

1977

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Edward P. Richards

Louisiana State University Law Center, richards@law.lsu.edu

Robert R. Sharp

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BBA 28180

MOLECULAR MOBILITIES OF SOLUBLE COMPONENTS IN THE AQUEOUS PHASE OF CHROMAFFIN GRANULES

ROBERT R. SHARP and EDWARD P. RICHARDS

Department of Chemistry, The University of Michigan, Ann Arbor, Mich. 48104 (U.S.A.)

(Received August 17th, 1976)

Summary

NMR relaxation times have been used to characterize molecular motion and intermolecular complexes in the aqueous phase of bovine chromaffin granules. Partially relaxed ^{13}C and proton spectra have been obtained at 3 and 25°C. T_1 measurements of five protonated carbons on epinephrine (C_2 , C_5 , C_6 , CHOH and NCH_3) give a correlation time of 0.15 (10^{-9}) s at 25°C for the catechol ring and methine carbon, while the effective correlation time for the NCH_3 group is somewhat shorter due to its internal degree of rotational freedom. Resonances of protonated carbons on the soluble protein chromogranin give very similar correlation times: 0.20 (10^{-9}) s for the peptide α -carbon and 0.2 (10^{-9}) s for the methylene sidechain carbons of glutamic acid. The correlation time (τ_R) of ATP was not measured directly using ^{13}C T_1 data due to the weakness of its spectrum, but its reorientation appears to be substantially slower than that of epinephrine or chromogranin. This conclusion is based on three observations: (1) the qualitative temperature dependence of T_1 for H_2 and H_8 on the adenine ring places τ_R for ATP to the right of the T_1 minimum, or $\tau_R \geq 1.0$ (10^{-9}) s; (2) ^{13}C resonances of ATP have anomalously low amplitudes compared with epinephrine resonances, a fact that is readily explained only if ATP undergoes substantially slower reorientation; and (3) a comparison of the T_1 data of H_8 in chromaffin granules and in a dilute aqueous solution, where τ_R for ATP can be measured directly, indicates that $\tau_R \approx 1.0$ (10^{-9}) s at 25°C in the granules. The relaxation data are consistent with the concept of a storage complex based on electrostatic interactions between a polyion (chromogranin) and its counterions (ATP and epinephrine), in which ATP cross-links cationic sidechains of the protein.

Introduction

High resolution NMR spectra of the ^1H , ^{13}C and ^{31}P resonances of intact chromaffin granules have recently been described [1,2]. These spectra demon-

strate the presence of a fairly fluid, essentially isotropic aqueous phase in the vesicle interior. This phase contains primarily epinephrine, chromogranin and ATP with little, if any, lipid. The nature of the intermolecular interactions among the soluble components has not been elucidated, although some form of complex appears necessary to explain the well-known stability of the granules with respect to epinephrine efflux [3–8]. Although they provide little direct information about the nature of the complex, the NMR spectra do provide several intriguing clues.

First, the γ - ^{31}P of ATP, and to a lesser extent the β - ^{31}P , are shifted downfield in the vesicles as would be consistent with ionic complexation at the phosphate esters. Second, a comparison of relative intensities in the ^{13}C spectra suggests that epinephrine and ATP have significantly different rotational correlation times in the granules and consequently are not present as a simple binary complex. Thirdly, chromogranin is present as a highly distended random coil polyelectrolyte. The protein does not form a gel, but probably interacts strongly with other ions in the aqueous phase (primarily epinephrine and ATP) through polyelectrolyte-counterion interactions.

In order to probe more directly the molecular nature of complexes in the aqueous phase we have measured ^{13}C and ^1H spin-lattice relaxation times (T_1 data) in the granules. T_1 data provide a direct measure of the correlation times for molecular reorientation experienced by a variety of nuclei on different solute molecules. Thus, these measurements provide a detailed picture of molecular motion in the vesicle interior. A comparison of correlation times of nuclei on different molecules also provides a useful criterion of intermolecular complexation.

