Pulse Radiolysis of Nucleic Acid Constituents and Related Compounds. II. Resolution of a Rapidly Decaying Transient Absorption in Cytosine Solutions[1]

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Abstract: Pulse radiolysis (10 MeV electrons) of N_2O saturated 5×10^{-4} *M* aqueous cytosine solutions, pH 10.8–12, results in a transient species which decays with a half-life of the order of 1 μsec. The decay follows pseudo-first-order kinetics and the half-life varies inversely with the OH^- concentration. The spectrum 1 μsec after the radiation pulse consists of two components. One resembles the spectrum obtained by addition of OH· to cytosine at pH 7. The other is similar to the spectrum obtained at pH 12.6 by reaction of cytosine with $O\cdot^-$ anion radical or of cytosine anion with OH· free radical. When decay of the short-lived transient is complete the spectrum is like the pH 12.6 spectrum. The short-lived transient is attributed to ionization of the cytosine–hydroxyl free-radical adduct by reaction with OH^- ion with a second-order rate constant $k = 3.5 \times 10^8\ M^{-1}\ sec^{-1}$. An upper limit for the value of the p*K* for the ionization reaction is established as 10.7.

Short-lived transients, with half-lives of the order of a microsecond or less, have been observed under several circumstances during pulse radiolysis of pyrimidine bases. For example, in mildly acidic (pH 4) argon or nitrogen saturated aqueous solutions of thymine the absorbance at λ 325 nm decays with a half-time of about 100 nsec.[4] This decay has been attributed to protonation of the anion radical formed when thymine reacts with a hydrated electron, e_{aq}^-. A rapid decay, which is being investigated further, has also been observed in neutral N_2O-saturated uracil solutions at λ ≈300 nm. Resolution of such rapid changes in absorbance is obviously essential if the properties of the organic radicals formed in the initial reactions between pyrimidine bases and the primary products of water radiolysis, e_{aq}^-, OH·, and H·, are to be determined.

In this paper we discuss a short-lived transient absorbance observed in N_2O-saturated alkaline aqueous solutions of cytosine. Identification of the reaction responsible for the decay has proved to be an important step in understanding the complex and only slightly less rapid changes which occur in spectra of radicals formed by OH· attack on several other pyrimidine bases in alkaline solutions. These latter reactions are discussed in a separate paper.

Experimental Section

The experimental procedures are described in the preceding paper.[5] Briefly, N_2O-saturated aqueous solutions of cytosine (5×10^{-4} *M*) were exposed to 4–20 nsec pulses of 10 MeV electrons, and changes in absorbance with time after the pulse were determined. Fresh solutions were used for each pulse. The pH was adjusted with dilute NaOH and H_2SO_4 or $HClO_4$ solutions. Control experiments with solutions containing NaOH but no cytosine or other OH· scavenger have been discussed previously.[6] An absorbance grows into control 0.01 *M* NaOH solutions over a 60-μsec period after the pulse which, with our present technique for mixing solutions, amounts to 0.0035 at 335 nm and 0.01 at wavelengths 550–600 nm. Such a slow reaction (believed to be between OH· or $O\cdot^-$ and CO_3^{2-} ion) cannot compete with the rapid reaction between OH· or $O\cdot^-$ and cytosine, and almost certainly does not occur in the cytosine solutions.

All values of absorbance are for an 8-cm light path.

In neutral N_2O-saturated 5×10^{-4} *M* cytosine solutions approximately 98% of the hydrated electrons reacts with N_2O (0.025 *M*, $k_{e_{aq}^- + N_2O} = 5.6 \times 10^9\ M^{-1}\ sec^{-1}$)[7] and is converted to OH· and the remainder with cytosine ($k_{e_{aq}^- + C} = 7 \times 10^9$).[8] In strongly alkaline solutions the percentage reacting with cytosine is probably even less because cytosine dissociates to give a negatively charged ion (p*K* = 12.2) which should react more slowly than the neutral molecule. The rate constant for the cytosine anion + e_{aq}^- reaction is not known, but comparable values for uracil[9] are $k_{U + e_{aq}^-} = 1.5 \times 10^{10}$ and $k_{U^- + e_{aq}^-} = 3 \times 10^9$. The possibility of transfer of electrons from N_2O^- to molecules such as cytosine has apparently been excluded by recent work of Adams, *et al.*[10] It thus seems unlikely that products resulting from electron capture or transfer to cytosine contribute significantly to the transient absorbances discussed below.

