

Biochimica et Biophysica Acta, 538 (1978) 155–163
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BBA 28349

MOLECULAR MOBILITIES IN CHROMAFFIN GRANULES

MAGNETIC FIELD DEPENDENCE OF PROTON T_1 RELAXATION TIMES

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(Received May 13th, 1977)

Summary

The magnetic field dependence of the NMR spin-lattice relaxation time of water protons in intact bovine chromaffin vesicles has been studied over the range 1.00–23.49 kG. The T_1 relaxation time shows a dispersion at field values near 20 kG. The observed proton resonance arises mainly from solvent protons ($^1\text{H}_2\text{O}$), but the relaxation rate, which is a weighted average over all sites with which the solvent protons rapidly exchange (i.e., NH and OH protons), is dominated by exchangeable protons in the most slowly moving soluble component. The field dependence of the T_1 dispersion demonstrates the existence of a site of exchangeable protons for which $\tau_r = 1.9 \pm 0.5$ ns at 3°C . This site is assigned to ATP and cationic groups to which its phosphate esters are complexed, since previously measured correlation times of epinephrine and the chromogranin backbone are nearly an order of magnitude too short to explain the T_1 dispersion. Quantitative estimates of the relative numbers of exchangeable protons on the different soluble components support this interpretation. The temperature dependence of T_1 of the peak due to exchangeable protons has also been measured over a temperature range -3 to 25°C . T_1 lengthens by about 30% over this range and exhibits no discontinuous behavior, as would be expected if a gel transition or structural alterations in the storage complex occurred. T_1 lengthens by less than 10% in chromaffin granule pastes that have been maintained at 25°C for 24 h, indicating considerable thermal stability in the storage complex. Possible effects on the solvent T_1 due to paramagnetic ions have been considered with the conclusion that they are probably negligible or of minor significance.

Introduction

NMR relaxation times have recently been used to study molecular motion in the aqueous phase of chromaffin granules [1,2]. Measurements of reorienta-

tional correlation times from ^{13}C T_1 values have shown that chromogranin is present as a random coil polyelectrolyte that does not form a gel in the vesicle interior. The correlation time of the backbone amide carbon of chromogranin is extremely short, $\tau_r \approx 0.2$ ns at 25°C , and is similar to the correlation time for overall tumbling of epinephrine, $\tau_r = 0.15 \pm 0.03$ ns. ATP, on the other hand, appears from several observations to have a substantially longer correlation time than either of these values. Quantitative estimates of its correlation time gave values of 1.0 ns at 25°C and 1.9 ns at 3°C , but these estimates were obtained from a comparison method and could be subject to substantial error. The experiments reported here provide a direct method for measuring correlation times of chemical sites that bear protons in rapid exchange with the solvent (primarily NH and OH). A site with a relatively long correlation time, $\tau_r = 1.9 \pm 0.5$ ns, has been found in bovine chromaffin granules. This site appears to consist of ATP and those cationic groups to which the ATP phosphate esters are bound.

In the present experiments, T_1 of the water protons in the aqueous phase is measured as a function of magnetic field strength. Protons of the solvent exchange rapidly with NH and OH protons on solute molecules, and the observed relaxation rate of the resonance due to labile protons is a weighted average of relaxation rates in the various chemical sites:

$$\left(\frac{1}{T_1}\right)_{\text{obs}} = \sum_i f_i \left(\frac{1}{T_{1,i}}\right) \quad (1)$$

where f_i is the fraction of the total pool of exchangeable protons in the i th site, and $T_{1,i}$ is the relaxation time in this site. Of course, the largest fraction of exchangeable protons (approx. 95%) in chromaffin granules is $^1\text{H}_2\text{O}$. $T_{1,i}$ in each site is a known function of magnetic field strength, H_0 [3]:

$$(T_{1,i})^{-1} = \frac{2\gamma^4\hbar^2}{5} I(I+1) \sum \frac{1}{r_{ij}^6} \left\{ \frac{\tau_{r,i}}{1 + \omega_H^2\tau_{r,i}^2} + \frac{4\tau_{r,i}}{1 + 4\omega_H^2\tau_{r,i}^2} \right\} + (T_{1,i})_{\text{trans}}^{-1} \quad (2)$$