The theory of spin-lattice relaxation is discussed in detail elsewhere [9]. T_1 is in general a double-valued function of the reorientational correlation time, τ_R , and can be written $T_1^{-1} = \phi^2 f_1(\tau_R)$. In the systems of interest here, relaxation results from coupling between neighboring nuclear magnetic dipoles, and ϕ^2 is the mean squared dipolar coupling energy. $f_1(\tau_R)$ is proportional to the Fourier amplitudes of the (time-dependent) dipolar interaction at the Zeeman transition frequencies. Proton T_1 data result from both intra- and intermolecular dipolar couplings. In the present experiments, the intermolecular contribution is suppressed by exchanging the solvent with $^2\text{H}_2\text{O}$. The intramolecular contribution is given by

$$(T_1)^{-1} = 2\gamma_{\text{H}}^4 \hbar^2 I(I+1) \sum_j r_{ij}^{-6} f_1^{\text{H}}(\tau_R) \quad (1\text{-a})$$

$$= 5.39(10^{59}) \sum_j r_{ij}^{-6} f_1(\tau_R), \quad (1\text{-b})$$

where

$$f_1^{\text{H}}(\tau_R) = \frac{1}{5} \left\{ \frac{\tau_R}{1 + \omega_{\text{H}}^2 \tau_R^2} + \frac{4\tau_R}{1 + 4\omega_{\text{H}}^2 \tau_R^2} \right\} \quad (1\text{-c})$$

γ_{H} is the proton gyromagnetic ratio, I is the nuclear spin quantum number, r_{ij} is an internuclear distance, and ω_{H} is the proton Larmor frequency. The functional dependence of T_1 on τ_R is plotted in Fig. 5. The transverse relaxation

time T_2 , is also plotted in the figure. T_2 is conveniently measured from the line-width, $W = (\pi T_2)^{-1}$ and is discussed fully in ref. 2. T_1 passes through a minimum in the neighborhood of $4\omega_H^2\tau_R^2 = 1$, and becomes proportional to τ_R to the right of this minimum. Thus, T_1 , unlike T_2 , is not a monotonically decreasing function of τ_R ; and unlike T_2 , T_1 is not sensitive to anisotropy in the local molecular environment that is associated with phase boundaries in heterogeneous media. Eqns. 1 assume that the medium is reasonably fluid ($\tau_R \leq 10^{-5}$ s), and that the motions of all nuclei i, j that contribute to the coupling are described by a single correlation time. For functional groups with internal rotational degrees of freedom, such as unhindered methyl groups, Eqn. 1 gives an effective correlation time which is always shorter than τ_R . For methyl groups, τ_{eff} lies in the range $\tau_R \geq \tau_{\text{eff}} \geq \tau_R/4$, where the lower limit corresponds to completely free internal rotation [10].

T_1 data of ^{13}C nuclei that are directly bonded to protons are strongly dominated by the nearest neighbor ^{13}C - ^1H interaction. For a ^{13}C nucleus directly bonded to N hydrogens [9,11]

$$(T_1)^{-1} = \frac{N\gamma_C^2\gamma_H^2\hbar^2}{r_{\text{CH}}^6} f_1^{\text{C}}(\tau_R) \quad (2-a)$$

$$= 2.40(10^{10}) Nf_1^{\text{C}}(\tau_R) \quad (2-b)$$

where

$$f_1^{\text{C}}(\tau_R) = \frac{1}{10} \left\{ \frac{\tau_R}{1 + (\omega_C - \omega_H)^2\tau_R^2} + \frac{3\tau_R}{1 + \omega_C^2\tau_R^2} + \frac{6\tau_R}{1 + (\omega_C + \omega_H)^2\tau_R^2} \right\} \quad (2-c)$$

The carbon-proton bond length is taken to be $1.09 (10^{-8})$ cm in Eqn. 2. Plots of T_1 and T_2 vs. τ_R for ^{13}C are shown in Fig. 3. Doddrell et al. [11] have computed the effects of internal rotation on ^{13}C T_1 data. In the extreme narrowing limit, which is characterized by $(\omega_C + \omega_H)^2\tau_R^2 \ll 1$, free internal rotation reduces τ_{eff} of a methyl carbon to 1/9 of the value, τ_R , that corresponds to no internal rotation.

Experimental

Chromaffin granules were prepared by differential centrifugation from homogenized bovine adrenal medullae as described previously [2]. All samples were prepared and kept at $3 \pm 2^\circ\text{C}$ until used. Reference samples containing epinephrine (1.0 M in $^2\text{H}_2\text{O}$, $p^2\text{H}$ 7.2) and ATP (0.5 M in $^2\text{H}_2\text{O}$, $p^2\text{H}$ 7.2) were prepared by weight and degassed by several cycles of the freeze-pump-thaw method.

