*STUDY OF CARBONIC ANHYDRASE USING PERTURBED ANGULAR CORRELATIONS OF GAMMA RADIATION**

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Abstract.—The angular correlation of the 150–247 kev gamma-ray cascade of ^{111m}Cd is strongly perturbed when this nucleus is bound to the enzyme carbonic anhydrase. A comparison of the perturbed angular correlation for the apoenzyme with that for native carbonic anhydrase confirms that the ^{111m}Cd binds at the active region of the enzyme. These results provide good evidence that the perturbed angular correlation reflects the effective molecular rotational correlation time at the metal binding site, and that this radioactive nucleus can be used as a rotational tracer to label biological macromolecules. The qualitative dependence of the perturbed angular correlation of the ^{111m}Cd cascade on the molecular rotational correlation time at the metal binding site is illustrated using a cadmium-complex solution at various temperatures.

Introduction.—A number of labeling techniques have recently been developed for the study of rotational correlation times, internal motions, and conformational changes in biological macromolecules.[1–3] For example, the depolarization or the decay of the fluorescence of small chromophores can be used to measure the rotational correlation time of the chromophore bound to a macromolecule.[1] The motion and orientation of stable free radicals bound to biomolecules are readily monitored by electron spin resonance,[2] and halide ions have been used as chemical probes for nuclear magnetic resonance studies of proteins labeled with metal atoms.[3]

These labeling techniques share a number of general features. Information on localized behavior of the macromolecule near the labeling site is usually accessible. In many instances, the labels can be incorporated into interesting regions of the macromolecule by using selective and specific chemical methods. Labels have been bound chemically to substrate or inhibitor molecules which subsequently interact with active regions of enzymes or antibodies.[4–6] On the other hand, there is always some uncertainty as to how much the label affects the system being studied.

When two gamma rays that are emitted successively in nuclear de-excitation are detected in coincidence, the coincidence counting rate $W(\theta, t)$ may depend strongly on the angle θ between their directions of propagation. This angular correlation in the directions of emission of a gamma-ray cascade can be perturbed by the interaction of nuclear moments in the intermediate state with fluctuating external fields. A study of the perturbed angular correlation of gamma radiation from radioactive nuclei can thus provide a measure of the nuclear relaxation time and thereby, in principle can yield the rotational correlation time, τ_c, of a mole-

cule to which the radioactive nucleus is bound. The use of a radioactive nucleus as a "rotational tracer" to label biological macromolecules thus offers the possibility of obtaining the information available with other labeling methods, but with the sensitivity, instrumental simplicity and *in vivo* applicability of radioactive tracer techniques.

Leipert *et al.*[7] have shown that the 173–247 kev gamma-ray cascade in ^{111}Cd following the decay of ^{111}In is strongly perturbed when the ^{111}In is bound to bovine serum albumin or poly-L-glutamic acid. Indeed, the angular correlation is sensitive to changes in pH, changes in urea concentration, addition of complexing reagents, and displacement of the $^{111}In^{3+}$ by Hg^{2+}. However, since the gamma-ray cascade observed in the daughter ^{111}Cd nuclei follows electron capture decay of ^{111}In at the binding site, the possibility that after-effects following decay might lead to detachment of Cd ions from these sites is a major concern. Furthermore, the nature of the In^{3+} binding sites in bovine serum albumin and poly-L-glutamic acid is unknown.

The energy level scheme of ^{111}Cd includes a 49-minute metastable state at 397 kev as shown in Figure 1.[8] It is straightforward to populate this state using the ^{110}Cd (n, γ) ^{111}Cd reaction, and to study the perturbed angular correlation of the 150–247 kev cascade. In this paper, the perturbed angular correlation of ^{111}Cd bound to the enzyme carbonic anhydrase is reported. By using the ^{111}Cd metastable state, the potentially large recoil effects from the ^{111}In electron capture decay are eliminated and only cadmium chemistry is involved.

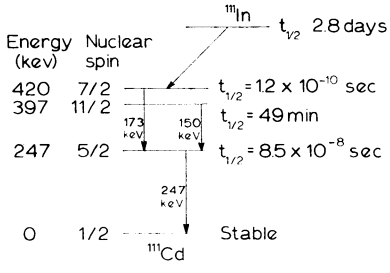

FIG. 1.—The 150–247 kev gamma ray cascade in ^{111}Cd from the 49-minute metastable state, and the 173–247 kev cascade following the electron capture decay of ^{111}In (data from ref. 8).

