of the Final Reaction Mixture of Guanosine with p-MOED

(a) Excitation spectra; (b) emission spectra. a_1 and b_1: a portion(1.0 ml) of 10 nmol/ml guanosine solution was treated according to the standard procedure. a_2 and b_2: the reagent blank.

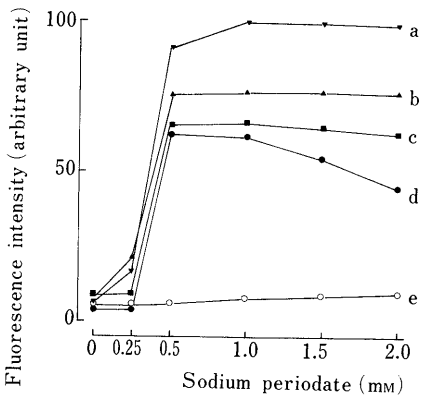


Fig. 2. Effect of Sodium Periodate Concentration on the Fluorescence Development from ATP, CTP, GMP and Guanosine

Portions (1.0 ml) of 10 nmol/ml solutions were treated as recommended at various concentrations of sodium periodate. (a) ATP; (b) CTP; (c) GMP; (d) guanosine; (e) the reagent blank.

deoxyribonucleosides and deoxyribonucleotides did not react with p-MOED even after periodate treatment, the two carbonyl groups derived from 2′,3′-diol of the ribosyl residue in ribonucleosides and ribonucleotides appear to participate in the fluorescence reaction.

ATP, CTP, GMP and guanosine were effectively oxidized with periodate at concentrations of *ca.* 0.5 mM and greater (Fig. 2); 1.0 mM was used as the optimum. pH had no effect on the oxidation reaction in the range of 2.0—6.0; pH 2.3 was employed, which was also optimum for the fluorescence reaction. The oxidation reaction came to completion within 4 min at room temperature (23—27 °C) and prolonged standing for at least 20 min had no effect; standing at room temperature (23—27 °C) for 5—10 min was selected for the standard procedure. Excess periodate interfered with the fluorescence reaction probably due to the degradation of p-MOED, and should therefore be decomposed with sodium sulfite. Maximum and constant fluorescence intensities were achieved in the sodium sulfite

concentration range of 0.5—2 mM; 1.0 mM solution was selected for the standard procedure.

Concentrations of p-MOED ranging from 10 to 30 mM in the reagent solution gave almost maximum and stable fluorescence intensities, but a higher concentration of the reagent caused an increase in the background fluorescence; 10 mM p-MOED was employed to obtain the highest ratio of the fluorescence intensity from each ribonucleoside or ribonucleotide to that of the reagent blank. p-MOED was slightly soluble in water but freely soluble in water-miscible organic solvents such as methanol, ethanol and isopropanol. Furthermore, these solvents accelerated the fluorescence reaction. Methanol accelerated the reaction most effectively over the concentration range of 20—30% (v/v) in the reaction mixture; we chose 20% in the reaction mixture, which was actually used as a solvent for p-MOED.

Hydrochloric acid at a concentration of 50 mM gave maximum fluorescence intensities for all the tested compounds and the blank value decreased with increasing concentration of hydrochloric acid (Fig. 3); 50 mM was selected as optimum (pH of the reaction mixture, 2.3).

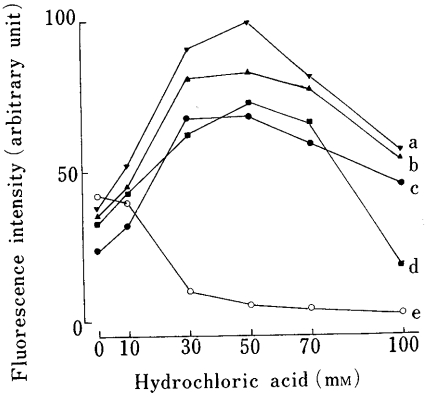


Fig. 3. Effect of Hydrochloric Acid Concentration on the Fluorescence Development from ATP, CTP, GMP and Guanosine

Portions (1.0 ml) of 10 nmol/ml solutions were treated as recommended at various concentrations of hydrochloric acid. (a) ATP; (b) CTP; (c) GMP; (d) guanosine; (e) the reagent blank.

Although 0.1 M citrate buffer (pH 2.3), 0.1 M tartarate buffer (pH 2.3), 30 mM sulfuric acid and 50 mM perchloric acid could also be used instead of the hydrochloric acid (pH of the reaction mixtures, 2.3), the observed fluorescence intensities were 88—97% of that given by 50 mM hydrochloric acid for the tested compounds.