NMR spectra were obtained from a JEOL JNM-PFT/100 Fourier transform NMR spectrometer interfaced to two different data systems. A Digilab NMR-3 data system was used initially to obtain a complete set of data (^1H at 3°C , 25°C and ^{13}C at 3°C , 25°C). Subsequently, the spectrometer was interfaced to a JEOL EC-100 computer system and a crystal filter was added, resulting in a substantial improvement in signal-to-noise factor. The proton measurements at 3 and 25°C and the ^{13}C measurements at 25°C were then repeated.

Relaxation times were determined from partially relaxed ($180^\circ - \tau - 90^\circ$ sample)_n spectra. Delays of 1.2–2.0 s were inserted between successive sequences in the ^{13}C measurements. These delays ensure essentially complete relaxation of all protonated carbons but not necessarily of the non-protonated carbons. Delays in the proton measurements were at least five times the longest T_1 in the spectrum. 90° pulse widths for both ^1H and ^{13}C were approx. 22 μs .

^{13}C T_1 data at 25°C have been computed from semilogarithmic plots of amplitude against time for five of the epinephrine-protonated carbons (C_2 , C_5 , C_6 , CHOH , NCH_3); typical least-square plots of the data are shown in Fig. 2. Data at 3°C were obtained without using a crystal filter and the spectra therefore have somewhat poorer signal-to-noise ratio. T_1 data at this temperature were computed from the null points, using $T_1 = t_{\text{null}}/\ln 2$. This procedure gives greater uncertainty in T_1 but is free from systematic error that can arise from an inaccurate value of the amplitude at t_∞ . Proton T_1 data were also computed from the null points, in this case because spectral overlap is relatively severe due to the much smaller magnitudes of proton chemical shifts. Null points of neighboring peaks can readily be estimated in the presence of moderate overlap that would cause systematic baseline error in semi-logarithmic plots. Estimated uncertainties in T_1 depend on peak intensity as well as on the degree of overlap, and are given in Table II.

Results

Partially relaxed ^{13}C spectra of isolated chromaffin granules at 25°C are shown in Fig. 1. A complete assignment of the carbon spectrum is given in ref. 2; the peaks of protonated carbons, which were used for the measurement of correlation times, are identified for convenience in the figure. The longest relaxation times are found for non-protonated carbons (denoted by dashed lines in the figure) in the downfield region. Among the epinephrine carbons, C_1 at 133 ppm and (C_3 , C_4), which are accidentally degenerate at 145 ppm, have somewhat longer T_1 data than do any of the protonated carbons, which include C_2 , C_5 , C_6 , CHOH , and NCH_3 . Similarly chromogranin resonances in the amide region near 178 ppm relax considerably more slowly than does the intense peak due to α -methine carbons of the protein backbone or any of the peaks in the sidechain methylene region. On the other hand, T_1 data of the protonated carbons of epinephrine are very similar to T_1 data of the protonated carbons of chromogranin, including the backbone methine carbons, indicating general similarity of the rotational correlation times of these molecules. Unfortunately, the ATP resonances are of anomalously low amplitude in the carbon spectra and are obscured by the baseline noise. As pointed out in ref. 2, this intensity loss would be expected if the correlation time for ATP were substantially longer than those for epinephrine or chromogranin.

^{13}C T_1 data have been measured for five of the epinephrine protonated carbons (C_2 , C_5 , C_6 , CHOH and NCH_3) and are listed in Table I. Correlation times corresponding to these values have been obtained by plotting the T_1 data on theoretical curves of Eqn. 2 which are shown in Fig. 3.

Since T_1 is a double-valued function of τ_R , an experimental criterion is necessary to decide whether the correlation time lies to the left or right of the T_1