Theoretical.—For the 173–247 kev gamma-ray cascade in ^{111}Cd following the decay of ^{111}In, as shown in Figure 1, $W(\theta, t)$ is given by[9]

$$W(\theta, t) = \frac{1}{\tau} e^{-t/\tau}[1 + A_2P_2(\cos\theta)], \quad (1)$$

where P_2 is the Legendre polynomial $(3\cos^2\theta - 1)/2$, τ is the mean lifetime of the intermediate nuclear state (for the 247 kev state of ^{111}Cd, $\tau = t_{1/2}/\ln 2 = 1.21.10^{-7}$ sec), t is the time interval between emission of the two gamma rays, and the coefficient $A_2 = -0.20$. For the 150–247 kev cascade starting from the 49-min metastable state, $W(\theta, t)$ is given by Equation (1) with $A_2 = +0.161$.[10] The finite solid angles subtended by the detectors reduce A_2 to an effective value somewhat lower than this.

The angular correlations of both the 173–247 and 150–247 kev cascades can be perturbed by interaction of the nuclear quadrupole moment of ^{111}Cd in the 247

kev state with fluctuating electric field gradients at the nucleus. In this case, the coefficient of P_2 (cos θ) in Equation (1) can be written as $A_2G_2(t)$.

The attenuation coefficient $G_2(t)$ depends only upon the delay time t between the emissions and upon the parameters describing the interaction in the intermediate nuclear state. The approximate behavior of $G_2(t)$ may conveniently be displayed by a plot of the anisotropy,

$$A(t) = \frac{W(\pi, t)}{W(\pi/2, t)} - 1 \approx \frac{3}{2} A_2G_2(t), \tag{2}$$

against the delay time t. The shape of this plot depends in detail on the relative magnitude of the molecular rotational correlation time, τ_c, as well as on the size of the nuclear quadrupole interaction, e^2qQ, in the intermediate state.[11]

For $\tau_c \ll (e^2\, qQ)^{-1}$, $G_2(t)$ for a random nuclear relaxation process§ varies as[11]

$$G_2(t) = e^{-\lambda_2 t}, \tag{3}$$

where

$$\lambda_2 = \frac{63}{1000} \overline{\overline{(e^2qQ)^2}}\, \tau_c/\hbar^2 \tag{4}$$

for a nuclear spin of $^5/_2$ and an axially symmetric electric field, and where the double bar denotes an ensemble average.

Experimental.—Excited ^{111}Cd was prepared by the reaction ^{110}Cd (n, γ) ^{111}Cd using the University of California, Berkeley, reactor. Because of the short half-life of the ^{111}Cd metastable state, three freshly irradiated samples of cadmium were required for each measurement. The angular correlation measurements were made with a four-detector fast-slow gamma-ray coincidence spectrometer using NaI(Tl) crystal detectors.[12] The slow side of the spectrometer discriminates the energies of the gamma rays involved in the cascade, gates the fast side of the spectrometer, and routes pulses into the appropriate sections of a multi-channel analyzer. The fast side of the system includes a time-to-amplitude converter to provide to the multi-channel analyzer a pulse with amplitude proportional to the delay time between the arrival of two gamma rays. The multi-channel analyzer then provides a direct display of $W(\theta, t)$ versus delay time.

Native carbonic anhydrase was obtained from Mann Research Laboratories and used without further purification. It was demonstrated to be enzymatically active by the method of Wilbur and Anderson.[13] Apo-carbonic anhydrase was prepared[14] by dialyzing 15 ml of 3×10^{-4} M carbonic anhydrase in 0.1 M acetate buffer at pH 5.0 for 48 hr against one liter of 10^{-2} M o-phenanthroline in the same buffer. The o-phenanthroline was removed by dialysis against three one-liter solutions of 0.1 M phosphate buffer at pH 6.1 containing 0.5 M NaCl. The concentration of the enzyme was determined spectrophotometrically using the extinction coefficient of 4.9×10^4 M^{-1} at 280 mμ.[15] Three samples of each substance were studied, each containing approximately a micromole of cadmium.

