In conclusion, we feel that our results convincingly demonstrate the potential of the selective ^{13}C enrichment technique for study of the dynamic properties of macromolecules in solution, an area of investigation to which X-ray diffraction techniques, for example, are not applicable. The selective enrichment approach in principle permits a detailed study of the dynamic state of any region of a native enzyme or other biological macromolecule by ^{13}C nmr. The conclusion that ^{13}C nmr line widths in deuterated macromolecules appear to be determined largely by scalar relaxation is important for realizing the potential of ^{13}C nmr spectroscopy in providing well-resolved nmr spectra of macromolecules. Deuterium decoupling using high power can eliminate the scalar relaxation of the second kind and thus give narrow signals whose widths are determined primarily by ^{13}C–^{2}H dipolar relaxation in favorable cases. This approach should permit detection of small chemical shift differences which reflect nonequivalence of side chains and changes in conformation.

Acknowledgments. Partial support of the portion of this research conducted at the University of California by the National Institutes of Health (GM 17450 and AM 13529), Research Corporation, Eli Lilly and Company, and Merck and Company is gratefully acknowledged. We thank Dr. George Hegeman for many valuable discussions about bacteriology and enzyme isolation, Dr. William Horsley for advice on isolation of α subunit, Dr. S. Ogawa for running the 220-MHz proton nmr spectra, Dr. Charles Yanofsky for providing the *E. coli* strains employed, and Drs. Mel Klein and Robert Connick for helpful discussions about magnetic resonance.

Specific and Fluorescent Modifications of Cytidine

Jorge R. Barrio[1] and Nelson J. Leonard*

Contribution from the Roger Adams Laboratory, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801. *Received September 5, 1972*

Abstract: Specific fluorescent modifications of cytidine have been obtained by reaction with several pyridinium and quinolinium hydrazides at pH 4.2 and 37°. In alkaline solution, and in general in neutral solution, the products showed a characteristic large absorption maximum at long wavelength where cytidine exhibited no absorption. A bathochromic effect was observed in the lowest energy transition of IIIa, as an example, and the wavelength was found to increase from neutral aqueous solution to 10% ethanol–chloroform. Methylation of IIIa by methyl iodide in sulfolane gave a 3-methylcytidine derivative, V, the structure of which was established by independent synthesis of V from nicotinic acid hydrazide methiodide (IIa) and 3-methylcytidine. The fluorescence of the modified cytidines showed structure and environment dependence. Compounds IIIc and IIId, by their ultraviolet absorption and fluorescent emission characteristics, present favorable possibilities for energy transfer studies with other fluorescing molecules, particularly in single-stranded oligo- and polynucleotides and nucleic acids.

Much of the current research on nucleic acid structure has centered on transfer ribonucleic acids. This attention is understandable since tRNAs constitute perhaps the most versatile class of nucleic acids in terms of the variety and complexity of the reactions in which they participate. The chemical reactions, moreover, can be valuable in sequence analysis, investigation of structure–function relationships, and primary, secondary, and tertiary structure analysis.[2]

Semicarbazide[3,4] and acyl hydrazides[5–7] react specifically with cytidine at pH 4.2 and 37°. In the interest of extending this useful reaction to realize specific fluorescent modification of cytidine and in seeking to achieve the goal of producing specific fluorescent modification of each tRNA base,[8–14] we have synthesized a series of pyridinium and quinolinium hydrazides, IIa–h, and examined the spectroscopic properties of their products with cytidine, IIIa–h.[15]

Experimental Section

Materials and Methods. Ethyl nicotinate (Aldrich), methyl isonicotinate (Chemicals Procurement Laboratories), 2,6-pyridine-

(1) Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina), 1970–1972.
(2) N. K. Kochetkov and E. I. Budowsky, *Progr. Nucl. Acid Res. Mol. Biol.*, **9**, 403 (1969).
(3) H. Hayatsu, K.-I. Takeishi, and T. Ukita, *Biochim. Biophys. Acta*, **123**, 445 (1966).
(4) H. Hayatsu and T. Ukita, *ibid.*, **123**, 458 (1966).
(5) K. Kikugawa, H. Hayatsu, and T. Ukita, *ibid.*, **134**, 221 (1967).
(6) K. Kikugawa, A. Muto, H. Hayatsu, K.-I. Miura, and T. Ukita, *ibid.*, **134**, 232 (1967).
(7) L. Gal-Or, J. E. Mellema, E. N. Moudrianakis, and M. Beer, *Biochemistry*, **6**, 1909 (1967).
(8) J. A. Secrist III, J. R. Barrio, and N. J. Leonard, *Biochem. Biophys. Res. Commun.*, **45**, 1262 (1971).
(9) J. R. Barrio, J. A. Secrist, III, and N. J. Leonard, *ibid.*, **46**, 597 (1972).
(10) J. A. Secrist III, J. R. Barrio, and N. J. Leonard, *Science*, **175**, 646 (1972).
(11) J. A. Secrist III, J. R. Barrio, N. J. Leonard, C. Villar-Palasi, and A. G. Gilman, *ibid.*, **177**, 279 (1972).
(12) J. A. Secrist III, J. R. Barrio, N. J. Leonard, and G. Weber, *Biochemistry*, **11**, 3499 (1972).
(13) J. R. Barrio, J. A. Secrist III, and N. J. Leonard, *Proc. Nat. Acad. Sci. U. S.*, **69**, 2039 (1972).
(14) N. J. Leonard, J. R. Barrio, and J. A. Secrist III, *Biochim. Biophys. Acta*, **269**, 531 (1972).
(15) We express our appreciation for the help of Dr. Kurt L. Loening, Nomenclature Director of Chemical Abstracts Service, in the naming of these products. Thus, compound IIIc is 4-[[(2,3-dihydro-2-oxo-1-β-D-ribofuranosyl-4(1*H*)-pyrimidinylidene)hydrazono]hydroxymethyl]-1-methylpyridinium hydroxide inner salt and IIIh is 2,6-pyridinedicarboxylic acid bis[2-(1,2-dihydro-2-oxo-1-β-D-ribofuranosyl-4-pyrimidinyl)-hydrazide]. The others are named accordingly.

