utilize red cells in bulk suspensions reflect the kinetic and kinematic balance of aggregation and dispersion processes. Direct manipulation of cells, as performed in these experiments, is required to separately quantitate such aggregation and dispersion processes.

Micromanipulation techniques were also useful in the separation of time-dependent, i.e., dissipative, processes from equilibrium processes. In general, solution viscosities and cell surface viscosities affect the rate at which cell contact and adhesion take place, but the data reported here were taken from pairs of cells that had reached mechanical equilibrium.

In conclusion, we have used the elastic deformation of the red blood cell at mechanical equilibrium to calculate the cell surface affinity in dextran solutions and plasma. The affinities are the reduction in free energy per unit of membrane area associated with formation of adhesive contact and are independent of cell geometry. The peak surface affinities of normal red blood cells were found to be 4.9×10^{-3} erg/cm^2 in D70, 2.2×10^{-2} erg/cm^2 in D150, and 2.0×10^{-3} erg/cm^2 in plasma. Neuraminidase-treated cells had higher affinities than normal cells: at least 2.8×10^{-2} erg/cm^2 in D70 and 1.8×10^{-3} erg/cm^2 in D28.

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A Method for Rapid, Continuous Monitoring of Solute Uptake and Binding†

Sol M. Gruner,* Gregory Kirk, Lekha Patel, and H. Ronald Kaback

ABSTRACT: A method has been developed for dynamically monitoring the free concentration of diffusible, tritiated solutes. The technique utilizes particles of a solid scintillator microencapsulated in gel beads that are permeable to diffusible label. Since tritium β radioactivity has an effective range in water of only a few micrometers, only label that is free to diffuse through the gel can excite the scintillator, while sequestered label is effectively excluded. Thus, the scintillation light output monitors the freely diffusible concentration of label exclusively. A simple, preliminary encapsulation technique is described and tested, and the theory behind the method is discussed with regard to the time resolution attainable for a given label concentration and type of encapsulation. The feasibility of the method is demonstrated by measuring the uptake of [^{3}H]tetraphenylphosphonium by *Escherichia coli* membrane vesicles in response to the generation of a membrane potential (interior negative).

In biological systems, it is frequently important to quantify uptake or binding of soluble substances by colloidal or macroscopic phases such as cells, subcellular particles, or macromolecules. The time course of the phenomenon is often an important parameter. Generally, uptake or binding is measured either directly by assaying the macroscopic phase after separation from the bathing medium or indirectly by determining the the free concentration of the solute. Although a wide variety of analytical techniques have been utilized for these measurements, no single method is applicable in every instance, and most of the methods either are relatively insensitive, exhibit poor time resolution, or are applicable to a limited group of solutes.

This paper describes the use of encapsulated scintillation beads for in situ monitoring of the free solution concentration of tritiated solutes. Advantage is taken of the fact that the β particle released when a tritium atom decays has a maximum range of about 7 μm in water. Consequently, a scintillator may be totally shielded from a tritiated source that is not freely diffusible. In the procedure, a finely divided scintillator is encapsulated in microbeads of a permeable gel. The gel pore size is selected to be large enough to allow for free diffusion of the tritiated solute in solution. As a result, the light output of the microbead suspension continuously monitors the free concentration of the tritiated species with no contribution from solute that is not freely diffusible.

Experimental Procedures

Materials

D-[^{3}H]Glucose (25 Ci/mmol) was obtained from New England Nuclear. [^{3}H]Tetraphenylphosphonium (bromide salt; 2.5 Ci/mmol) was prepared by the Isotope Synthesis Group of Hoffmann-La Roche Inc. under the direction of Arnold Liebman. All other materials were of reagent grade and purchased from commercial sources.