Results.—The anisotropy of $^{111m}Cd^{2+}$ in 0.5 M sodium chloride solutions buffered to pH 6.1 with 0.1 M phosphate is shown in Figure 2*a* where the experimental anisotropy $A(t)$ is plotted against the delay time, t, in nanoseconds. The anisotropy is only weakly perturbed, characteristic of the free ion in solution.[7, 9] The decay of the anisotropy is approximately linear as might be expected from Equations (3) and (4) when both e^2qQ and τ_c are small. The anisotropy for buffered $^{111m}Cd^{2+}$ in the presence of 3×10^{-4} M native carbonic anhydrase is

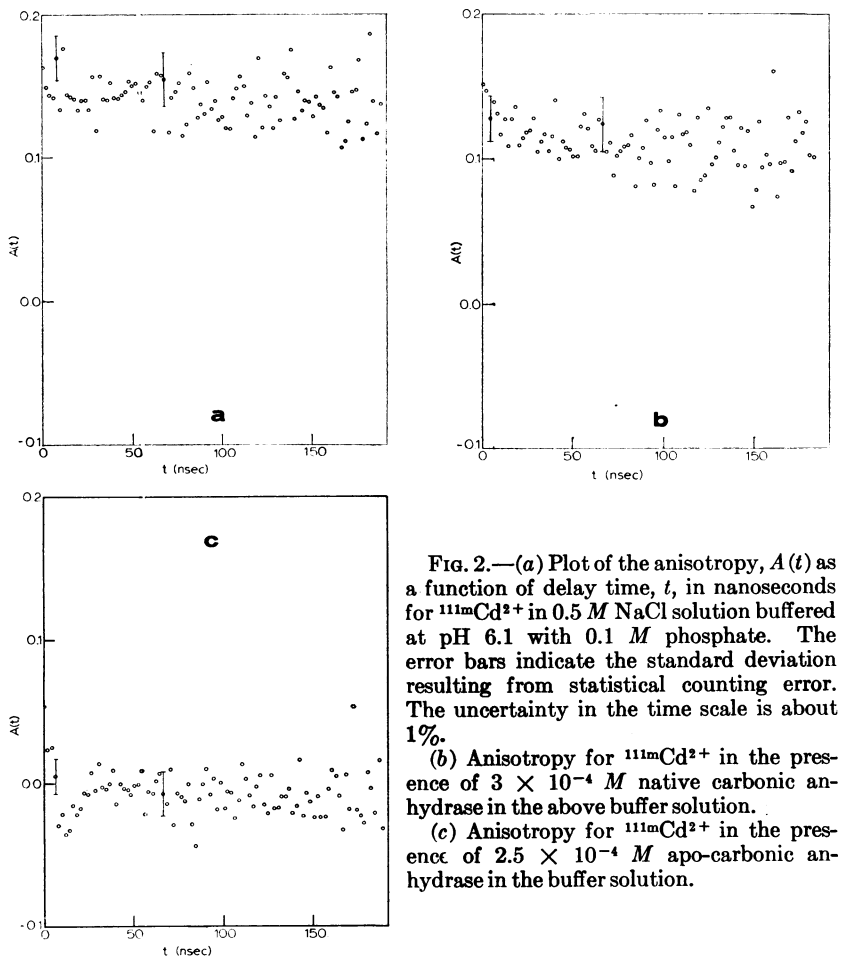

FIG. 2.—(*a*) Plot of the anisotropy, $A(t)$ as a function of delay time, t, in nanoseconds for $^{111m}Cd^{2+}$ in 0.5 M NaCl solution buffered at pH 6.1 with 0.1 M phosphate. The error bars indicate the standard deviation resulting from statistical counting error. The uncertainty in the time scale is about 1%.

(*b*) Anisotropy for $^{111m}Cd^{2+}$ in the presence of 3×10^{-4} M native carbonic anhydrase in the above buffer solution.

(*c*) Anisotropy for $^{111m}Cd^{2+}$ in the presence of 2.5×10^{-4} M apo-carbonic anhydrase in the buffer solution.

shown in Figure 2*b*. The samples of native enzyme were prepared by adding to 4 ml of the enzyme solution 0.5 ml of a 2×10^{-3} M solution of radioactive cadmium chloride. The anisotropy plot is again characteristic of the free ion in solution, although its lower value may indicate that a small fraction of the Cd^{2+} is bound to the native enzyme.