Table I. Microanalytical Data

Compd	Formula	Mol wt	Mp, °C	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N	pK_a
IIIa	$C_{16}H_{19}N_5O_6 \cdot 2H_2O$	413.39	200–202	46.48	5.61	16.94	46.14	5.62	17.09	5.9
IIIb	$C_{22}H_{24}BrN_5O_6$	534.37	150,[a] 165[b]	49.45	4.52	13.11	49.80	4.91	13.43	5.9
IIIc	$C_{16}H_{19}N_5O_6 \cdot 0.25H_2O$	381.86	285–286	50.32	5.15	18.33	50.28	5.39	18.10	5.5
IIId	$C_{22}H_{24}BrN_5O_6$	534.37	144,[a] 160[b]	49.45	4.52	13.11	49.85	4.92	13.25	5.2
IIIe	$C_{14}H_{22}N_5O_6 \cdot 2H_2O$	392.39	147,[a] 174[b]	42.85	6.68	17.85	43.22	6.59	17.48	6.7
IIIf	$C_{16}H_{19}N_5O_6 \cdot 2H_2O$	413.39	131–132	46.48	5.61	16.94	46.72	5.49	16.58	7.8[5]
IIIg	$C_{20}H_{21}N_5O_6 \cdot 2H_2O$	463.45	190,[a] 210[b]	51.83	5.43	15.11	52.08	5.10	14.55	6.3
IIIh	$C_{25}H_{29}N_9O_{12}$	647.56	235–236	46.37	4.51	19.47	46.57	4.64	19.40	9.7
V	$C_{17}H_{22}IN_5O_5 \cdot H_2O$	537.31	144–146	38.00	4.50	13.03	38.40	4.42	12.98	7.9

[a] Shrinkage. [b] Decomposition.

dicarboxylic acid monohydrate (Aldrich), Girard P Reagent (1-(carboxymethyl)pyridinium chloride hydrazide, Aldrich) (IIf), and Girard T Reagent ((carboxymethyl)trimethylammonium chloride hydrazide) (IIe) were purified before use. Cytidine and adenosine were purchased from Calbiochem, uridine and guanosine from Sigma Chemical Co. Melting points were determined using a Thomas–Hoover melting point apparatus and are uncorrected. Electronic absorption spectra were recorded on a Cary Model 15 spectrophotometer. For quantitative measurements, a specific amount of material was placed inside a volumetric flask and dissolved in the appropriate amount of distilled water or 1 *N* HCl. When spectra were to be determined in water at several values of pH, three equal aliquots were withdrawn and placed in volumetric flasks, and the pH was adjusted with 1 *N* NaOH or 1 *N* HCl. The same solutions were used for emission and excitation studies immediately after running the electronic absorption spectra. All electronic absorption, fluorescence emission, and fluorescence excitation spectra were determined against an appropriate blank. Fluorescence emission and fluorescence excitation spectra were recorded on a Hitachi Model MPF-2A fluorescence spectrophotometer. Nmr spectra were measured on a Varian A-60 spectrometer. Chemical shifts are reported in parts per million on the δ scale and refer to to 10–20% solution (w/v) with TMS as an internal reference unless otherwise stated. Homogeneity and R_f of the products were established by thin layer chromatography on silica gel (Eastman Chromagram sheets). Microanalyses were performed by Mr. Josef Nemeth and his staff, who also weighed samples for quantitative electronic absorption studies. The pK_a determinations were run for us at the Lilly Research Laboratories, Eli Lilly and Co., and appreciation is due Mr. George Maciak.

Nicotinic acid hydrazide methiodide (IIa),[16a] isonicotinic acid hydrazide methiodide (IIc),[16b] 1-benzylpyridinium-4-carboxyhydrazide bromide (IId),[17] and 2,6-pyridinedicarboxydihydrazide (IIh)[18] were prepared by published procedures.

1-Benzylpyridinium-3-carboxyhydrazide bromide (IIb), prepared in similar manner,[17] was purified by recrystallization from methanol–ether to yield 66% of product, mp 160–161°, having a confirmatory nmr spectrum.