Methods

Scintillator Preparation. Poly(vinyltoluene)-based NE102 plastic scintillator microspheres of 1–10-μm diameter (Nuclear Enterprises, Inc., San Carlos, CA) were chosen because of both their size and their relative immunity to most aqueous saline

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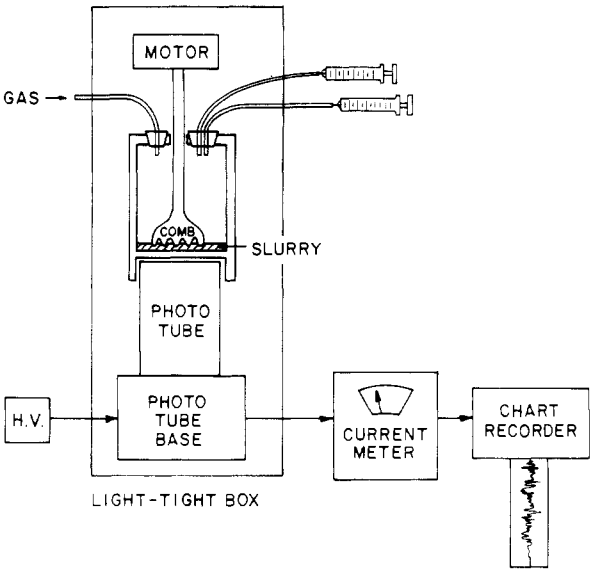

FIGURE 1: Schematic of apparatus used to detect light output of scintillator bead–macrophase slurry.

solutions. Two grams of the beads was suspended in 20 mL of 3% Triton X-100 by vortexing and gentle sonication. The detergent was required because the beads are difficult to wet. The detergent was then removed by five or six washes. For each wash, the beads were sedimented by centrifugation at 6000*g* for 10 min. After each sedimentation, the supernatant was discarded, and the beads were resuspended in 30 mL of distilled water. After the final wash the resuspended beads were allowed to settle overnight, and the supernatant was carefully removed. The resultant slurry was roughly 10% beads by volume. The following quantities were then used, for each milliliter of settled bead slurry, to produce a polyacrylamide gel: 300 mg of acrylamide and 1.5 mg of *N,N'*-methylenebis(acrylamide) were dissolved in the slurry. Polymerization was catalyzed by the addition of 10 μL of 10% (w/v) ammonium persulfate solution and 2 μL of *N,N,N',N'*-tetramethylethylenediamine (TEMED). The polymerized gel was cut into pieces, suspended in distilled water, and then sheared in a blender for several minutes to a uniform consistency of fine particles. Microscopic examination of the product revealed irregularly shaped bits of gel (mean size $\sim$0.1 mm) encasing clumps of scintillator. The scintillator to gel volume ratio was $\sim$10%. The gel particles were allowed to settle after which the supernatant was discarded. The final slurry was refrigerated until used.

Scintillation Detection. Radioactively induced scintillation was detected with the apparatus illustrated in Figure 1. Several milliliters of a mixture of the macrophase to be monitored (e.g., vesicles), a tritiated substrate, and the gel–bead slurry were contained, to a depth of 1–2 mm, in a polished Lucite cylinder,[1] which was coupled with optical grease to the face of an RCA 8850 photomultiplier (RCA Electronics, Harrison, NJ). The photomultiplier was operated at −2000 V (photoelectron gain = 8×10^6). The current output of the tube was read directly on a Keithley 610C electrometer. The electrometer output was also recorded on a chart recorder. Typical dark currents for the phototube, after dark adaptation, were $\sim 10^{-9}$ A. The slurry was continuously stirred at 1 rps by a motor-driven comb cut from a thin Teflon sheet. A long shaft fitted to the motor assured that the motor fields did not affect the photomultiplier. The entire apparatus was enclosed in a light-tight box. Various tubes, fitted to the Lucite cylinder and extended through the light-tight box wall, allowed for gas flow and substrate injection, as needed.

Membrane Vesicles. Membrane vesicles were prepared from *Escherichia coli* ML 308-225 ($i^-z^-y^+a^+$) as described (Kaback, 1971; Short et al., 1975).