Most of the radicals observed are produced by reaction of neutral or anionic forms of OH· and cytosine ($k_{OH\cdot + C} = 3\text{–}5 \times 10^9$),[11] in yields[12] equal to ($g(OH\cdot + g(e_{aq}^-)) \approx 5.2$. A small yield of H· ($g(H\cdot) = 0.55$) contributes about 10% of the radicals. Studies with thymine suggest that the products formed by H· and OH· reactions with pyrimidine bases have similar absorption spectra,[5,6] and that the extinction coefficient of the H· reaction product is no more, and probably less, than that of the OH· product. The rates

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(1) Supported in part by the Division of Biology and Medicine of the U. S. Atomic Energy Commission through contracts with the University of California and with Gulf General Atomic Inc.

(2) University of California, Los Angeles, Calif.

(3) Gulf General Atomic Inc.

(4) L. M. Theard, F. C. Peterson, and L. S. Myers, Jr., to be published elsewhere.

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Table I. Intensity, Components, and Rate of Decay of Short-Lived Transient Absorbance in Pulse-Irradiated N_2O-Saturated Alkaline Aqueous Cytosine Solutions, λ 335 nm

pH	Radiation dose (rads)	Max early absorbance per 1000 rads at 335 nm		$R_1\cdot/(R_1\cdot + R_2\cdot)$	Absorbance per 1000 rads of $R_2\cdot$ after $R_1\cdot$ decay	$t_{1/2}$ for $R_1\cdot$ decay (μsec)	k (10^8 M^{-1} sec^{-1})
		Obsd	Of $R_1\cdot$				
10.8	800	0.045	0.042	0.72	0.012	3.3	3.5
11.2	750	0.038	0.033	0.57	0.013	1.0	4.1
11.7	900	0.022	0.013	0.22	0.014	0.5	3.0
12.3	1100	0.016	0.005	0.09	No decay		

of decay of the H· and OH· products are also similar. These considerations suggest that the small amount of cytosine–H· reaction product does not grossly distort spectra or alter conclusions regarding the OH· product.

Results

The short-lived transient absorbance in N_2O-saturated aqueous cytosine solutions, pH 11.4, is illustrated in the two upper oscillograms of Figure 1. The transient absorbance can be observed at wavelengths near 335 (and also 450 nm) but it is not present in tracings made at 400 nm (third oscillogram, Figure 1). Its occurrence and decay rate depend markedly on pH. At pH 7 and 12.3 it does not appear. At pH 10.8 the initial transient absorbance is intense and it decays with a half-life of several μsec. As the pH is increased toward 12.3 the initial intensity decreases and the rate of decay increases.

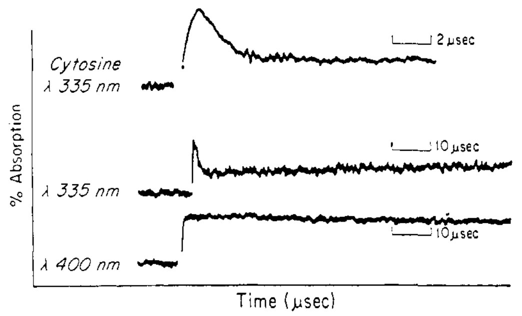


Figure 1. Oscillograms showing short-lived transient absorbance in N_2O-saturated 5 × $10^{-4}$$M$ aqueous cytosine solutions, pH 11.4, at λ 335 nm, and appearance of trace at a wavelength (400 nm) at which parent and daughter have the same extinction coefficient.

Table I presents the results of an analysis of the series of oscillograms on which the above qualitative comments are based. The oscillograms were obtained from solutions irradiated at different pHs, using an oscilloscope sweep speed of 2 μsec/div and a wavelength of 335 nm. Computation of results in several of the columns will be discussed below in conjunction with a proposed reaction scheme. The comparatively slow rise time, 300 nanosec, should lead to slightly low values for the intensity of the initial absorbance, and slightly high values for the half-life, especially at pH 11.70.