Anal. Calcd for $C_{13}H_{14}BrN_3O$: C, 50.64; H, 4.54; N, 13.63. Found: C, 50.73; H, 4.57; N, 13.67.

1-Methylquinolinium-4-carboxyhydrazide iodide (IIg) was made by first treating methyl 4-quinolinate[19] with methyl iodide in methanol at 25° for 12 hr to form the **methiodide,** mp 174–176°.

Anal. Calcd for $C_{12}H_{12}INO_2$: C, 43.79; H, 3.68; N, 4.26. Found: C, 43.94; H, 3.84; N, 4.48.

The intermediate was then treated with 10% excess hydrazine hydrate in methanol, and the product was recrystallized from water by addition of ethanol and ether, mp 220–222°. Confimatory nmr spectra were obtained for these compounds.

Table II. Quantitative Electronic Absorption Data of Hydrazides and Their Reaction Products with Cytidine

Compd	1 *N* HCl λ_{max}	1 *N* HCl $10^{-3}\epsilon$	Acid pH 2.8 ± 0.1 λ_{max}	Acid pH 2.8 ± 0.1 $10^{-3}\epsilon$	Neutral[a] pH 6.2 ± 0.1 λ_{max}	Neutral[a] pH 6.2 ± 0.1 $10^{-3}\epsilon$	Alkaline pH 10.8 ± 01 λ_{max}	Alkaline pH 10.8 ± 01 $10^{-3}\epsilon$
IIa			264	5.2	264	5.2	255	7.1
							305	4.6
IIb			260	5.5	260	5.5	256	6.2
							306	4.3
IIc			268	6.0	268	6.0	253	5.2
							372	5.0
IId			267	6.3	267	6.3	255	5.8
							380	5.8
IIe							217[b]	7.1
IIf			259	4.9	259	4.8	258	4.6
IIg	324	8.4	322	8.5	322	8.5	318	7.5[c]
							408	2.0[c]
IIh			224	10.4	223	10.4	220[b]	10.2
			273	5.5	274[b]	6.1	273	6.2
IIIa	275[b]	12.2	270	11.2	260	9.4	257	9.5
	292	17.9	340	5.4	340	16.3	345	18.3
IIIb			274	13.2	265	11.5	258	10.7
			340	6.0	344	11.2	347	17.8
IIIc			220	14.2	227	12.9	227	13.3
			275	12.8	264	11.8	264	12.3
			376	6.9	400	14.4	403	15.9
IIId			275	15.3	266	13.0	265	13.6
			375	5.4	408	10.9	412	11.8
IIIe			278	11.8	238	8.2	247	8.5
					282	10.8	307	17.6
					307	10.0		
IIIf			267	12.9	260	11.8	260	12.3
			278	12.4	267	12.0	267	12.2
					280[b]	10.8	306	15.1
					306[b]	7.4		
IIIg	292	16.9	278	11.8	310	17.2	308	19.0
	322[b]	9.4	322	10.0	420	3.5	422	4.2
IIIh			225[b]	21.6	225[b]	21.6	242	17.4
			282	25.6	278	24.4	335	30.9
V	275[b]	12.0	272	11.7	272	11.6	258[b]	10.6
	292	16.9	286[b]	11.6	286[b]	11.4	331	10.4

[a] Any small changes which may affect the pH of the solution will cause changes in the spectrum. [b] Shoulder. [c] Decomposition.

Anal. Calcd for $C_{11}H_{12}IN_3O \cdot H_2O$: C, 38.06; H, 4.06; N, 12.10. Found: C, 38.10; H, 4.15; N, 11.90.

Nicotinic acid *N'*-methylhydrazide methiodide (IIi) was obtained by treatment of methyl nicotinate methiodide with 30% excess methylhydrazine in sulfolane at 45° for 24 hr. The product was precipitated several times from methanolic solution with ether and finally recrystallized from ethanol, mp 141–142°, having a confirmatory nmr spectrum.

Anal. Calcd for $C_9H_{12}IN_3O$: C, 32.76; H, 4.09; N, 14.33; I, 43.34. Found: C, 33.00; H, 4.11; N, 14.13; I, 43.28.

Reaction of Hydrazides IIa–h with Cytidine. General Procedure.

(16) (a) D. D. Libman, D. L. Pain, and R. Slack, *J. Chem. Soc.*, 1328 (1954); (b) H. Yale, K. Losee, J. Martins, M. Holsing, F. M. Perry, and J. Bernstein, *J. Amer. Chem. Soc.*, **75**, 1933 (1953).

(17) J. Cymerman-Craig, J. L. Garnett, and D. M. Temple, *J. Chem. Soc.*, 4057 (1964).

(18) J. Supniewski, T. Bany, and J. Krupinska, *Bull. Acad. Pol. Sci., Cl. 2*, **3**, 55 (1955); *Chem. Abstr.*, **50**, 7800*h* (1956).

(19) H. Meyer, *Monatsh. Chem.*, **22**, 115 (1901).