The anisotropy for $^{111m}Cd^{2+}$ in the presence of 2.5×10^{-4} M apo-carbonic anhydrase in the same buffer is shown in Figure 2*c*. The correlation is strongly perturbed. The shape of the plot suggests that time-independent quadrupole interactions are mainly responsible for the perturbation of the angular correlation. Under these conditions, $\tau_c > (e^2 qQ)^{-1}$ and the nuclear spin system is effectively "immobilized." Figure 2*c* then indicates that the cadmium ion is rigidly bound to the apoenzyme, and that the motion of the metal-enzyme complex is slow compared to $1/(e^2qQ)$ for ^{111}Cd in the 247 kev state. The particular time dependence for $A(t)$ in this case is similar to that observed in polycrystalline solids,[11] but with the significant difference that $A(t)$ [and thus $G_2(t)$]

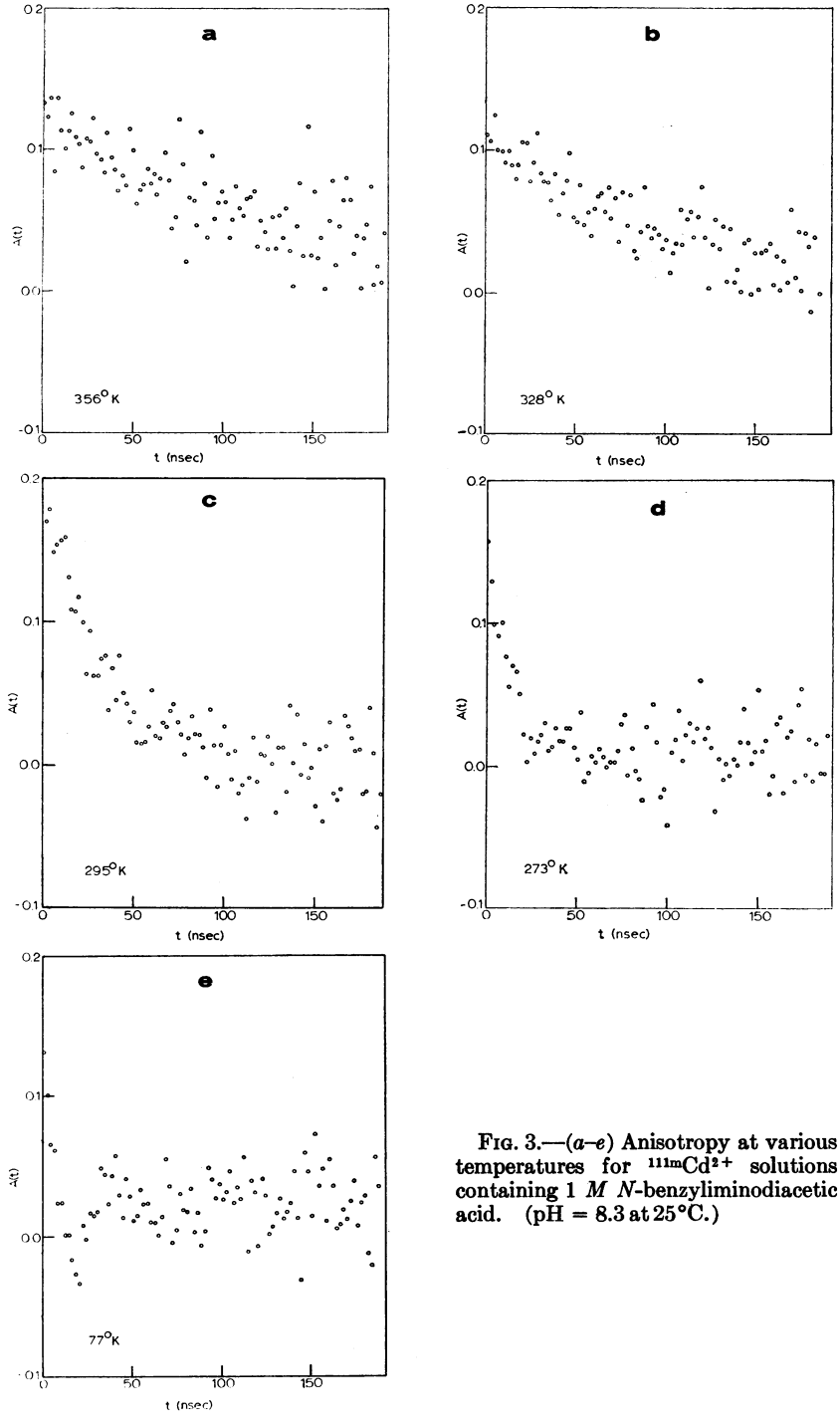

FIG. 3.—(*a–e*) Anisotropy at various temperatures for $^{111m}Cd^{2+}$ solutions containing 1 *M* *N*-benzyliminodiacetic acid. (pH = 8.3 at 25°C.)

goes to zero for $t \gg (e^2qQ)^{-1}$, while for a poly-crystalline solid $G_2(t)$ approaches the "hard-core" value of $^1/_5$.

