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Thermodynamic Fluctuations in a Reacting System—Measurement by Fluorescence Correlation Spectroscopy

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The temporal correlations of thermodynamic concentration fluctuations have been measured in a chemically reactive system at equilibrium by observing fluctuations of the fluorescence of a reaction product. The experiment yields the chemical rate constants and diffusion coefficients and shows the coupling among them. Data are reported for binding of ethidium bromide to DNA.

The time correlations of thermodynamic concentration fluctuations in reactive multicomponent systems at equilibrium are determined by the kinetics of chemical reactions and diffusion processes. Purely diffusive fluctuations have been measured with great success by quasielastic light scattering[1] and extension to reaction kinetics has prompted several experiments[2] and attracted considerable theoretical attention.[3] However, it now appears that homogeneous chemical kinetics are not amenable to scattering studies because the dielectric-constant changes that reveal the fluctuations are usually too small. In contrast, optical absorbance coefficients and fluorescent quantum yields frequently display large changes. Hence we chose to observe intrinsic concentration fluctuations using a fluorescent indicator.

We report here direct observations of fluctuations about thermodynamic equilibrium in a reactive multicomponent system of biophysical interest. We have studied the reversible binding to DNA of ethidium bromide (EtBr), a dye that inhibits nucleic acid synthesis.[4] The complex of dye and DNA is strongly fluorescent[5]; thus fluctuations in the number of molecules of the complex in a small volume defined by a beam of exciting radiation are indicated by fluctuations of the total fluorescent power.[6]

The principal chemical reaction between the DNA (A) and the EtBr (B) to form the fluorescent complex (C) is supposed to be a single-step bimolecular process with rate constants k_f and k_b (in fact the system is more complex, as will be seen):

$$A + B \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C. \tag{1}$$

The equilibrium constant is defined in terms of the equilibrium concentrations $\bar{C}_A$, $\bar{C}_B$, and $\bar{C}_C$ as $K = k_f/k_b = \bar{C}_C/\bar{C}_A\bar{C}_B$. Thermodynamic concentration fluctuations decay via the chemical reaction as $\exp(-Rt)$, where the inverse relaxation time R is[7]

$$R = k_f(\bar{C}_A + \bar{C}_B) + k_b. \tag{2}$$

Since diffusion provides alternative relaxation paths, the correct description of the fluctuation spectrum requires a set of coupled differential equations which for ideal solutions assume the form

$$\partial\,\delta C_j/\partial t = D_j\nabla^2\,\delta C_j + \textstyle\sum_k T_{jk}\,\delta C_k, \tag{3}$$

where $\delta C_j \equiv \delta C_j(\vec{r}, t)$ are the local concentration fluctuations of the three reactants of Eq. (1); the D_j are corresponding diffusion coefficients, and the T_{jk} are the elements of the matrix of linear chemical interaction coefficients implied by Eq. (1).[3]

In our experiment, fluctuations of the concentration C_C of the fluorescent complex cause the fluctuations in a photodetector current. The autocorrelation function of the photocurrent fluctuation $\delta i(t) = i(t) - \langle i(t)\rangle$, with $|\tau|$ written τ, is $G_i(\tau) = \langle \delta i(t+\tau)\,\delta i(t)\rangle$. Neglecting shot noise for simplicity, the photocurrent due to fluorescence induced by the exciting radiation $I(\vec{r})$ is

$$i(t) = g\epsilon Q\int_{-\infty}^{\infty} I(\vec{r})C_C(\vec{r}, t)\,d^3r, \tag{4}$$

where g accounts for quantum efficiency, photomultiplier gain, and geometrical and filtering losses. Q and ϵ are the fluorescence quantum efficiency and extinction coefficient for the EtBr-DNA complex.

In contrast with quasielastic scattering experiments, there is no spatial coherence; the coherence time of the broad emission spectrum is $\sim 10^{-14}$ sec and a random fluorescent emission delay of $\sim 10^{-9}$ sec uncouples the fluorescence from the coherent exciting radiation. Thus fluorescent emission from different molecules is uncorrelated so that intensities are added. Thus

$$G_i(\tau) = (g\epsilon Q)^2 \int I(\vec{r}) I(\vec{r}') \langle \delta C_C(\vec{r}, t)\, \delta C_C(\vec{r}, t+\tau) \rangle\, d^3r\, d^3r'. \tag{5}$$

$G_i(\tau)$ is evaluated in terms of solutions of Eq. (3) and statistical weights determined by the equipartition theorem.[8] We will report details elsewhere.