Table III. Thin Layer Chromatography on Silica Gel. R_f Values (× 100) of Hydrazides, Nucleosides, and Reaction Products

Compd	Solvent system[a] A	B	C
IIa	4	7	17
IIb	7	20	48
IIc	2	5	17
IId	8	23	52
IIf	4	9	4
IIg	6	10	*b*
IIh	43	50	10
IIIa	6	2	10
IIIb	19	6	39
IIIc	6[c]	1	11[c]
IIId	28[c]	12[c]	40[c]
IIIe	9	4	11
IIIf	4	2	9
IIIg	10	5	20
IIIh	16[c]	4	4[c]
Cytidine	49	25	38
Uridine	66		40
Adenosine	65		58
Guanosine	55		32

[a] Solvent systems are (A) methanol–water (9:1); (B) 1-butanol–acetic acid–water (77:13:10); (C) 2-propanol–ammonia–water (7:1:2). [b] Decomposes. [c] Fluoresces.

To 486 mg (2 mmol) of cytidine was added 10% excess above the millimolar equivalent of the hydrazide dissolved in 2.4 ml of 2 *M* AcOH, and the mixture was adjusted to pH 4.2 with NH_4OH or AcOH. The mixture was maintained at 37° with magnetic stirring and, when the reaction was complete as judged by tlc (7–10 days) (Table IV), it was neutralized with 4 *M* LiOH. The solution was stirred with 10–15 ml of acetone, the upper acetone layer was removed, and the acetone treatment was repeated four more times. The semisolid residue was dissolved in MeOH and precipitated with acetone or EtOAc. The product was filtered and purified by recrystallization (IIIa and IIIc, H_2O–acetone, IIIh, H_2O–MeOH) or reprecipitation (MeOH–acetone). If further purification was necessary, a slightly acid solution of the product was loaded onto a Dowex 50W-X8 (H^+ form) column (2.0 × 20 cm) which was thoroughly washed with water followed by elution with 0.02 *M* NH_4OH. The fractions containing the product (checked by spectroscopy, see Table II) were combined, and the solvent was evaporated below 40°. The residue was dissolved in MeOH and precipitated with EtOAc or acetone. After reprecipitation two more times from MeOH–acetone, 150–200 mg of the pure product was obtained (see Tables I–V).

Methylation of IIIa. A solution of 413 mg (1 mmol) of IIIa·$2H_2O$ in 1 ml of water and 10 ml of sulfolane was heated with excess methyl iodide (500 mg) at 60–70° for 30 min. The product was precipitated with MeOH–Et_2O. After recrystallization from MeOH–EtOAc, and washing with ethyl acetate, 400 mg of pure product V (hygroscopic) was obtained, mp 144–146° dec, identical in all properties with the compound obtained from IIa + IV.

Table IV. Nmr Chemical Shifts for 5-H and 6-H in III

Compd	Doublets: 5-H $(CD_3)_2SO$	5-H $(CD_3)_2SO$, D_2O	5-H $(CD_3)_2SO$, D_2O CF_3COOD	6-H $(CD_3)_2SO$	6-H $(CD_3)_2SO$, D_2O	6-H $(CD_3)_2SO$, D_2O, CF_3COOD
IIIa	6.48	6.50	6.20	7.95	7.98	7.98
IIIb	6.35[a]	6.35[a]	6.30	7.90	7.90	8.10
IIIc	*b*	*b*	6.30	*b*	*b*	8.10
IIId	6.45	6.55	6.30	7.82	7.90	8.20
IIIe	6.15	6.30	6.30	7.80	7.90	8.30
IIIg	6.34	6.49	6.27	*c*	*c*	*c*
IIIh	5.90	6.10	6.40	8.00	8.00	8.50
IV			6.30[d]			8.32[d]
V	6.31	6.31	6.63	7.71	7.70	8.31

[a] Broad. [b] Low solubility. [c] The resonance was partially obscured by that of the aromatic quinolinium protons. [d] For 3-methylcytidine methosulfate (IV), the 3-methyl resonance in $(CD_3)_2SO$, D_2O, CF_3COOD is at δ 3.55 compared with 3.58 for that of 3-methyl in V.