ω_H , which is the only field-dependent quantity on the right-hand side, equals γH_0 , where γ is the proton magnetogyric ratio. $\tau_{r,i}$ is the rotational correlation time in the i th site, I is the proton nuclear spin ($I = 1/2$), and r_{ij} is the internuclear distance between the i th proton and neighboring protons j . $(T_{1,i})_{\text{trans}}^{-1}$ is the intermolecular term and is given approximately by

$$(T_{1,i})_{\text{trans}}^{-1} = 2\pi N\gamma^4\hbar^2/5(D_{\text{H}_2\text{O}} + D_i) \approx 2\pi N\gamma^4\hbar^2/5D_{\text{H}_2\text{O}}.$$

D_i is the self-diffusion coefficient of the i th site, and N is the number of protons per cm^3 . It is evident from Eqn. 2 that $T_{1,i}$ is field dependent when the correlation time of the i th site satisfies the condition $2\omega_H\tau_{r,i} \gtrsim 1$. As the field is raised from low to high field in this region, $1/T_{1,i}$ falls from its limiting value $(1/T_{1,i})^0$ at low field toward an asymptotic dependence on H_0^{-2} at fields sufficiently high that $2\omega_H\tau_{r,i} \gg 1$. This behavior is illustrated by the theoretical plots in Fig. 1. According to Eqn. 1 the contribution of each site to the observed relaxation rate is proportional to $1/T_{1,i}$ of that site. A site that contains a very small fraction of exchangeable protons may dominate the observed T_1 if the relaxation time in that site is much shorter than that of the solvent. In this way, the effect of relatively immobile sites with short T_1 is greatly amplified.

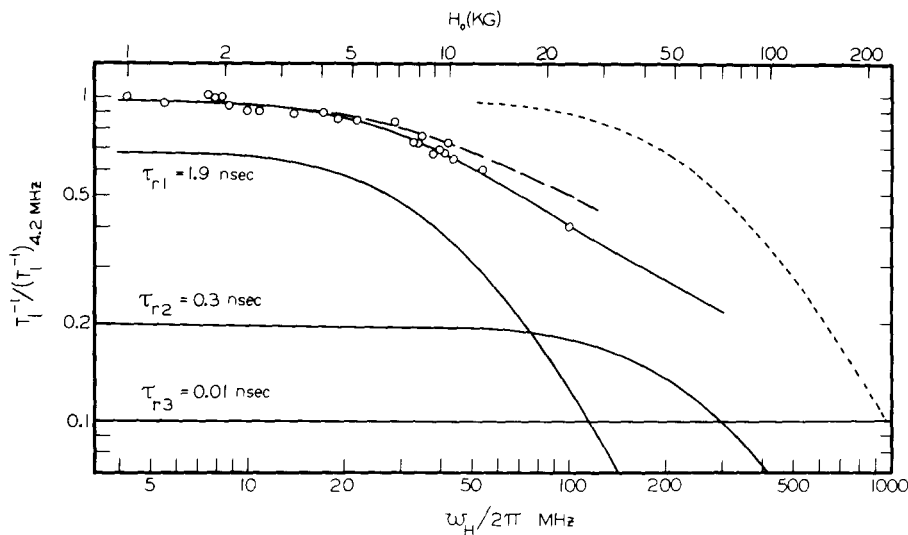


Fig. 1. Magnetic field dependence of the spin-lattice relaxation time of protons in the aqueous phase of bovine chromaffin granules at 4°C. Relaxation rates are normalized relative to T_1^{-1} (4.2 MHz) = 1. The abscissa gives the magnetic field strength (H_0) and the NMR frequency, ($\omega_H/2\pi$) MHz. The broken line (---) is the closest fit consistent with all correlation times less than or equal to 0.3 ns. Dashed line (----) assumes three sites with $\tau_{r1} = 1.9$ ns, $\tau_{r2} = 0.3$ ns and $\tau_{r3} = 0.005$ ns and relative relaxation efficiencies of 58%, 32% and 10%. Solid lines (—) are a three-site fit with the same correlation times as above and relaxation efficiencies of 70%, 20% and 10%.