Results and Discussion

Theoretical. The tritium isotope (^{3}H) has the desirable characteristics of decaying via the emission of a low-energy electron (E_{max} = 18.6 keV; mean weighted energy = 6.3 keV) and of being readily incorporated into many organic compounds.[2] The emitted electron is primarily responsible for the excitation of optical transitions in scintillating materials. The range, *R*, appropriate to the tritium β-ray spectrum is $R \simeq 1$ μm in materials with the density of water.[3] Thus, an effective aqueous shield to this radiation need be only a few micrometers thick. The short range of the electron suggests that its detection is a surface area problem. Consequently, one chooses to maximize the surface area to volume ratio of the detecting scintillator while simultaneously constraining the scintillator volume to be sufficient to fully stop the average incident electron. Together these facts argue that the scintillator be small beads of a few micrometers in diameter.

It is desired to segregate the scintillator beads from the colloidal or macroscopic phase being monitored. The latter—e.g., cells, vesicles, or macromolecules—shall be referred to as the *macrophase* to distinguish it from the scintillator beads. This is desired so that a tritiated molecule sequestered by the macrophase is unable to excite a bead of scintillator. A suitable permeable coating is a carbohydrate or polyacrylamide gel coating. For the following discussion, it is assumed that the coating is a gel. The time response of the system for monitoring uptake or binding of the tritiated substrate is limited by the time it takes the substrate to diffuse between the macrophase and the scintillator beads. The time response is optimized by maintaining a concentrated slurry of coated scintillator and the macrophase. Under these conditions, it is highly probable that the macrophase is in contact with a given coated scintillator bead. Thus, it is desired that the gel coating be thick enough to effectively stop most of the β rays incident upon it from a tritium atom on the outer surface. The gel coating is largely water; consequently, a thickness of a few micrometers is sufficient.[3]

An idealized coated scintillator bead is shown in Figure 2. Its time response may be calculated by assuming a sudden change in the concentration of the tritiated label external to the gel coating and computing the diffusion kinetics which follow. Specifically, assume that at time $t = 0$ the concentration, $n(r,t)$, of the tritiated label suddenly increases from $n = 0$ to a constant value of $n = n_0$ for radii greater than r

[1] In retrospect, a silica cylinder would have been easier to clean. Silica is superior to glass in that glass is a weak scintillator with a substantial ^{40}K background.

[2] Although only ^{3}H is explicitly discussed in this article, the β emissions of ^{14}C and a few other isotopes are of sufficiently low energy that advantage may be taken of encapsulated scintillators. However, the thickness of the required encapsulation grows rapidly with the β energy.

[3] The term "range" is used throughout this paper. The term has various definitions in the literature. As used in this paper, the term means the thickness of the implied material (usually water) that, when interposed between a layer of tritium atoms and a plane surface, reduces the energy deposited beyond the surface to some nominal fraction of the energy of the β electrons emitted. We calculate the range to be $\simeq 1$ μm for reduction of the emitted energy by 90% (Kirk & Gruner, 1982). Note, however, that the essential features of this paper would remain unaltered even if a substantially longer range were selected.

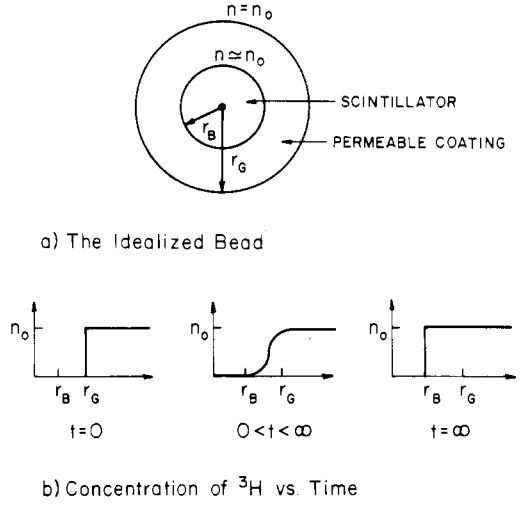

FIGURE 2: (a) Idealized coated scintillator bead. (b) Concentration of a diffusible substance in permeable coating region of a bead graphed as a function of distance from bead center for three different times. At $t = 0$, the concentration exterior to the coating increases suddenly to a value n_0. After a long time, the concentration in the bead coating equilibrates to a value very nearly equal to n_0.