The absorption spectrum of radicals 1 μsec after the pulse in pH 11.4 solutions is shown in Figure 2, curve A. By 4 μsec after the pulse, when decay of the short-lived transient absorbance is essentially complete, the spectrum has changed to that shown in curve B. This latter curve is similar to the spectrum obtained when the pH is 12.6. For comparison, the spectrum of radicals formed by irradiation at pH 7 is given by the smooth curve, C. (This curve has been displaced. A radiation dose of 1000 rads gives an absorbance of 0.058 (8-cm light path) at 335 nm. Points are discussed below.) The spectra at pH 7 and 12.6 are both fully grown within 1 ½ μsec after the pulse, and they show negligible change in shape during the next few microseconds, except for a secondary growth at the higher pH in the wavelength range 300–350 nm.

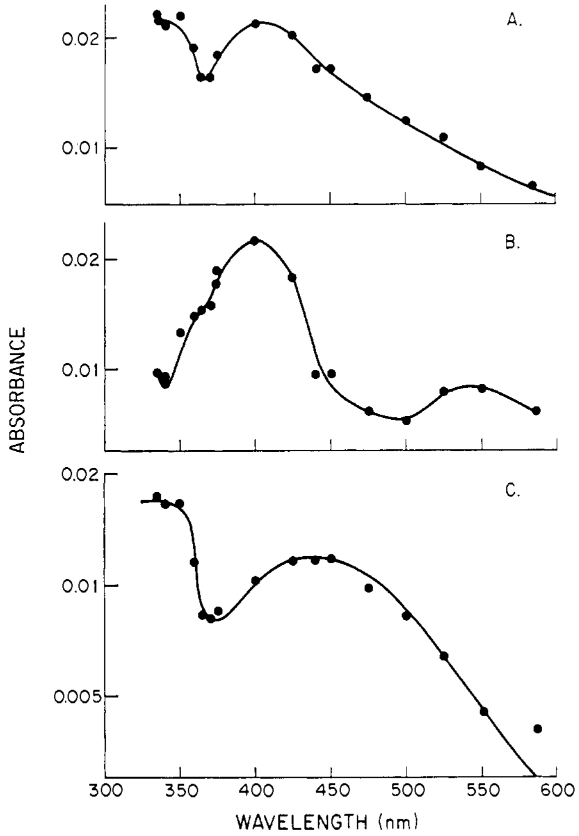


Figure 2. Absorption spectra obtained in N_2O-saturated 5 × 10^{-4} M aqueous cytosine solutions, pH 11.4, after a pulse of 10 MeV electrons: A, 1 μsec after the pulse; B, 4 μsec after pulse, C, points, calculated values ($A - \frac{1}{2}B$); curve, spectrum obtained by irradiation at pH 7, intensity adjusted to match points.

Discussion

Comparison of the spectra of Figure 2 suggests that spectrum A may be a composite of B and C, indicating that the pH 11.4 spectrum at 1 μsec after the pulse is the resultant spectrum of a mixture of radicals of the kinds formed at pH 7 ($R_1\cdot$) and 12.6 ($R_2\cdot$). This possibility was tested by subtracting from points defining spectrum A absorbances equal to ⅓, ½, ⅔, etc. of the absorbances of points defining spectrum B. Subtraction of ½B gives the points shown near spectrum C. The points fall remarkably close to curve C, the spectrum which has the shape of the spectrum of $R_1\cdot$ radicals. If this identification is accepted, then it follows that all $R_1\cdot$ radicals disappear and the concentration of $R_2\cdot$ doubles during the period 1–4 μsec after the pulse. The absorbance of $R_2\cdot$ at 4 μsec is the same as that observed at pH 12.6 before any secondary reactions occur, showing that the radical concentrations in the two systems are the same in spite of the disappearance of $R_1\cdot$ at pH 11.4, and equal to the total yield of radicals at pH 12.6.