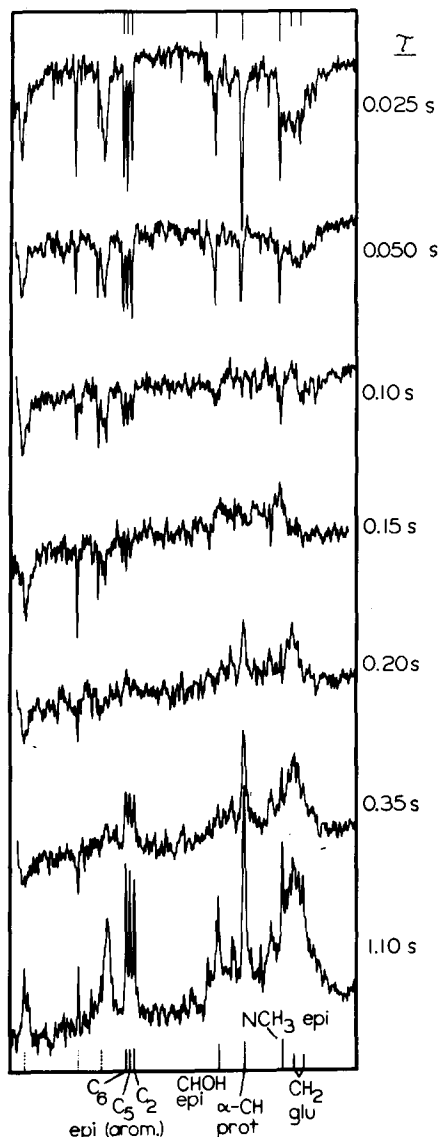


Fig. 1. Partially relaxed ^{13}C spectra of isolated chromaffin granules at 25°C . Assignments are given for protonated carbons used in the analysis of correlation times. Dashed lines denote non-protonated carbons: (from left to right) the amide carbon region of chromogranin, (C_3 , C_4) of epinephrine and C_1 of epinephrine.

minimum. Appropriate criteria are provided by two independent observations. First the ^{13}C linewidths, which are reported in ref. 2, place an absolute upper limit on τ_{R} . These limiting values, which are obtained by neglecting the effect of motional anisotropy on the linewidth, demonstrate that $\tau_{\text{R}} \leq 2$ ns for all epinephrine carbons and that $\tau_{\text{R}} < 7$ ns for the chromogranin backbone [2]. Thus, the solution of Eqn. 2 for which $\tau_{\text{R}} \approx 200$ ns is clearly excluded. Incidentally, this comparison also shows that T_2 in the aqueous phase is strongly dominated by effects of motional anisotropy due to the influence of phase bound-

TABLE I
 ^{13}C T_1 DATA AND CORRELATION TIMES IN CHROMAFFIN GRANULES

Molecule	Carbon	3°C		25°C	
		T_1 (s)	τ_R (ns)	T_1 (s)	τ_R (ns)
Epinephrine	C-2	0.14 ± 0.06	0.32	0.31 ± 0.04	0.15
	C-5	0.14 ± 0.06	0.32	0.31 ± 0.04	0.15
	C-6	0.14 ± 0.06	0.32	0.34 ± 0.04	0.135
	$^{13}\text{CHOH}$	0.14 ± 0.05	0.32	0.25 ± 0.04	0.18
	N^{13}CH_3	0.29 ± 0.10	0.053 ^a	0.32 ± 0.06	0.047 ^a
Chromogranin	$\alpha\text{-CH}$	0.12 ± 0.04	0.37	0.23 ± 0.03	0.20
	Glu- βCH_2			0.07–0.20	0.12–0.33
	Glu- γCH_2			0.07–0.20	0.12–0.33

^a τ_{eff} as defined in the text.

aries on molecular reorientation. If the effects of motional anisotropy were negligible, then T_1 would equal T_2 . Since $T_2 \approx T_1/10$, anisotropic effects dominate the linewidth, although as shown elsewhere [2], the absolute anisotropy, which is measured by $\langle 3 \cos^2\theta' - 1 \rangle$ is still very small.

A second observation that demonstrates that τ_R is to the left of the T_1 minimum is the temperature dependence of measured T_1 data. Since τ_R increases monotonically with temperature, T_1 exhibits opposite temperature dependence on the two sides of the minimum. Experimentally, the ^{13}C T_1 data uniformly lengthen with increasing temperature indicating that both epinephrine and the

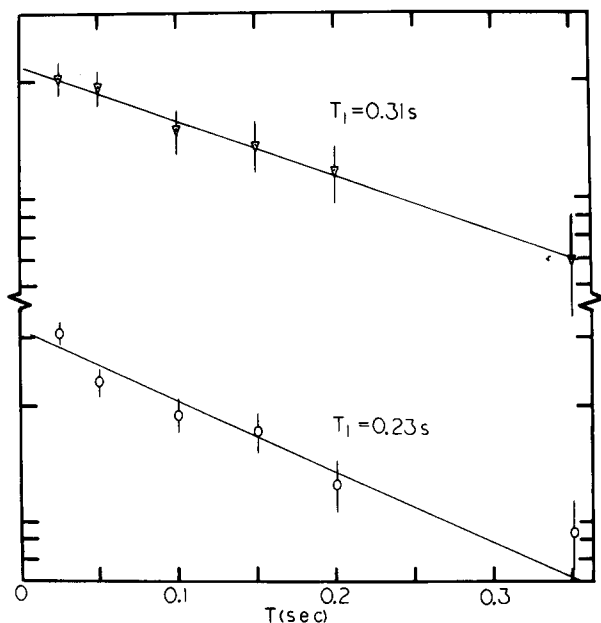


Fig. 2. Semilogarithmic plots of partially relaxed amplitudes: ∇ , ^{13}C -2 of epinephrine and \circ , the backbone $\alpha\text{-}^{13}\text{CH}$ peak of chromogranin.