The convolution of the spatial concentration correlation function with the Gaussian profile of the cylindrical laser beam leads to characteristic diffusion times of the form $\tau_j = w^2/4D_j$, where $j = A, B$; w is the beam radius at $I(w)/I_{\max} = e^{-2}$. Hence the solutions of Eq. (3) may be reduced to convenient form using the expansion parameter $(D_B - D_A)Rw^2 \ll 1$. Noting that $D_A = D_C \ll D_B$ and specializing to an excess of DNA sites, i.e., $\bar{C}_B \sim \bar{C}_C \ll \bar{C}_A \sim \bar{K}^{-1}$, we obtain the form of $G_i(\tau)$ useful for our experiments:

$$G_i(\tau) = \frac{g^2\epsilon^2 Q^2 P^2 l \bar{C}_C}{\pi w^2} [A_0(\tau) + A_+(\tau) + A_-(\tau)], \tag{6}$$

where P is the total power in the incident light beam, l is the length of the illuminated (cylindrical) volume, and

$$A_0(\tau) = K\bar{C}_B (1 + \tau/\tau_A)^{-1},$$

$$A_+(\tau) = \frac{K\bar{C}_A}{1+K\bar{C}_A} \frac{1}{1+\tau/\tau_+} \left(1 + \frac{2}{\tau_B R} \frac{1}{1+K\bar{C}_A} \frac{1}{1+\tau/\tau_+}\right),$$

$$A_-(\tau) = \frac{1}{1+K\bar{C}_A} \frac{e^{-R\tau}}{1+\tau/\tau_-} \left(1 - \frac{2}{\tau_B R} \frac{K\bar{C}_A}{1+K\bar{C}_A} \frac{1}{1+\tau/\tau_-}\right),$$

with characteristic times $\tau_+ = \tau_B(1+K\bar{C}_A)$, $\tau_- = \tau_B(1+K\bar{C}_A)/K\bar{C}_A$.

The time dependence of $A_0(\tau)$ is determined entirely by the slower diffusing species through τ_A; A_+ reflects diffusion of the small molecule as slowed by chemical interaction with the slower diffusers; A_- is dominated by the exponential factor due to the chemical relaxation of Eq. (2). The convolution with $I(\vec{r})$ in Eq. (4) introduces factors of the form $(1+\tau/\tau_j)^{-1}$ replacing the more usual exponential form.

The mean photocurrent is $\langle i(t) \rangle = g\epsilon Q l P \bar{C}_C$ and the relative root-mean-square fluctuating photocurrent is

$$\frac{\delta i_{\rm rms}}{\langle i(t) \rangle} = \frac{[G_i(0)]^{1/2}}{\langle i(t) \rangle} = (\pi w^2 l \bar{C}_C)^{1/2}. \tag{7}$$

In our experiments $\pi w^2 l \sim 10^{-8}$ cm^3 and $\bar{C}_C \sim 10^{-7}$ M or $\sim 10^{14}$ molecules/cm^3 so that $\delta i_{\rm rms}/\langle i(t) \rangle \sim 10^{-3}$.

To measure $G_i(\tau)$ we excited the EtBr-DNA fluorescent complex with the 514.5-nm line of a stabilized argon laser, usually at 1.5 mW power to minimize photodecomposition with the beam focused to $w = 5.5$ μm in a cell of thickness $l = 150$ μm. The orange fluorescence was collected by a parabolic reflector, passed through a saturated $K_2Cr_2O_7$ solution filter to reject the laser light, and collected on an S-20 photocathode. The photocurrent fluctuations were analyzed, after filtering out the steady component, with two 100-channel SAICOR autocorrelators. From 10^4 to 10^7 intensity samples were recorded for each time delay. The response of the entire system was proven with a stable white-light source that could be weakly modulated at $\sim 0.01\%$ rms. The optical properties of the DNA-EtBr complex are $\epsilon = 3.8$ (mM cm)$^{-1}$ at 514.5 nm,[9] $Q = 14\%$.[10] In the free dye, Q is only $\sim 0.7\%$.[5] Our calf thymus DNA was phenol extracted and sonicated to a molecular weight of 2×10^5. The experiments reported here were all made at 22°C in a suitable buffer (10^{-4} M Na-ethylene-diamine-tetra-acetic acid, 10^{-2} M tris-HCl, pH 8, 10^{-1} M NaCl.