These observations can be explained readily by a conversion of $R_1\cdot$ to $R_2\cdot$. It should be possible to monitor such a conversion by following changes in absorbance with time at wavelengths at which the molar extinction coefficient of $R_1\cdot$ is much larger than that of $R_2\cdot$. Curves B and C show that this holds at $\lambda \sim 330$ and ~ 450 nm. It is at these wavelengths that the short-lived transient absorbance is observed. The short-lived transient absorbance therefore can be attributed to the conversion reaction. Since the half-life of the transient is inversely related to the OH^- ion concentration (Table I), the conversion reaction must be base catalyzed.

The possibility that $R_1\cdot$ is eliminated by a second-order radical–radical reaction can be excluded by kinetics. The initial concentration of $R_1\cdot$, assuming $R_1\cdot$ to be the only initial product, is about 6×10^{-6} M/1000 rads. A half-time of 1 μsec would require the extremely high rate constant $2k = 2 \times 10^{11}\ M^{-1}\ \text{sec}^{-1}$. This is probably larger than the diffusion limit for radicals the size of the ones involved, and is 100 times larger than previously reported values for such reactions.[5]

The reactions suggested to explain the short-lived transient absorbance and associated spectral changes can be expressed schematically as follows (C = cytosine). At pH 7

$$\text{C} + \text{OH}\cdot \longrightarrow R_1\cdot \tag{1}$$

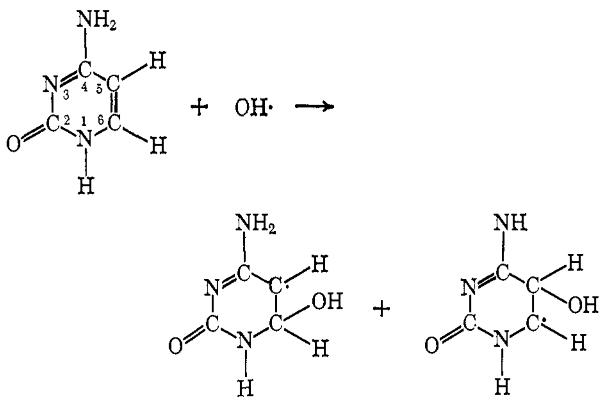
At pH 12.6

$$\text{C}^- \text{ (or C)} + \text{OH}\cdot \text{ (or O}\cdot^-) \longrightarrow R_2\cdot \tag{2}$$

At intermediate pH values, both reactions, the proportion of (2) increasing with pH, and the added reaction

$$R_1\cdot \longrightarrow R_2\cdot \tag{3}$$

This scheme is consistent with the chemistry of reactions of cytosine with OH·. At pH 7 OH· reacts with cytosine mainly by addition to the 5,6 double bond (reaction 1) to give a mixture of the 5-yl and 6-yl radicals.[13] In strongly alkaline solutions cytosine dissociates as an acid (pK = 12.2) in a reaction which is accompanied by a tautomeric change and a change in the absorption spectrum. At pH 11.4 and 12.6, 14 and 70% of the cytosine molecules are present in the anionic form. In this pH range some of the OH· free radicals are also ionized (pK = 11.9).[14] The following variants of reaction 4 may occur with these anions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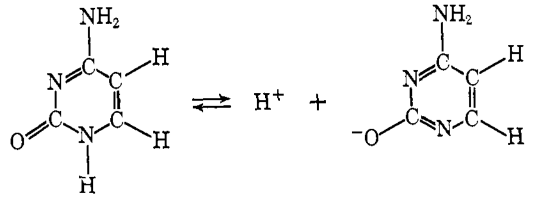
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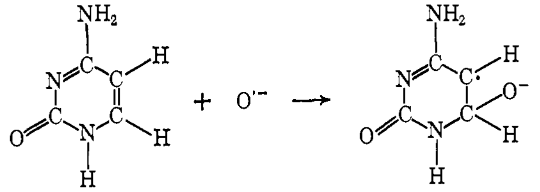
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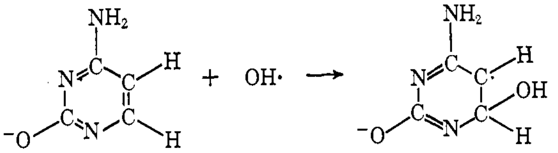
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Reaction of $O\cdot^-$ with the cytosine anion should be slower than reaction 6 or 7 because of charge repulsion, and probably is not important so long as there are appreciable quantities of the undissociated forms. The relative proportions of reactions 4, 6, and 7 will depend on the relative concentration of reactants, which in turn depend on the pH, and on the rate constants. The rate constants are not known for cytosine in alkaline solution, but ionization of thymine to the singly charged form reduces the rate constant for the net reaction of thymine with OH· (or $O\cdot^-$) from 7.4×10^9 at pH 7 (analogous to reaction 4) to $3.9 \times 10^9\ M^{-1}\ \text{sec}^{-1}$ at pH 11, analogous to reaction 7.[6] This suggests that at the higher pH's reaction 4 is more prominent than is indicated by the proportions of the different species.