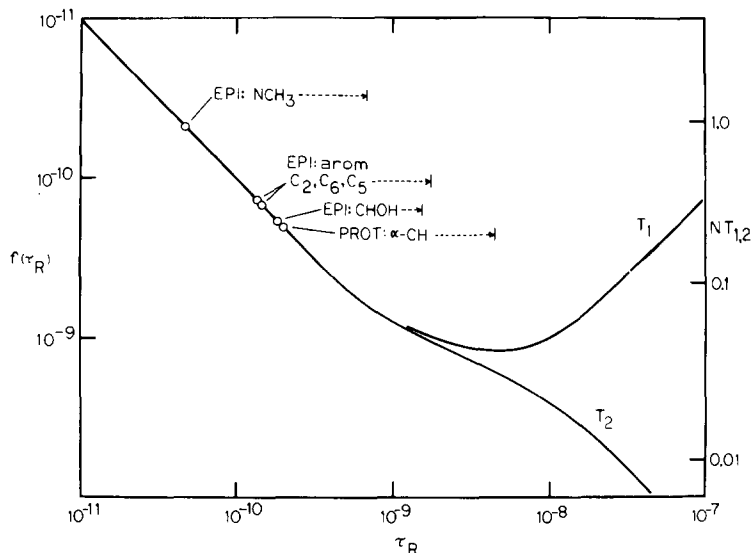


Fig. 3. Reorientational correlation times computed from ^{13}C T_1 data at 25°C . Solid lines are theoretical plots of Eqn. 2 of the text and Eqn. 2 of ref. 2 computed for a field strength of 23.49 kG. Dashed arrows indicate upper limits to the correlation times consistent with linewidths reported in ref. 2.

chromogranin backbone have correlation times to the left of the minimum. The N^{13}CH_3 resonance of epinephrine shows little temperature dependence. This behavior is often seen for methyl groups with relatively free internal rotation.

Among the various chromogranin resonances, the ($\alpha\text{-CH}$) carbons overlap to a large extent and give rise to an intense, well defined peak in the methine region. Since the α -carbon is protonated it provides a very convenient motional probe of the peptide backbone. Unfortunately, this peak does overlap with the epinephrine NCH_2 group which gives rise to a minor portion of the total observed intensity. The epinephrine contribution to this peak is probably 20–25%, assuming that the epinephrine methylene intensity is similar to the intensities of the nearby epinephrine CHOH and NCH_3 groups. In spite of this overlap, the $\alpha\text{-CH}$ peak certainly provides a reasonable measure of the correlation time of the chromogranin peptide backbone. This value, $\tau_R = 0.20$ ps at 3°C , is very short and shows beyond question that chromogranin is a random coil protein in a highly fluid medium.

T_1 data of carbons on the chromogranin sidechains have also been estimated roughly from the null points of two peaks that correspond to the β - and γ -methylene resonances of glutamic acid. T_1 data of these resonances are uncertain within a factor of two, but they imply correlation times in the same range ($\tau_R \sim 0.25$ ps) as were found for the peptide α -carbons and for epinephrine.

Partially relaxed proton spectra of chromaffin granules that were exhaustively exchanged with $^2\text{H}_2\text{O}$ have also been obtained. T_1 values for various chemical sites on epinephrine, ATP and chromogranin were measured at 3 and 25°C and are listed in Table II. The partially relaxed ^{13}C spectra provide a much more direct method for computing correlation times than do proton spectra, since proton relaxation is not necessarily dominated by nearest-neighbor interac-

TABLE II
 PROTON T_1 DATA AND CORRELATION TIMES IN CHROMAFFIN GRANULES

Molecule	Proton	3° C		25° C	
		T_1 (s)	τ_R (ns)	T_1 (s)	τ_R (ns)
Epinephrine	aromatic	0.22 ± 0.03	0.23^a	0.33 ± 0.04	0.15^a
	NCH ₂	0.085 ± 0.025	$0.14^a; (0.31)^b$	0.10 ± 0.03	$0.12^a; (<0.27)^b$
	NCH ₃	0.22 ± 0.06	—	0.26 ± 0.07	—
ATP	H ₂	0.35 ± 0.07	—	0.29 ± 0.06	—
	H ₈	0.22 ± 0.06	$1.9^{+1.0} a$ —0.9	0.16 ± 0.07	$1.0^{+1.0} a$ —0.5
Chromogranin	CH ₃	0.14 ± 0.04	—	0.17 ± 0.04	—
	CH ₂	0.10 ± 0.03	$(<0.37)^b$	0.10 ± 0.03	$(<0.37)^b$

^a Correlation times computed by comparison with T_1 data in a reference solution, using a procedure described in the text.