Measurements of $G_i(\tau)$ for two "pure" dyes, rhodamine 6G and EtBr, demonstrated the method for nonreactive species. Accord with each factor of Eq. (7) was verified. The observed $G_i(\tau) \propto (1+\tau/\tau_D)^{-1}$, where $\tau_D = w^2/4D_D$ is determined by the diffusion coefficients D_D of the pure dyes. We obtained $D_D \cong 1.5\times10^{-6}$ cm^2/sec for both dyes.

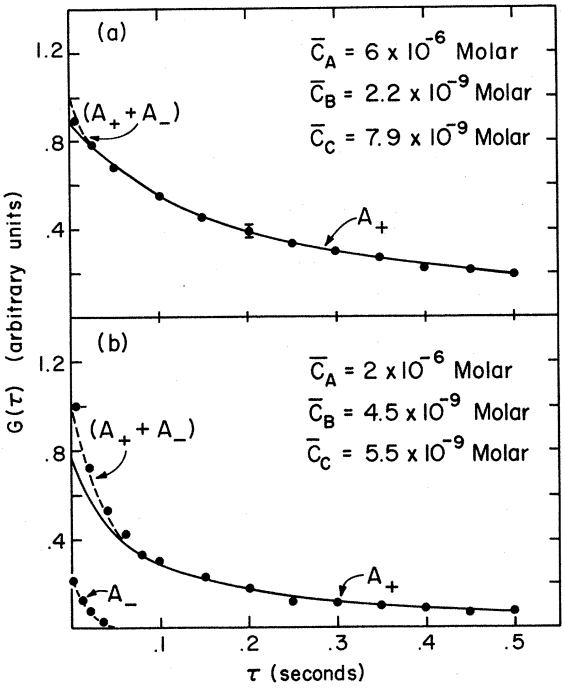

FIG. 1. Typical data points for $G_i(\tau)$ at two values of $\bar{C}_A$, fitted by Eq. (6).

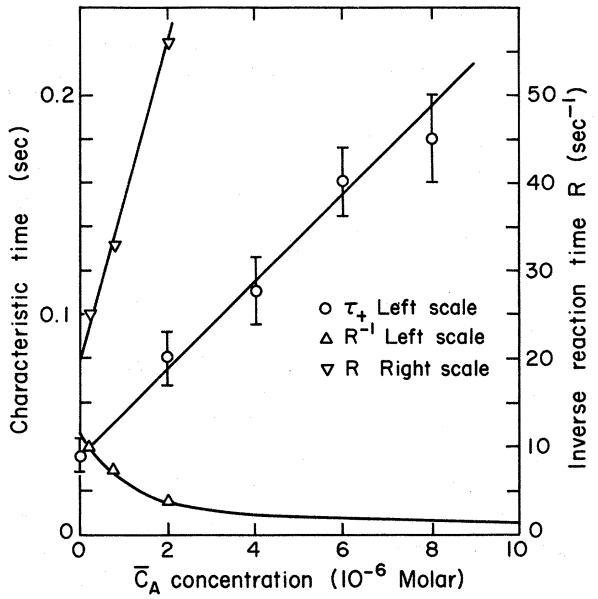

FIG. 2. Magnitude and variations of τ_+ and R^{-1} with $\bar{C}_A$, which yield the chemical parameters of the reaction; see text.

Although $\tau_D \cong 36 \pm 7$ msec, the uncertainties of w^2 and thus D_D may be ~ 50%. These values of D_D are reasonable.[11]

To derive the kinetic parameters, $G_i(\tau)$ was measured as a function of $\bar{C}_A$ from 2×10^{-7} to 8×10^{-6} M. Over this range the relative amplitudes of $A_+(0)$ and the $A_-(0)$ change considerably. At the *higher* $\bar{C}_A$, the term $A_+(\tau)$ dominates, as illustrated by the fit to measured points in Fig. 1(a), so that a set of values of τ_+ may easily be determined as a function of $\bar{C}_A$ as shown in Fig. 2, yielding a value for k_f/k_b.