The products of reactions 6 and 7 are tautomers, and the less stable should be converted readily to the other by reaction with OH^- ion and water. By analogy with reaction 5 we suggest that the product of reaction 7 is the more stable of the tautomers. This structure has more conjugated double bonds in the ring than the product of reaction 4, and should have a different absorption spectrum.

The neutral radical formed in reaction 4, like the parent molecule, should undergo ionization in alkaline solutions. The pK value should be smaller than that of cytosine because of the added hydroxyl group. The product of ionization is the more stable tautomer formed by reactions 6 and 7, presumably the product shown in reaction 8.

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(13) (a) M. N. Khattack and J. H. Green, *Intern. J. Rad. Biol.*, **11**, 131, 137, 577 (1966) (these authors refer to earlier papers); (b) M. G. Ormerod and B. B. Singh, *ibid.*, **10**, 533 (1966); (c) Cl. Nicolau, M. McMillen, and R. O. C. Norman, *Biochim. Biophys. Acta*, **174**, 413 (1969).

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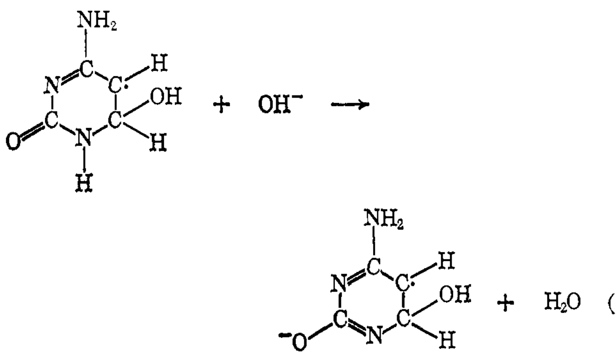
(8)

Reactions 6 and 7 may give the 6-yl as well as the 5-yl radical. The above discussion of tautomerism and ionization holds for 6-yl radicals also.

Referring to the reaction scheme, reaction 1 may be associated with 4, 2 with 6 and 7, and 3 with 8. The identification of $R_2\cdot$ must be taken as tentative because the radiation chemistry of cytosine in alkaline solutions has not been studied in detail, and other possibilities cannot be ruled out by reference to known products. The identification is reasonable, however, and analogous reactions of $OH\cdot$ and $O\cdot^-$ with double bonds are well known in other chemical systems. The identification of reaction 8 with 3, and observation of the short-lived transient absorbance at pH > 10.7 require a pK value less than 10.7 for reaction 8.

The data in Table I permit several requirements of this reaction scheme to be tested. According to the scheme, the number of radicals formed initially, ($R_1\cdot + R_2\cdot$), is a constant for a given radiation dose, and we are concerned with the case in which reaction 3 is fast compared with radical decay, so that the total number of radicals does not change appreciably during the period under consideration. If these conditions hold (1) the maximum absorbance per 1000 rads in oscillograms obtained at 335 nm, A_M, should decrease as the pH is increased. Column 3 of Table I shows that this requirement is met. The decrease is caused by a reduction in the proportion of $R_1\cdot$ formed as the pH is increased through the range in which cytosine and $OH\cdot$ are becoming ionized. The change in products lowers the absorbance because the extinction coefficient of $R_1\cdot$ at 335 nm is greater than that of $R_2\cdot$. If only $R_1\cdot$ were formed, A_M would be 0.058 (pH 7 result); if only $R_2\cdot$ were formed it would be 0.012 (pH 12.6 result). In addition, at the higher pH values A_M is further reduced by conversion of some of the $R_1\cdot$ formed initially to $R_2\cdot$ (reaction 3) during the 1.5-μsec period required for the absorbance to reach its maximum value.