^b Upper limits to correlation times computed by considering only the relaxation contribution arising from dipolar couplings of geminal protons.

tions, and reliable values of $\sum_i r_{ij}^{-6}$ may be difficult to obtain. In the present instance, however, the proton spectra provide the only available probe of molecular motion of ATP. Two protons on the adenine base, H₂ and H₈, are readily monitored and appear downfield of the aromatic resonances of epinephrine in a spectral region that is essentially free of interfering resonances (Fig. 4). Unfortunately, the ribose protons of ATP are obscured by the intense ²HHO peak and the α -CH peak of chromogranin, and are unsuitable for study. H₂ and

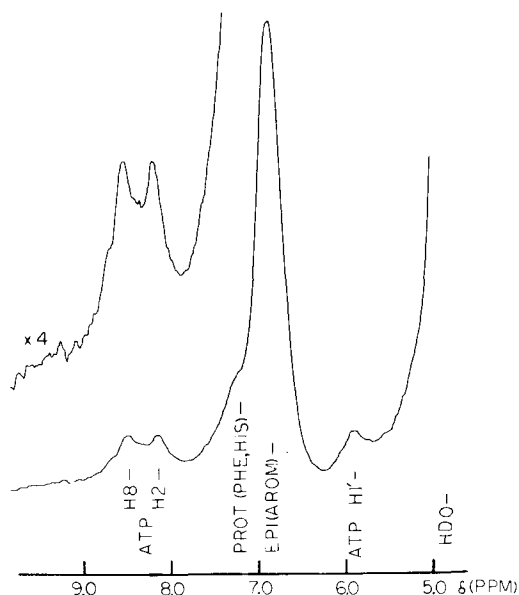


Fig. 4. Proton spectrum of chromaffin granules in the region downfield of the ²HHO peak.

H_8 , however, are quite informative in that the temperature dependence of their T_1 data is qualitatively different from that of any of the resonances of epinephrine or chromogranin. Whereas T_1 data of the latter molecules uniformly lengthen with increasing temperature, T_1 data of H_2 and H_8 decrease with increasing temperature. The magnitude of this effect is near the limits of experimental uncertainty but was reproduced on different batches of chromaffin granules. Such behavior is indicative of an ATP correlation time to the right of the T_1 minimum for protons, or $\tau_R \geq 1.0$ ns at 25°C.

Another way of estimating τ_R for ATP is to compare the proton T_1 data in chromaffin granules with the T_1 data in a reference solution, for which the absolute value of τ_R can be measured directly by ^{13}C NMR. In this way the ratio of correlation times (or more precisely of the functions $f_1(\tau_R)$) in the two media is given by the inverse ratio of T_1 data while the absolute value of τ_R is calibrated by ^{13}C data in the reference solution. In order that the measured correlation time characterize reorientational motion of ATP, however, it is necessary that the monitored proton be relaxed primarily through intramolecular dipolar couplings. This condition should be satisfied quite well by H_8 on the adenine base of ATP. In the low energy anti conformation of ATP, this proton sits in a pocket above the furanose ring in close proximity to four ribose protons, H_2' , H_3' , H_5' and H_5'' . Dipolar couplings to these protons almost certainly dominate T_1 in $^2\text{H}_2\text{O}$ solutions, where intermolecular relaxation contributions arising from the solvent protons are effectively suppressed.

Using the H_8 proton to monitor ATP reorientation, we compute correlation times of 1.0 ns at 25°C and 1.9 ns at 3°C (data from the reference solution are given in Table III). These estimates are about seven times longer than the directly measured correlation times for epinephrine and the chromogranin backbone. The former value is very near the T_1 minimum (see Fig. 5) and is consistent with both the NOE behavior and the anomalous temperature dependence of ATP resonances cited above. It should be noted, however, that this procedure is indirect in that it "calibrates" the geometric factor, $\sum_j r_{ij}^{-6}$, by measurements in a reference solution. It could be subject to substantial error if, in the two media, the adenine moiety is in different conformations with respect

TABLE III

CARBON AND PROTON T_1 DATA AND MOLECULAR CORRELATION TIMES IN REFERENCE SOLUTIONS CONTAINING EPINEPHRINE (1.0 M IN $^2\text{H}_2\text{O}$, DEGASSED) AND ATP (0.5 M IN $^2\text{H}_2\text{O}$, DEGASSED)

T_1 data measured at $25 \pm 2^\circ\text{C}$.