The fluorescence reaction occurred at temperatures higher than *ca.* 120 °C. The maximum and stable fluorescence intensities were attained by heating at 120 °C for 15—25 min, at 130 °C for 10—20 min or at 140 °C for 5—15 min. The background fluorescence, however, increased with prolonging reaction time. Therefore, heating at 140 °C for 10 min was selected as optimum. The fluorescence in the final mixture was stable for at least 2 h in daylight at room temperature, and pH in the range of 2.0—12.0 had no effect on the fluorescence spectra and intensity of the mixture.

The calibration graphs for the compounds were linear over the concentration range of 0.1—50 nmol/ml. The precision was established by repeated determinations of 10 nmol/ml solutions of ATP, CTP, GMP and guanosine. The relative standard deviations were 1.9, 1.8, 1.5 and 2.1%, respectively ($n=10$ in each case).

Fluorescence from Diverse Ribonucleosides, Ribonucleotides and Reducing Sugars Ribonucleosides including those having modified bases and ribonucleotides other than the tested compounds fluoresced under the recommended conditions (Table II). Reducing sugars also yielded fluorescences, of which the intensities were lower than those from ribonucleosides and ribonucleotides. Some examples are given in Table II. The excitation (maxima, around 340 nm) and emission (maxima, around 470 nm) spectra of the fluorescences from those compounds were almost identical to those of guanosine.

Reaction of Other Substances The following substances did not fluoresce under the recommended conditions at concentrations as high as 100 nmol/ml: nucleic acid bases (adenine, guanine, cytosine, uracil and thymine), deoxyribonucleosides (thymidine, 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, 2′-deoxyuridine and 3′-deoxyguanosine), deoxyribonucleotides (2′-deoxygua-

Table II. Relative Fluorescence Intensities Obtained by the Reaction of Various Ribonucleosides, Ribonucleotides and Reducing Sugars with p-MOED, and Their Detection Limits[a]

Compound	RFI[b]	LD[c] (pmol/ml)	Compound	RFI[b]	LD[c] (pmol/ml)
Nucleosides					
Adenosine	144	160	ADP	153	170
Cytidine	154	150	ATP	147	180
Guanosine	100	230	CTP	122	210
Uridine	135	170	GMP	107	210
Xanthosine	139	160	Adenylyl-(3′—5′)-uridine	103	250
Inosine	105	220	Sugars		
Pseudouridine[d]	352	60	D-Ribose	57	460
5-Methylcytidine[d]	89	250	D-Glucose	20	660
1-Methylguanosine[d]	143	160	D-Arabinose	36	630
N^2-Methylguanosine[d]	186	120	D-Mannose	41	550
7-Methylguanosine[d]	52	490	Lactose	43	530
1-Methylinosine[d]	182	120	*N*-Acetylglucosamine	25	900
Nucleotides			D-Galactose	36	630
AMP	155	170	D-Fructose	30	750

a) Portions (1.0 ml) of 10 nmol/ml solutions of various compounds were treated according to the standard procedure. *b*) The fluorescence intensity from guanosine was taken as 100. *c*) Limit of detection. Defined as the concentration in the sample solution which gave a fluorescence intensity twice that of the blank. *d*) Modified nucleosides.

Table III. Excitation and Emission Maxima of the Fluorescence Produced by the Reaction of Catechol Compounds with p-MOED, and Their Relative Fluorescence Intensities[a]

Compound	Excitation maximum (nm)	Emission maximum (nm)	RFI[b]
Guanosine	340	470	100.0
Norepinephrine	330	390	1.0
Epinephrine	330	390	1.2
Dopamine	330	405	1.5
3,4-Dihydroxyphenylacetic acid	330	390	2.1
3,4-Dihydroxyphenylethylene glycol	330	460	3.0
3,4-Dihydroxybenzylamine	330	390	1.8
Pyrogallol	330	390	1.8
2-Hydroxyestrone	335	420	8.4
4-Hydroxyestrone	330	390	19.2

a) Portions (1.0 ml) of 10 nmol/ml solutions of the substances were treated according to the recommended procedure. b) The fluorescence intensity obtained with 10 nmol/ml guanosine was taken as 100.0.

nosine-5′-monophosphate and 2′-deoxyadenosine-5′-triphosphate), 2- or 3-deoxy sugars (2-deoxy-D-ribose, 3-deoxy-D-ribose and 2-deoxy-D-glucose), hypoxanthine, xanthine, histamine, spermidine, creatine, creatinine, acetaldehyde, benzaldehyde, pyruvic acid, phenylpyruvic acid, and 17 L-α-amino acids. Only catechol compounds fluoresced slightly under the present conditions, and their fluorescence excitation and emission maxima were at much shorter wavelengths, except for 3,4-dihydroxyphenylethylene glycol (Table III).

This paper provided the first fluorometric method of determination that works on all ribonucleosides and ribonucleotides. This method is comparable in sensitivity to other fluorometric methods[1-4,7-9] selective for some nucleosides and nucleotides but not to the colorimetric methods.[5,6,10,11] The method presented here should be useful for the determination of ribonucleosides and ribonucleotides in biological samples.

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