Table V. Fluorescence Emission and Fluorescence Excitation Spectra

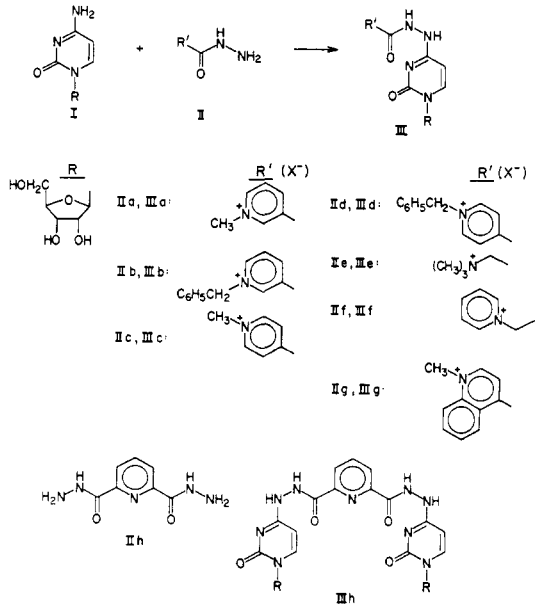
Compd	Fluorescence emission: Maximum, nm	Excitation, nm	Fluorescence excitation: Maximum, nm	Emission, nm
IIIa	No fluorescence (2.8, 6.2 and 10.7)[a]			
IIIb	No fluorescence (2.8, 6.2 and 10.7)[a]			
IIIc	550 (10.7)[a]	403	400 (10.7)[a]	550
	550 (6.2)	400	400 (6.2)	550
	530 (2.8)	372		
IIId	550 (10.7)[a]	412	410 (10.7)[a]	550
	550 (6.2)	408		
	520 (2.9)	375		
IIIe	390 (10.7)[a]	307		
	390 (6.2)	307		
	360 (2.9)	278		
IIIf	Slight fluorescence (2.7, 6.2 and 10.7)[a]			
IIIg	460 (2.8)[a] slight fluorescence (6.2 and 10.8)	322	338 (2.8)[a]	460
IIIh	460 (10.7) slight fluorescence (2.8 and 6.2)	335		

[a] pH values are given in parentheses.

Results and Discussion

The specific and quantitative modification of cytidine with pyridinium and quinolinium hydrazides can be accomplished by stirring a solution of the nucleoside with the reagent at pH 4.2 and 37° (Scheme I). Pure

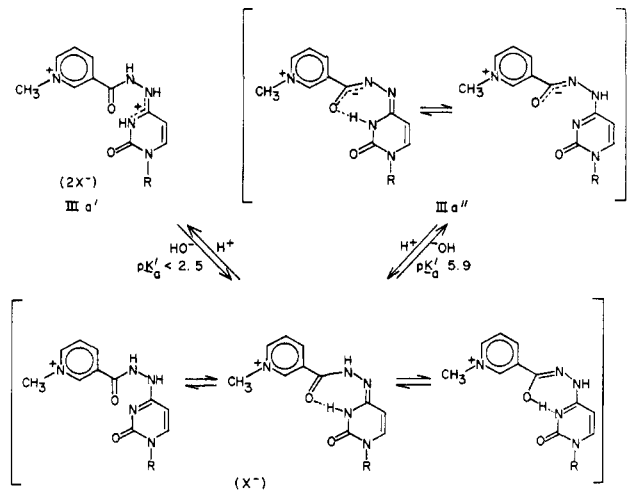
Scheme I



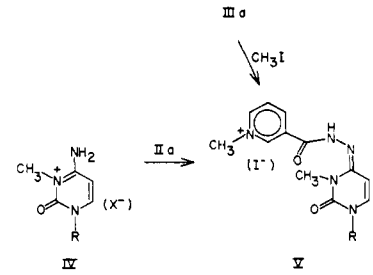
product was isolated either by reprecipitation and recrystallization or through the use of a Dowex 50W-X8 (H^+ form) column, which was washed with water followed by elution with 20 m*M* NH_4OH to obtain the product. The mode of reaction of N^1-substituted cytosine and cytidine 2′(3′)-phosphate has already been proved[5] using Girard P reagent (IIf) and comparing the reaction product with an authentic sample obtained from Girard P reagent and the 1-substituted 4-thiouracil. We used Girard P reagent (IIf) as a model reagent to test the reaction with cytidine. The ultraviolet spectra of the product (IIIf) at different pH's showed similar characteristics to those reported by Kikugawa, *et al.*,[5] for the product with cytidine 2′(3′)-phosphate. No reaction occurs between the major ribonucleosides adenosine, guanosine, thymidine, and uridine and the reagents IIa–h within 90 hr, whereas, for example, cytidine reacts with IIc to give a new ultraviolet-absorbing and fluorescent compound after 40 hr, as followed by thin layer chromatography (Table III).[20] With the exception of compound IIIf (11% decomposition at pH 10.8), the products III showed stability in aqueous solution over the pH range 2.8–10.8 during 48 hr, while the reagents II were stable at pH 2.8 and neutral pH but showed decomposition at pH 10.8 during the same period.

The products were isolated as their zwitterionic forms, as exemplified by IIIa′′, when the reagent used had an iodide counterion (IIa,c,g) and as the quaternary bromide IIIb,d when the reagent had a bromide counterion (IIb,d). Purification of the product through the use of a Dowex 50W-X8 (H^+ form) column and elution with NH_4OH provide the zwitterionic forms of the compounds also (*i.e.*, IIIe,f). A similarity in nmr and uv spectra linked the products in structure. The resonances of the pyrimidin-2-one and pyridinium protons in general were broad[21] and poorly resolved when the compounds were dissolved in $(CD_3)_2SO$, reflecting the tautomeric equilibria as proposed for IIIa and IIIa′′, with possible hydrogen bonding (Scheme II). Pro-