$= r_G$ (Figure 2b). The light output of a bead will be proportional to the concentration of label within range. As time proceeds the concentration of label for $r_B < r < r_G$ will rise until, eventually, it stabilizes at an equilibrium concentration nearly equal to n_0 for a gel that is mostly water.

The concentration, $n(r,t)$, may be calculated by solving the diffusion equation appropriate to the geometry and boundary conditions given above. The concentration may then be integrated over the range of the β rays to determine the integrated light output, $I(t)$, expected from a scintillator bead at time t. For times short compared to the time it takes the solution to reach equilibrium the light output is calculated to be (Kirk & Gruner, 1982)

$$I(t) = 4(\pi Dt)^{1/2} r_G^2 n_0 \tag{1}$$

for $t \ll R^2/D$, where $R = r_G - r_B \equiv$ gel coating thickness.

Recall that n approaches n_0 asymptotically for $r_B < r < r_G$ and for times greater than R^2/D. As equilibrium is established, the light output tends toward

$$I(t = \infty) = 4\pi R r_G^2 n_0 \tag{2}$$

for $R \ll r_G$.

Thus

$$I(t)/I(t = \infty) = [Dt/(\pi R^2)]^{1/2} \tag{3}$$

for $t \ll R^2/D$.

The above treatment should be taken as being only an approximate description of the time response for the light output, $I(t)$. This follows not only because of the simplifications used but also because the inhomogeneities and encapsulation details in any real bead population will likely dominate the uncertainties in the time response. The treatment does, however, allow reasonable numerical estimates. It predicts an initial $t^{1/2}$ time dependence and a characteristic time response

$$t_r \simeq R^2/D \tag{4}$$

For $R = 1$ μm and the diffusivity of medium-weight organic molecules [100–500 daltons, which implies $D \simeq 10^{-5}$ cm^2/s in water—see Weast (1970)] this suggests a time response of $t_r \simeq 1$ ms. Thus, quantitative monitoring of the free solution concentration of the label, if the concentration is large enough (see below), seems feasible on time scales of several milliseconds. The onset of a *change* in this concentration can probably be detected on even shorter time scales.

The time resolution and the accuracy of the determination of the concentration will be limited by the statistics of tritium decay and of light detection. Essentially, one wishes to determine the fluctuation in the detected light signal in a given time interval for a given concentration of free solution tritiated label. The total number of tritium atoms decaying in a time interval, Δt, that will excite scintillation is given by $N_{\Delta t}$:

$$N_{\Delta t} = A(V_G/V_F)\kappa\Delta T \tag{5}$$

where A = total tritium activity (decays per second), V_G = total volume of fluid in range of the scintillator, V_F = total volume of the free fluid, and κ = the probability that a decaying tritium within range of the scintillator excites a scintillation $\simeq 0.23$ (Kirk & Gruner, 1982).

A numerical example is appropriate. Assume one is dealing with 0.5 nmol of substrate, each tritiated at one nonexchangeable site. (Tritiation at one site implies an activity of about 25 Ci/mmol.) If V_G/V_F is taken to be about 1%, then

$$N_{\Delta t} \simeq 1000\Delta t \text{ decays} \tag{6}$$

Tritium decay obeys Poisson statistics. If one were able to count all $N_{\Delta t}$ decays, the fluctuation in the measurement would be given by $(N_{\Delta t})^{1/2}$. Thus, in a 0.1-s interval, the intrinsic accuracy would be

$$\text{noise/signal} = (N_{\Delta t})^{1/2}/N_{\Delta t} = 1/(N_{\Delta t})^{1/2} = 10\%$$

If one expects to detect the scintillation signal via a photomultiplier, then the number of signal photoelectrons emitted from the photocathode, N_e, would be the next weakest link in the statistical chain. This number is given by

$$N_e = N_{\Delta t}\epsilon_s(\bar{E}/E_\gamma)\sigma\epsilon_c \tag{7}$$

where ϵ_s = efficiency of the scintillator, $\bar{E}$ = the expectation value of the β-ray energy deposited in the scintillator [its value is approximately 6 keV (Kirk & Gruner, 1982)], E_γ = average energy of a photon output by the scintillator, σ = light collection efficiency, and ϵ_c = net photocathode efficiency over the scintillation spectrum.