Epinephrine	Proton	T_1 (s)	Carbon	T_1 (s)	τ_R (ns)
1.0 M	CH_2N	0.28	CH_2N	0.55	0.042
	CH_3N	0.54	CH_3N	1.18	0.013 ^a
	$\text{C}_2, \text{C}_5, \text{C}_6$	0.54	$\text{C}_2, \text{C}_5, \text{C}_6$ HCOH	0.51 1.17	0.09 0.04
ATP	H_8	0.27	C_8	0.107	0.38 ± 0.04
0.5 M	H_2	0.56	C_2	0.099	0.41 ± 0.04

^a τ_{eff} as defined in the text.

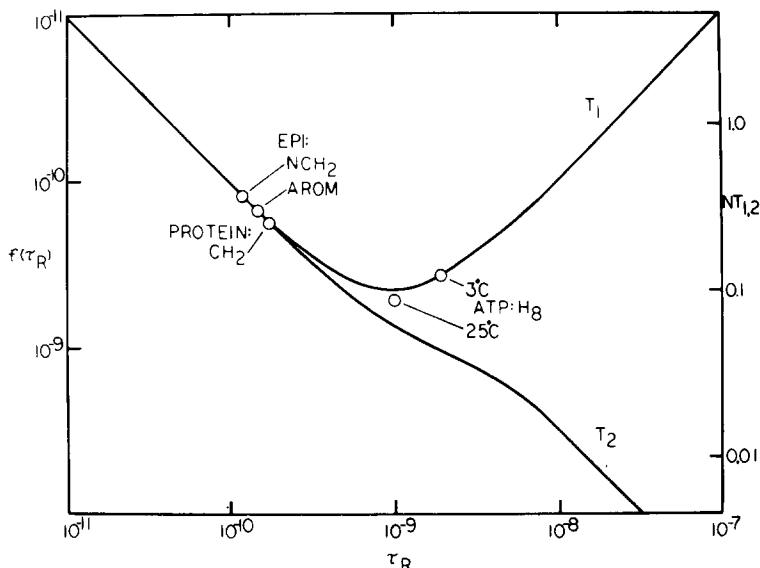


Fig. 5. Reorientational correlation times computed from proton T_1 data. All data taken at 25°C except as otherwise noted. Solid lines are plots of Eqn. 1 of the text and Eqn. 1 of ref. 2, computed for a field strength of 23.49 kG. The NCH_3 correlation time is τ_{eff} as defined in the text.

to the ribose ring. When applied to epinephrine, however, this method gives values of τ_R that are entirely consistent with directly measured values obtained from the ^{13}C spectra (see Table II and Fig. 5).

The region between 1.40 and 2.40 ppm in the proton spectrum contains four intense peaks due to methylene protons on the amino acid sidechains of chromogranin. Relaxation times of these resonances likewise do not provide a direct probe of sidechain motion, since the factor $\sum r_{ij}^{-6}$ in the T_1 expression contains comparable contributions from dipolar couplings of both geminal and vicinal protons. Only the geminal couplings are accurately known. A rough estimate of the methylene correlation time can be obtained by neglecting the vicinal couplings and using an average T_1 to describe relaxation in the methylene region (see Table II). This approximation gives $\tau_{R,av} \leq 0.37$ ns. It provides an upper limit on the average correlation time, which it probably overestimates by a factor of about two. It is in reasonable agreement with the range of correlation times, $\tau_R = 0.12\text{--}0.33$ ns, calculated from ^{13}C resonances of the β - and γ -methylene groups of glutamic acid.