Scheme II



Scheme III



tonation of the molecules by addition of CF_3COOD in general improved the resolution and shifted the resonances, particularly of 5-H and 6-H in the pyrimidin-2-one moiety (Table IV). The tautomeric possibilities of IIIa′ are limited. Compound IIIc in $(CD_3)_2SO$ with 10% CF_3COOD exhibited a pair of doublets at δ 6.30 and 8.10 (J = 7.0 Hz) assignable to these 5 and 6 protons, respectively, and a pair of doublets at δ 8.45 and 8.97 (J = 6.0 Hz) for the α and β protons on the pyridinium ring. For comparison, the nmr spectrum of isonicotinic acid hydrazide methiodide (IIc) in $(CD_3)_2SO$ showed doublets at δ 8.32 and 9.16 (J = 6.0 Hz) due to the α- and β-pyridinium protons. Similarly, isonicotinic acid N'-methylhydrazide methiodide (IIj) showed the same AB pattern at δ 8.18 and 8.98.

Methylation of IIIa by methyl iodide in sulfolane gave a monomethyl derivative. The ultraviolet absorption spectrum of this product in 1 *N* HCl (pH < 1) was very similar to that of IIIa in 1 *N* HCl[22] but different at pH 2.8 and in neutral and alkaline solution. The observed electronic absorption spectra are in agreement with the structure assignment as a 3-methylcytidine derivative, 3-[[(2,3-dihydro-3-methyl-2-oxo-1-β-D-ribofuranosyl-4(1*H*)-pyrimidinylidene)hydrazono]hydroxyl-

(20) It is assumed that reagents of type II will also react with other natural C compounds such as deoxycytidine, $N^4,O^{2'}$-dimethylcytidine, 5-methylcytidine, 2′-*O*-methylcytidine, and 2-thiocytidine: R. H. Hall, "The Modified Nucleosides in Nucleic Acids," Columbia University Press, New York, N. Y., 1971, pp 120–131.

(21) G. C. Y. Lee, J. H. Prestegard, and S. I. Chan, *J. Amer. Chem. Soc.*, **94**, 951 (1972).

(22) T. Ueda and J. J. Fox, *ibid.*, **85**, 4024 (1963).

methyl]-1-methylpyridinium iodide (V, lactam tautomer shown).[22-27] The structure was fully established by independent synthesis from nicotinic acid hydrazide methiodide (IIa) and 3-methylcytidine methosulfate (IV, $X^- = CH_3SO_4^-$) followed by conversion of the counterion to iodide. The conditions used for the reaction between IIa and IV were similar to those used for the synthesis of IIIa–h, but the optimum pH for the reaction was near 8.7, the pK_a of IV. No reaction was observed between nicotinic acid *N'*-methylhydrazide methiodide (IIi) and cytidine at pH 4.2 or in the presence of sodium bisulfite at pH 7.5.[28]

The CONH group (hydrazide portion of the molecule) in IIIa, as an example, is more acidic (pK_a = 5.9) (see Table I) than that in its precursor IIa (pK_a = 10.15) and than that in V (pK_a = 7.9). The absence of a 3-H in V is a partial cause since a qualitatively similar effect has been observed in comparing the conjugate acids of cytidine (pK_a = 4.15)[29] and 3-methylcytidine (pK_a = 8.7).[22,23] The possibility of stabilization by O–H–N^3 hydrogen bonding (IIIa''), in addition to the electron-withdrawing effect of the pyrimidin-2-one ring, can explain the difference in acidity between IIIa and IIa.

Further information, in addition to that available from nmr spectra and pK_a determinations, can be obtained from consideration of the electronic absorption spectra of compounds IIIa–h and V at various pH values (Table II). In alkaline solution, and in general in neutral solution (pH 6.2), the products showed a characteristic large absorption maximum at long wavelength where cytidine exhibited no absorption. The parent hydrazides IIa–d,g also have, in alkaline solution, a typical absorption band at long wavelength that corresponds to the zwitterionic form of the quaternary hydrazide with acidic dissociation of the CONH group.[30,31] In the corresponding cytidine derivatives (III), the absorbance in alkaline solution is at longer wavelength and higher extinction, which requires a longer conjugated system, as in IIIa''. A comparison of the wavelength of the lowest energy transition for the 3-pyridinium isomer IIIa (340 nm at pH 6.2 and 345 nm at 10.8) *vs.* the 4-pyridinium isomer IIIc (400 nm at pH 6.2 and 403 nm at 10.8), and IIIb (344 nm at pH 6.2 and 347 nm at pH 10.8) *vs.* IIId (408 nm at pH 6.2 and 412 nm at pH 11.8), indicates the importance of conjugation through the entire molecule, including, for the 4-pyridinium isomers (IIIc and IIId), the possible contribution of anhydro base structures, for the location of this particular absorption.[32] The electronic absorption spectra of IIIa and V are given in Figure 1, from which it is obvious that the spectra of the *dicationic* species of IIIa (IIIa', see Scheme II) and V are similar, and therefore the structures of the dications are similar, as mentioned earlier. The fact that the spectrum of the neutral species of IIIa differs from that of V indicates the contribution of 4-aminopyrimidin-2-one forms of IIIa and IIIa'' in the tautomeric equilibria (Scheme II).