Equation 7 is dependent on the details of the light-detecting apparatus. For a given scintillator and detection apparatus, all the parameters can be readily specified. For example, using the value of $N_{\Delta t}$ computed above, and reasonable values for the remaining constants (the values chosen were applicable to the test system described in the next section), one arrives at

$$N_e = (1000\Delta t)(0.03)(6000/3)(0.3)(0.15) = (2.7 \times 10^3)\Delta t \text{ photoelectrons}$$

Note that $N_e/\Delta t$ is roughly comparable to the photoelectron dark current expected from a good photomultiplier and roughly 8 times the fluctuation in that dark current integrated over 0.1 s. It, therefore, represents a respectable signal. The overall fluctuations will, of course, be greater due to the fluctuations in $N_{\Delta t}$.

By setting $N_{\Delta t} = 1$ in eq 7, one may compute an average photoelectron contribution from a single tritium decay. For the example at hand it is about three electrons per decay. This number results primarily from the low value of the product $\epsilon_s\sigma$. It is likely that a careful state of the art construction can improve this substantially. For example, placing a mirror above the slurry would almost double the light collection efficiency. In this case, there would be sufficient photoelectrons per decay to pulse height discriminate against the photocathode dark current, and the counting statistics would be more closely determined by the fluctuations in the number of tritium decays.

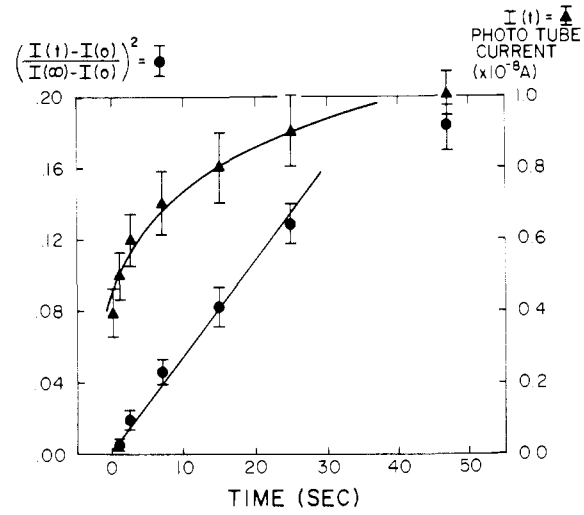

FIGURE 3: Time response of photomultiplier current, $I(t)$, upon addition of 11 μCi of tritiated glucose (▲). Also shown is the background-subtracted quadratic ratio of the response to the equilibrium ($t = \infty$) response (●). A straight-line fit to these data indicates that the light output increases as $t^{1/2}$ for early times. It is expected that light output eventually falls from a $t^{1/2}$ dependence, since $t^{1/2}$ is unbounded.

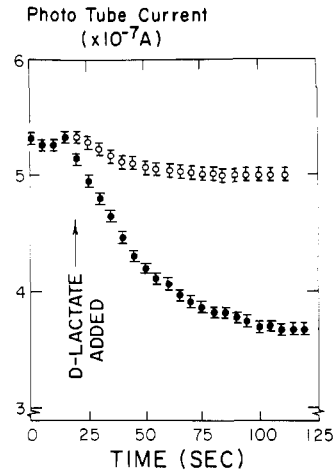

FIGURE 4: Photomultiplier current output in response to addition of D-lactate to a slurry of coated scintillator, *E. coli* ML308-225 membrane vesicles, and [^{3}H]TPP$^+$ in the absence (●) and presence (○) of valinomycin (see text for details).

Experimental. There is an extensive literature on both scintillators (Birks, 1964) and microencapsulation techniques (Zaborsky, 1973). Our interests, however, were not in microbead process development but in constructing a simple monitor that would demonstrate feasibility. Consequently, commercially available plastic scintillator microspheres were embedded into a block of gel and the gel block was sheared in a blender (see Methods).