At the *low* $\bar{C}_A$, the major term $A_-(\tau)$ is dominated by the exponential factor due to reaction kinetics. This range yields an estimate of k_b, the limiting value of R as $\bar{C}_A + \bar{C}_B \to 0$. Given trial estimates from the limiting cases, intermediate concentration data as in Fig. 1(b) can be analyzed reiteratively to improve the estimates of K and R. The derived dependence of τ_+ and R on $\bar{C}_A$ is exhibited in Fig. 2. The characteristic time τ_- is not reliably obtained as an independent experimental result. The dependence of τ_+ and τ_- on w^2 in Eq. (6) was confirmed in the limiting cases.

Equation (1) oversimplifies the mechanism of binding. Surface sites for EtBr in DNA[5] and puzzling kinetic complexities[12] have been observed. We attribute to these complications the observation that the measured value of $[G_i(0)]^{1/2}$ has approximately $\frac{1}{3}$ of the calculated magnitude while $\langle i(t)\rangle$ remains rather consistent with the pure-dye experiments and calculations. The reduced amplitude of $G_i(0)$ can be interpreted as a shift of some of the fluctuation spectrum out of the range 50 μsec $< \tau <$ 2 sec.

With Eq. (6) and the typical data set shown in Fig. 2, the dependence of R on C_A yields $k_b = 20 \pm 7$ sec^{-1} and $k_f = (1.8 \pm 0.8)\times10^7$ sec^{-1} M^{-1}, whence $K = k_f/k_b$ gives $4\times10^5 \leq K \leq 20\times10^5$ M^{-1}. The dependence of τ_+ on C_A independently determines K with greater precision as $K = (5.4 \pm 1)\times10^5$ M^{-1}. By conventional fluorescent titration in the same apparatus we obtained $K = (6 \pm 1)\times10^5$ M^{-1}. Using $K = 5.4\times10^5$ M^{-1} and the most precise datum on R at the largest C_A, the best values of the kinetic parameters are obtained as $k_b = 27$ sec^{-1} and $k_f = 1.5\times10^7$ sec^{-1} M^{-1}. Independent values of $k_b = 40 \pm 10$ sec^{-1} and $k_f = 2\times10^7$ sec^{-1} M^{-1} have been determined by conventional temperature perturbation methods at 32°C.[13] A reasonable correction for the small temperature dependence leads to agreement well within the combined uncertainties.

We conclude that our method of fluorescence correlation spectroscopy has successfully revealed the ubiquitous fluctuations around equilibrium in a reactive system with sufficient precision to identify and measure chemical reaction kinetics as well as diffusion and to demonstrate

the coupling between them. We confirm that conventional kinetic equations provide a good description of the intrinsic thermodynamic concentration fluctuations in our archetypal reactive system. The precision of our method can surely be improved and it may be extendable to other indicators such as optical adsorption or Raman scattering. The extremely small numbers of molecules ($\sim 10^4$) needed for a measurement suggest interesting applications in surface physics and biophysics.

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Evidence for Isotopic Impuritons in Solid Helium

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Measurements of the spin diffusion coefficient D and the NMR linewidth T_2^{-1} are reported for a ^{3}He impurity in solid ^{4}He at molar volume 21 cm^3. For $x_3 < 3\times10^{-2}$, $Dx_3 = (1.2\pm0.4)\times10^{-11}$ cm^2 sec^{-1}, where x_3 is the fractional impurity concentration, and for $x_3 < 2\times10^{-3}$, $T_2x_3 = (1.1\pm0.3)\times10^{-4}$ sec. These results conform with a model of "impuriton" excitations or "mass fluctuation waves" in which the quasiparticles move like a gas.

In the last few years several workers[1,2] have pointed out that by applying elementary quantum-mechanical ideas to impurities (and other point imperfections) that can tunnel in a solid, one is led to postulate a set of wavelike excitations in which the impurities are delocalized. Isotopic impurity in solid helium is an ideal system in which to search for such excitations because the large zero-point energy of the particles leads to a temperature-independent tunneling frequency J, which for pure solid ^{3}He at low temperatures is about 10^8 sec^{-1} at the melting pressure, decreasing rapidly with increasing pressure.[3]

The question of what properties of the system would be strongly affected by these impurity waves, or "impuritons" as we shall call them, is not a simple one. Since together with phonons, the impuritons are the excitations of the system at low temperatures, formally all physical properties are affected, but in many cases not in a