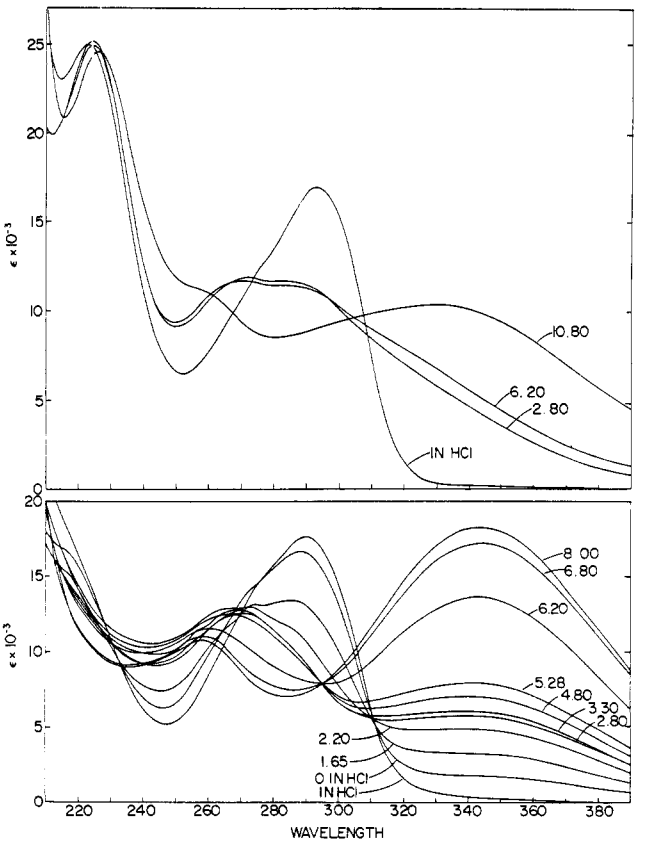


Figure 1. Top panel: Ultraviolet absorption spectra of V in water at different pH's. Bottom panel: Variation in ultraviolet absorption of IIIa with pH in phosphate buffers. From pH 8.0 to 10.8 little change was noted in the spectrum.

Compound IIIa was chosen to study solvent and pH effects. A clear isosbestic point was observed at 295 nm upon variation of the pH from 2.8 to 10.8, corresponding to the equilibrium shown in Scheme II. Above pH 8–9, little change was noted in the ultraviolet spectrum. However, below pH 2.5 the aminopyrimidin-2-one moiety is 3-protonated and no absorption maximum is observed above 300 nm. A clear isosbestic point was observed at 311 nm. The possible tautomeric forms shown for IIIa and IIIa'' in Scheme II are intended to be representative rather than exclusive, but their existence is favored by the nmr and uv spectra presented thus far. We do not mean to exclude intermolecular hydrogen bonding from consideration. The ground state of IIIa'', zwitterionic form, is highly polar and would be expected to be considerably stabilized by polar solvents and by general electrostatic effects, as well as by specific hydrogen bonds to oxygen or nitrogen atoms. The wavelength of the lowest energy transition for compound IIIa was found to increase in the order: neutral aqueous solution, 340 nm; and basic pH, 345 nm; ethanol, 363 nm; 10% ethanol–chloroform, 376 nm; dimethyl sulfoxide,[33] 380 nm, consistent with this expectation for the $\pi \rightarrow \pi^*$ transition.

Fluorescence Properties. The fluorescence characteristics of the compounds III at 25° and at different

(23) P. Brookes and P. D. Lawley, *J. Chem. Soc.*, 1348 (1962).
(24) W. Szer and D. Shugar, *Acta Biochim. Pol.*, **13**, 177 (1966).
(25) J. A. Haines, C. B. Reese, and Lord Todd, *J. Chem. Soc.*, 1406 (1964).
(26) R. H. Hall, *Biochem. Biophys. Res. Commun.*, **12**, 361 (1963).
(27) R. L. C. Brimacombe and C. B. Reese, *J. Chem. Soc. C*, 588 (1966).
(28) K. Kai, Y. Wataya, and H. Hayatsu, *J. Amer. Chem. Soc.*, **93**, 2089 (1971).
(29) J. J. Fox and D. Shugar, *Biochim. Biophys. Acta*, **9**, 369 (1952).
(30) A. Albert, *Experientia*, **10**, 370 (1953).
(31) A. Albert, *Nature (London)*, **177**, 525 (1956).
(32) A. R. Katritzky, H. Z. Kucharska, and J. D. Rowe, *J. Chem. Soc.*, 3093 (1965).
(33) E. M. Kosower, *J. Amer. Chem. Soc.*, **80**, 3253 (1958).

pH's, which are given in Table V, showed structure dependence. Thus, whereas compound IIIg retains the fluorescence properties of the parent hydrazide IIg, compounds IIIc and IIId show emission characteristics (λ_{em} 550 nm) due to their much longer wavelength absorption bands. The fluorescence excitation maximum for IIIc was found at 400 nm (pH 6.2 or 10.7), corresponding within limit of error to the longest wavelength ultraviolet absorption maximum for this compound. Moreover, the observed smooth fluorescence emission of IIIc is a mirror image of the absorption band. The fluorescent cytidine derivatives are, in general, detectable down to concentrations of the order of 10^{-5} to 10^{-6} M, with a quantum yield lower than 0.01.