The light output of the system was calibrated by the injection of 11 μCi of tritiated glucose in a 1–2-mm bed of scintillator–gel slurry. The scintillator volume was estimated as 1% of the total volume. An estimate of the expected output current, given the photoelectron gain of 8×10^6, can be computed on the basis of eq 5 and 6. The examples chosen after eq 5 and 6, in fact, pertain to the geometry at hand, given the realization that the scintillator encapsulation procedure does not enter directly into these equations. The predicted current was $N_e/\Delta t = (2700)(8 \times 10^6)(1.6 \times 10^{-19}) \simeq 3 \times 10^{-9}$ A. The measured current was actually $(1.5 \pm 0.3) \times 10^{-8}$ A where the error refers to the estimated root mean square fluctuation through the bandwidth of the electrometer. Given the variation in the sizes and shapes of the gel particles, a factor of 5 difference is not considered to be significant. It appeared that 10% changes in the signal should be detectable with only 10 μCi of total activity.

The system time response was measured by injecting 20 μCi of tritiated glucose into the slurry as it was being stirred. A plot of the output current, $I(t)$, is shown in Figure 3. Also shown is a plot demonstrating that

$$[I(t) - I(0)]/[I(\infty) - I(0)]^2 \simeq \alpha t$$

where $t < 50$ s. This indicates that the output light intensity initially varies as $(t)^{1/2}$, as predicted by the diffusion model embodied in eq 3. Note that the model leading to eq 3 is also valid for microbeads of scintillator dispersed in much larger bits of gel, provided that R is now taken to be the size of the gel bits. Thus, it is predicted that

$$\alpha = D/(\pi R^2) = 0.02$$

where $R \simeq 0.01$ cm and D = diffusivity of glucose = 0.67×10^{-5} cm^2/s. The value of α measured from the slope of Figure 3 is 0.005/s. The difference between the measured and predicted values is not unreasonable, given the variance in the sizes of the gel pieces.

So that the utility of the encapsulated scintillator beads in monitoring a biological phenomenon could be tested, accumulation of [^{3}H]tetraphenylphosphonium (TPP$^+$) by *E. coli* ML 308-225 membrane vesicles in response to the generation of a transmembrane electrical potential ($\Delta\Psi$, interior negative) was examined (Schuldiner & Kaback, 1975; Ramos et al., 1976, 1979, Ramos & Kaback, 1977; Felle et al., 1980). The data presented in Figure 4 show the change in photomultiplier current observed during an experiment in which the specimen chamber (cf. Figure 1) contained a slurry of *E. coli* membrane vesicles (16 mg of protein) and 0.5 mL of chopped scintillator–gel in 50 mM potassium phosphate, pH 6.6 (total volume 10 mL). Addition of [^{3}H]TPP$^+$ to a concentration of 8 μM (total radioactivity approximately 160 μCi) resulted in a base-line current of 5.3×10^{-7} A with small fluctuations. On addition of lithium D-lactate to a final concentration of 20 mM, there was a sharp decrease in output current that fell to within 10% of the minimal value of 3.6×10^{-7} A in 50 s. Although not shown, the photomultiplier output then remained constant for about 20 min when the reaction mixture was purged with nitrogen; subsequently, current output returned to the control level. When a similar experiment was performed in the presence of the ionophore valinomycin (final concentration 2 μM), the decrease in current was only about one-tenth of that observed in the absence of the ionophore (Figure 4). This effect is consistent with the known ability of the ionophore to dissipate $\Delta\Psi$ in the presence of potassium (Shuldiner & Kaback, 1975; Ramos et al., 1976; Ramos & Kaback, 1977).

These results clearly demonstrate that the method, crude though it is at this initial stage, is capable of measuring changes in free solute concentration considerably more rapidly than the more commonly used technique of flow dialysis (Ramos et al., 1976, 1979). Furthermore, the signal strength and time response are within reasonable limits for this crude system, and the decrease in signal with time conforms to the characteristic $t^{1/2}$ behavior.