Discussion

Among the various correlation time measurements discussed above, the most accurate are those based on the T_1 data of several protonated carbons of epinephrine and on the T_1 data of the α -methine carbons of chromogranin. Uncertainty in these correlation times is approx. $\pm 10\%$ at 25°C , and $\pm 30\%$ (due to poorer signal to noise ratio because of the lack of a crystal filter) at 3°C . All of the epinephrine resonances, with the exception of those on the N-methyl group, which has an internal degree of rotational freedom, indicate a uniform

reorientational correlation time of 0.15 ns at 25°C and 0.32 ns at 3°C for the catechol moiety and the β -methine carbon. The proton data in Table II show that τ_R for the NCH₂ group is similar in magnitude. Comparing these correlation times with those in a 1.0 M aqueous solution of epinephrine (Table III), it is evident that the reorientation of epinephrine is slowed by a factor of 2–4 in the intragranular medium relative to a simple aqueous solution. This difference is surprisingly small. Furthermore, it must account for the combined effects of viscosity changes and molecular immobilization due to specific complex formation in the chromaffin granule aqueous phase. Thus, the aqueous phase is clearly highly fluid. The viscosity of this phase is at most four times that of water, since in the absence of complex formation, τ_R varies directly as viscosity over kT [9]. Furthermore, epinephrine almost certainly interacts strongly with the other soluble components. It diffuses freely through the chromaffin vesicle membrane at 0°C and yet is maintained indefinitely in the storage granules in an osmotically inactive form in the presence of large concentration gradients [3,4,12]. The low osmotic activity of epinephrine in intact vesicles implies some restriction to its rotational motion, and hence the measured τ_R values suggest that the viscosity of the aqueous phase is very similar to that of dilute aqueous solutions.

The fluidity of the medium is also mirrored in the very short correlation times of the chromogranin backbone and sidechains. τ_R for the α -methine carbon on the peptide backbone is quite similar to that of the epinephrine catechol moiety (of course this value represents an average for all methine carbons in the peptide chain). Correlation times of methylene groups on the amino acid side chains are known less accurately than that of the α -methine carbon, but are in the same general range: $\tau_R = 0.12$ – 0.33 ns at 25°C. These values emphasize the nature of chromogranin as a random coil polyelectrolyte that lacks secondary structure.

Perhaps the most interesting finding is that the correlation time for ATP (as measured by the H-8 proton on the adenine base) appears to be substantially longer than correlation times of either epinephrine or chromogranin. The inference of an ATP correlation time near 1.0 ns rests on three observations. First, the T_1 data of both H₂ and H₈ increase with decreasing temperature, which is possible only if the correlation time is to the right of the T_1 minimum (i.e. $\tau_R \geq 1.0$ ns). The temperature dependence of the T_1 data of these protons was measured on two different granule preparations and reproducibly showed qualitatively different behavior than the T_1 data of other resonances. Secondly, the amplitudes of ATP peaks in the carbon spectra are anomalously low with respect to the amplitudes of epinephrine peaks. The only two obvious reasons for this difference are loss of nuclear Overhauser enhancement (NOE) or differential broadening of ATP resonances relative to epinephrine. Either reason implies a longer correlation time for ATP. Doddrell et al. [11] have calculated the dependence of Overhauser enhancement on τ_R for ¹³C resonances at 25.1 MHz. Very little loss of NOE occurs when $\tau_R \lesssim 0.4$ ns, but the enhancement drops rapidly toward unity in the region $1.0 \text{ ns} < \tau_R < 3.0 \text{ ns}$. Thus, from the correlation times in Tables I and II, maximum enhancements are expected for epinephrine and chromogranin but significantly reduced enhancements are expected for ATP. And thirdly, a difference in correlation time for ATP and

epinephrine is consistent with our comparison of the T_1 data of H_8 in a reference solution and in chromaffin granules.

The conclusion that ATP is significantly less mobile than either epinephrine or chromogranin raises intriguing questions concerning the nature of the intermolecular complexes responsible for the low osmotic activity of epinephrine. Measured correlation times show that chromogranin is a random coil polyelectrolyte and therefore strongly suggest that the complex may be of the type formed between a polyion and its counterions. Electrostatic interactions inside the polyion domain are much larger than those between simple ions and are certainly adequate to lower substantially the osmotic activity of low molecular weight counterions [13,14]. It is also natural that epinephrine and ATP would play very different roles in such a complex due to their different charges. Specifically, the multiple negative charges of ATP could lead to site binding of positively charged amino acid sidechains and consequent cross-linking of the protein. Localization of ATP at cross-linked sites would account at least qualitatively for its low rotational mobility relative to epinephrine and the peptide backbone. The possible relevance of such complexes to the hormone storage mechanism will be considered at greater length elsewhere.

Acknowledgements

This work was supported in part by Institutional Research Grant No. IN-40P to the University of Michigan from the American Cancer Society. Stimulating discussions with Dr. Edward Domino are gratefully acknowledged.

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