Figures 3*a–e* show the anisotropy at various temperatures for a series of cadmium solutions containing 1 *M* *N*-benzyliminodiacetic acid. Since the correlation time for the motion of the metal ion-*N*-benzyliminodiacetic acid complex is a function of temperature, these plots provide qualitative examples of the behavior of the perturbed angular correlation as a function of the rotational properties of the complex. The plot for the frozen solution is clearly similar to the plot for the apo-enzyme shown in Figure 2*c*, but the finite hard-core value of $G_2(t)$ for large t in this case indicates that the condition $\tau_c \gg (e^2qQ)^{-1}$ is satisfied for the frozen solution.

The quadrupolar interaction in the Cd^{++}-*N*-benzyliminodiacetic acid complex can be estimated from the data shown in Figure 3*e*.[16] Values of λ_2 can be obtained by fitting exponential curves to the data in Figures 3*a–d*, and Equation (4) can then be used to calculate rotational correlation times for these samples. This procedure yields $\tau_c = 9 \times 10^{-11}$ seconds for the complex under the conditions of Figure 3*a*, with values appropriately larger at the other three temperatures.

Discussion.—The comparison of the delayed coincidence spectra for $^{111m}Cd^{2+}$ in the presence of native and apo-carbonic anhydrase indicates that at pH 6.1 the cadmium ion is immobilized at the zinc binding site of the enzyme. The shapes of the anisotropy plots for the free and immobilized nuclear spin systems are similar to the behavior of spectra of Cd^{++}-*N*-benzyliminodiacetic acid complexes as a function of molecular rotational correlation time.

The shapes of the delayed coincidence spectra obtained with the ^{111}In decay scheme[7] are consistent with the results obtained using the 49-minute metastable state of ^{111}Cd. Thus, when ^{111}In is bound to bovine serum albumin at pH 5.7, the ^{111}Cd nuclear spin in the 247 kev state of the daughter nucleus is effectively immobilized. The main features of the angular correlation of the ^{111}Cd cascade do not seem to be affected by recoil from the ^{111}In electron capture decay, or by the change from indium to cadmium chemistry. The perturbed angular correlation of both the 173–247 kev and the 150–247 kev cascade of ^{111}Cd indeed appear to reflect the effective molecular rotational correlation time at the metal binding site.

Although using the ^{111}Cd metastable state eliminates any problem that might arise from recoil in the ^{111}In electron capture decay, and only cadmium chemistry is involved, the ^{111}Cd metastable scheme has several obvious problems. The 49-minute half-life of the state is, of course, inconveniently short. Furthermore, ^{111m}Cd produced by the (n, γ) reaction cannot be chemically separated from the inert cadmium target material. The presence of the large excess of inactive cadmium limits the ultimate sensitivity of ^{111m}Cd as a label. ^{111}In produced by the $^{111}Cd(p, n)$ ^{111}In reaction can be separated from the cadmium target material, and ^{111}In electron capture decay has a convenient 2.8 day half-life. The results reported here for ^{111m}Cd provide support for continued exploration of the use of the more practical ^{111}In decay scheme as a rotational tracer for the study of biological macromolecules.

The radioactive rotational-labeling technique shares a number of general ad-

vantages and problems with other labeling techniques. Information on localized behavior of macromolecules or membranes near the labeling site is usually available. Thus, the labels are sensitive to changes in conformation, such as helix-coil transitions and allosteric effects. In many cases, labels can be bound chemically to substrate or inhibitor molecules which can subsequently be incorporated into interesting regions of macromolecules. The ^{111}In label can for example be bound with a tight chemical complexing agent such as EDTA which is covalently linked with an active group such as a sulfhydryl reagent. With this general scheme, concern with the details of indium chemistry and binding constants can be eliminated, and the chemical selectivity of the method then depends on the choice of the active group covalently linked to the complex. On the other hand, as with all of the labeling methods there is uncertainty as to how much the label affects the system being observed. The compelling features for the development of the radioactive rotational label include the potential sensitivity of the method, which is of the order of 10^{-12} molar for ^{111}In, and the fact that the method can easily be applied *in vivo*.

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§ For a random relaxation process, the autocorrelation function of matrix elements governing transitions between substates of the intermediate nuclear state varies as $e^{-|\tau|/\tau_c}$.

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