It is concluded that even this simple approach is a useful starting point for the development of more sophisticated methodology. The relatively slow time resolution of the sheared scintillator–gel results from the large mean size of the particles. Since the diffusion time varies inversely with the square of dimensions of the gel particle, considerably better time resolution can be achieved by producing smaller particles.

Ultimately, one wishes to separately encapsulate individual scintillator beads. Thus, the procedure described has the advantages of simplicity, but the encapsulated scintillator used here is only a crude approximation of the ideal (Figure 2). We are currently exploring alternative procedures for encapsulation and detection.

Finally, it is noted that many cells or cell clusters are substantially larger than a few micrometers across. If the tritiated solute is incorporated into the bulk of such an object, then the object can act as its own shield for large fractions of its internal volume. For example, 73% of the volume of a spherical cell 10 μm in diameter is more than 1 μm removed from the cell surface. In such cases, uncoated scintillator microspheres, when mixed with the cell suspension, will monitor the bulk absorption of the tritiated solute with kinetics limited only by the time required for the solute to be incorporated into the cell.

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Conformation of the Deoxydinucleoside Monophosphate dCpdG Modified at Carbon 8 of Guanine with 2-(Acetylamino)fluorene†

B. Hingerty and S. Broyde*

ABSTRACT: Minimized conformational potential energy calculations were performed for dCpdG modified with the carcinogen 2-(acetylamino)fluorene (AAF). The major adduct, linked via a covalent bond between guanine C-8 and N-2 of AAF, was investigated. The 12 variable torsion angles and both deoxyribose puckers were independent flexible parameters in the energy minimizations. Three categories of low-energy conformers were calculated in which the guanine was syn and nearly perpendicular to the plane of the fluorene: (1) forms in which fluorene is stacked with cytidine (included among these is the global minimum energy conformation); (2) conformers which preserve guanine–cytidine stacking while placing the fluorene in a base-pair obstructing position; (3) conformers which maintain guanine–cytidine stacking and place the fluorene at the helix exterior, without interfering with base pairing. The Z form is important in this group. In addition, a low-energy conformation with guanine anti, but still nearly perpendicular to fluorene, was computed. Molecular models were constructed for the most important conformations incorporated into larger polymers. These indicated that the fluorene–cytidine stacked forms induce a severe kink in the B helix. Conformers with guanine–cytidine stacking and AAF in a base-pair obstructing position place the AAF at the B-type helix interior with little distortion in the helix direction. Conformers with the guanine–cytidine stack in which AAF does not affect base pairing place the fluorene at the Z or alternate helix exterior. It is suggested that base sequence, extent of modification, and external conditions such as salt concentration determine which of a number of possible conformational effects is actually induced by AAF. The variety of observed experimental results with AAF-modified DNA may reflect these various conformational possibilities.

The carcinogen *N*-acetoxy-2-(acetylamino)fluorene (AAAF) links covalently to DNA. In vitro, the major adduct (about 80%) results from a linkage between C-8 of guanine and N-2 of AAAF (Kriek et al., 1967), while a minor adduct results from a linkage between N-2 of guanine and C-3 of AAAF (Kriek, 1972; Westra et al., 1976; Yamasaki et al., 1977). The acetoxy is eliminated, yielding the products C8(G)-N2(AAF) and N2(G)-C3(AAF). In vivo these same two adducts are observed (Kriek, 1972), but a large fraction of the C8(G) adducts are deacetylated to give C8(G)-N2(AF) (Irving & Veazey, 1969; Kriek, 1972, 1974; Kriek & Westra, 1980). Uncharacterized adenine adducts, present in small amounts, have also been reported in vitro (Kapuler & Michelson, 1971; Kriek & Rietsema, 1971; Levine et al., 1974). In vivo, the major C8(G)-N2(AAF) adduct is largely repaired, while the minor N2(G)-C3(AAF) adduct is persistent (Howard et al., 1981, and references cited therein).

A great deal of experimental work has been done in an effort

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