The contributions of $R_1\cdot$ and $R_2\cdot$ to the maximum absorbance can be determined as follows: A_M is given by $A_M = A_{R_1\cdot} + A_{R_2\cdot} = 0.058a + 0.012b$, where $A_{R_1\cdot}$ and $A_{R_2\cdot}$ are the absorbances of $R_1\cdot$ and $R_2\cdot$, and a and b are the fractions of $R_1\cdot$ and $R_2\cdot$ present; $a = R_1\cdot/(R_1\cdot + R_2\cdot)$ and $b = R_2\cdot/(R_1\cdot + R_2\cdot)$. Since $(a + b) = 1$, one can readily solve for a ($a = (A_M - 0.012)/0.046$) and compute the values given in column 5. The absorbance due to $R_1\cdot$, $A_{R_1\cdot}$, is $0.058a$ (column 4). The absorbance due to $R_2\cdot$ is, of course, the difference between columns 3 and 4. The values of a and $A_{R_1\cdot}$ are somewhat smaller than expected from the equilibrium concentrations of the reactants. At pH 10.8, for example, cytosine is 4% and $OH\cdot$ 7% ionized. If only reactions 1 and 2 occur, a mixture of products should be formed which contains about 90% $R_1\cdot$ and has $A_{R_1\cdot} \approx 0.052$ instead of 0.042. Reaction 3 cannot account for the discrepancy because it is too slow at pH 10.8 to affect the concentration of $R_1\cdot$ significantly during the observation period. It thus appears that $R_2\cdot$ must be produced by a reaction other than 2 or 3, possibly one involving an intermediate species formed during conversion of e_{aq}^- to $OH\cdot$ by N_2O. The possible occurrence of such a reaction does not affect conclusions regarding the short-lived transient, but it does have the effect of making the $O\cdot^-$ concentration appear larger than the concentration in equilibrium with $OH\cdot$ and H^+ ion.

(2) The absorbance, when decay of the short-lived transient is essentially complete, *i.e.*, when all $R_1\cdot$ has changed to $R_2\cdot$, and before a significant amount of tertiary reactions involve $R_2\cdot$, should be the same in all solutions irrespective of pH. This requirement is met very well by the data in column 6 which show an extreme variation in absorbance of only 0.002.

(3) The disappearance of $R_1\cdot$ (reaction 3) at any given pH above about 10 should follow pseudo-first-order kinetics and the half-life should be inversely proportional to the OH^- ion concentration.

The contribution at any given time by $R_1\cdot$ to the total absorbance can be calculated by the relation given above, taking A_t = observed absorbance at the given time. Alternatively, a quantity proportional to the $R_1\cdot$ absorbance can be calculated simply by subtracting 0.012 from A_t. (To correct for possible systematic shifts in the curves, the actual observed values of $R_2\cdot$ absorbance (Table I, column 6) were used in our computations.) Points calculated in this way fall very close to straight lines on semilogarithmic paper over the entire pH range, indicating that the disappearance of $R_1\cdot$ does indeed follow first- or pseudo-first-order kinetics. The half-lives (Table I, column 7) vary inversely with OH^- ion concentration, as required, and values of the second-order rate constant for reaction 3, $k = 0.693/(t_{1/2} \times OH^-)$, are essentially constant with an average value $(3.5 \pm 0.4) \times 10^8\ M^{-1}\ \mathrm{sec}^{-1}$.

The satisfactory way in which the data meet the above requirements provides strong support for the general reaction scheme given in equations 1, 2, and 3. The identification of these general reactions specifically with reactions 4, 6 and 7, and 8, respectively, while not proven, provides a set of reactions for the formation of cytosine radicals initially present and for their adjustment to solution conditions which is consistent with the data and with presently available knowledge of cytosine reactions.

Acknowledgment. J. D. Zimbrick is grateful to the U. S. Public Health Service, Institute of General Medical Sciences, for a postdoctoral Fellowship award.