Another virtue of this method of measuring correlation times is that the field dependence of the T_1 dispersion depends only on the correlation times. Other factors that determine absolute values of T_1 , in particular the bound fraction f_i and the geometric factor $\sum r_{ij}^{-6}$, do not influence the center frequencies of the T_1 dispersions, but rather affect the relative contributions of each site to the total relaxation rate in the low field limit.

In general, paramagnetic metal ions can also produce a magnetic field dependence in the solvent T_1 if solvent molecules exchange rapidly with sites in the inner coordination sphere of the metal. This effect tends to complicate the straightforward interpretation of field-dependent T_1 values given by Eqn. 2. Small concentrations of transition metals, most notably iron and copper [4,5], are known to be present in chromaffin granules. The effects of these metals on observed T_1 values are considered in detail below, and it is concluded that they are very probably negligible.

Experimental

Bovine chromaffin granules were prepared as described previously [1]. Proton relaxation times were measured on a sample that was washed four times in 150 mM KCl, but not exchanged with $^2\text{H}_2\text{O}$. All steps in the procedure were carried out at 0–5°C, and NMR measurements were made at $3 \pm 1^\circ\text{C}$.

T_1 values were measured using a Bruker B-KR 322s variable frequency (4–60 MHz) pulsed NMR spectrometer described elsewhere [6,7]. The standard inversion-recovery sequence (180° — τ — 90° —sample) was used to measure pro-

ton T_1 values. The measurement at 100 MHz utilized a JEOL JNM/PFT 100 Fourier transform NMR spectrometer interfaced to a Digilab NMR-3 data system.

Results

Proton T_1 values of bovine chromaffin granules were measured over a range of magnetic fields, 1.00–23.49 kG, corresponding to proton resonance frequencies from 4.25 to 100.0 MHz. The data, which are given in Table I, are relaxation times of the total integrated intensity of the aqueous phase only and exclude the rapidly relaxing lipid component reported in ref. 1. In an undeuterated sample, the signal due to exchangeable protons (of which the main component is due to $^1\text{H}_2\text{O}$) comprises 91% of the total observed intensity. Thus, the contribution of nonexchangeable protons in the aqueous phase is small and can for the present purposes be neglected.

The proton T_1 values show a dispersion at field strengths above about 3 kG; at the highest field, 23.49 kG, T_1 drops to 40% of its limiting low field value at 1.00 kG. This field dependence of T_1 indicates the presence of at least one site of exchangeable protons with long τ_r . In analysing these data quantitatively, we seek answers to three questions. First, can the observed T_1 dispersion be explained in terms of sites on epinephrine or chromogranin? Correlation times for both these molecules are known fairly accurately from ^{13}C data. Secondly, if a site with longer correlation time is required, does this site have a correlation time in reasonable agreement with our previous estimate of τ_r for ATP? And thirdly, is the fraction of exchangeable protons associated with ATP and the cationic side chains to which it may be complexed sufficiently large to account for the observed T_1 dispersion?