Compounds IIIc, IIId, and IIIh, especially in zwitterionic form, show a clear enhancement of fluorescence when adsorbed on silica gel (Table III). Since the emission maximum of IIIc is shifted in going from water (550 nm) to dimethyl sulfoxide (578 nm), corresponding to the bathochromic shift in the ultraviolet absorption, the influence of the environment on the fluorescence of this molecule is clear and may be of advantage. The incorporation of these fluorophores into single-stranded oligo- and polynucleotides and nucleic acids at original cytidine sites would result in changes in fluorescence properties depending upon the nature of the "solvent" in the region of the modified cytidine and upon interactions[34] with other portions of the larger matrix.

For application as converters of cytidine units to fluorescent modifications, compounds IIIc and IIId offer the best possibilities. Excitation is possible at 400–410 nm, well outside the range of absorption of proteins and nucleic acids. In addition, the absorption bands are in a favorable region for energy transfer studies with other dyes. Specifically, the fluorescent modification of adenine residues with chloroacetaldehyde[8-14] introduces fluorescence emission at about 410 nm and may be an excellent donor, since it has a sufficiently long fluorescence lifetime (~23 nsec) and the emission band of the 1,N^6-ethenoadenylate overlaps the absorption band of IIIc or IIId. Thus, if the spacing and relative orientation of the oscillators permitted strong interaction, the energy transfer should be efficient.[35]

The bifunctional reagent IIh reacts with two molecules of cytidine to give IIIh, which is fluorescent and emits at 460 nm at pH's above its pK_a' when excited at 335 nm. The appearance of fluorescence implies reaction with two cytidine moieties and may therefore signify intramolecular reaction when the cytidines have the proper spacing (bifunctional reagents with other distances between hydrazide functions can be engineered). Alternatively, it may be possible to use the reagent to bind together polynucleotide chains containing exposed cytidine units. In general, the fluorescence data for IIIc, IIId, and IIIh indicate that these derivatives of cytidine may provide advantages in further studies of tRNA structure and function by chemical modification.

Acknowledgment. We wish to thank Professor Gregorio Weber for valuable discussion and for providing the necessary facilities for fluorescence determinations. This work was supported by Research Grant GM-05829 from the National Institutes of Health, U. S. Public Health Service.

(34) A. J. Pesce, C. G. Rosēn, and T. L. Pasby, "Fluorescence Spectroscopy," Marcel Dekker, New York, N. Y., 1971.

(35) L. Stryer, *Radiat. Res. Suppl.*, **2**, 432 (1960).

p-Alkoxybenzyl Alcohol Resin and *p*-Alkoxybenzyloxycarbonylhydrazide Resin for Solid Phase Synthesis of Protected Peptide Fragments

Su-Sun Wang

Contribution from the Chemical Research Department, Hoffmann-La Roche Inc., Nutley, New Jersey 07110. *Received August 9, 1972*

Abstract: Two new resins—*p*-alkoxybenzyl alcohol resin ($HOCH_2C_6H_4OCH_2C_6H_4$ resin) and *p*-alkoxybenzyloxycarbonylhydrazide resin ($H_2NNHCOOCH_2C_6H_4OCH_2C_6H_4$ resin)—were prepared. The former resin is suitable for the synthesis of protected peptide fragments possessing a free carboxyl group while the latter is useful for the synthesis of protected peptide hydrazides. Applications of these resins in the syntheses of Z-Leu-Leu-Val-Phe, Z-Phe-Val-Ala-Leu-$HNNH_2$, Asp-Arg-Val-Tyr-Val-His-Pro-Phe, Z-Lys(Z)-Phe-Phe-Gly, and Z-Lys(Z)-Phe-Phe-Gly-Leu-Met-NH_2 are described.

Recent developments in solid phase peptide synthesis have been reviewed by Merrifield[1,2] and discussed by others.[3,4] The method has been widely and quite successfully utilized for the rapid and convenient synthesis of numerous polypeptides. However, the products obtained by this technique are, in general, rather difficult to purify. Although in certain instances, effective purification can be achieved by selective proteolysis[5] or affinity chromatography,[6]

(1) R. B. Merrifield, *Advan. Enzymol.*, **32**, 221 (1969).
(2) G. R. Marshall and R. B. Merrifield in "Biochemical Aspects of Reactions on Solid Supports," G. R. Stark, Ed., Academic Press, New York, N. Y., 1971, p 111.
(3) E. Wünsch, *Angew. Chem.*, **83**, 773 (1971).
(4) J. Meienhoffer, 163rd National Meeting of the American Chemical Society, Boston, Mass., April 1972, M15.
(5) B. Gutte and R. B. Merrifield, *J. Biol. Chem.*, **246**, 1922 (1971).
(6) H. Taniuchi and C. B. Anfinsen, *ibid.*, **244**, 3864 (1969).