An attempt was first made to fit the T_1 frequency dependence using relaxation components with $\tau_r \leq 0.35$ ns. All of the soluble components that are observable in ^{13}C spectra of chromaffin granules (namely, epinephrine, the backbone α -carbon of chromogranin and the glutamic acid sidechain resonances) have correlation times shorter than or equal to that value at 3°C. Thus this situation would be descriptive of chromaffin granules if τ_r for ATP and the cationic groups to which it is presumably complexed likewise were no longer than 0.35 ns. Theoretical curves computed from this assumption did not produce a

TABLE I

SPIN-LATTICE RELAXATION TIME AGAINST NMR FREQUENCY FOR THE PROTON RESONANCE OF THE AQUEOUS PHASE OF BOVINE CHROMAFFIN GRANULES AT 3°C

f (MHz)	T_1 (s)	f (MHz)	T_1 (s)	f (MHz)	T_1 (s)
4.250	0.23	14.008	0.26	37.898	0.35
5.550	0.24	17.250	0.26	39.898	0.34
7.605	0.23	19.250	0.27	41.198	0.34
8.005	0.23	21.994	0.27	42.198	0.32
8.402	0.23	29.003	0.27	44.098	0.36
8.805	0.25	33.098	0.32	54.117	0.38
10.001	0.25	34.098	0.32	100.000	0.58
11.000	0.25	35.098	0.30		

satisfactory fit of the data. The closest possible fit (shown as the dotted line in Fig. 1) is obtained by setting all $\tau_{r,i} = 0.35$ ns. This correlation time predicts a decrease of only 10% in $(T_1)^{-1}$ at 100 MHz, whereas a decrease of 60% is actually observed. Thus the observed dispersion requires the presence of a site for which $\tau_{r,i}$ is substantially longer than 0.35 ns.

Since the correlation times of epinephrine and the chromogranin backbone have been measured directly and are known to be shorter than 0.35 ns, the T_1 dispersion apparently arises from exchangeable protons on ATP as well as from cationic groups that may neutralize the multiply charged phosphate esters. Other observations support a relatively long correlation time for ATP [1], and ^{31}P chemical shifts indicate that the β - and γ -phosphate esters are complexed in intact chromaffin granules [8].

The observed frequency dependence of T_1 was readily reproduced by assuming: (1) that the site of long τ_r is ATP; (2) that ATP has a correlation time near 1.9 ns at 3°C as found previously [2]; and (3) that it is complexed at its phosphate esters by the cationic side chains (*lys, his, arg*) of chromogranin. Relative numbers of exchangeable protons on epinephrine, chromogranin, ATP and water are listed in Table II. Correlation times for the different components are also listed in Table II. Correlation times for epinephrine and for the chromogranin amide protons are measured values from the partially relaxed ^{13}C spectra. The correlation time for ATP is taken to be 1.9 ns, the value previously inferred from proton T_1 measurements. Side chains of chromogranin are assumed to have two correlation times, which correspond respectively to basic residues (*lys, arg, his*) that complex the phosphate esters of ATP, for which $\tau_{r,i} = 1.9$ ns, and acidic residues that have similar correlation times to the glutamic acid sidechains, $\tau_r \approx 0.3$ ns. There is no quantitative way to assign T_1

TABLE II

FRACTIONS OF LABILE PROTONS (NH AND OH) AND CORRELATION TIMES ASSOCIATED WITH DIFFERENT COMPONENTS OF THE AQUEOUS PHASE OF BOVINE CHROMAFFIN GRANULES

(a) Ref. 16; (b) Ref. 17; (c) based on the molecular weight (77 000) and amino acid composition of chromogranin A as given in ref. 16, soluble protein assumed to be 77% of total protein (ref. 18); (d) (Sharp, R.R. and Sen, R., unpublished results); (e) taken from ref. 19, accounting for the fact that the dielectric relaxation time, which describes reorientation of a vector, is three times τ_r , which describes reorientation of a rank two tensor (ref. 3, pp. 298–300).

Component	% dry wt. ^a	% wet wt. ^b	Mol/100 g wet wt.	Labile H per molecule	Labile H (mol/100 g wet wt.)	Labile H (mol) ($\text{H}_2\text{O} = 1.0$)	$\tau_{r,i}$ ^d (ns) at 3°C
Catecholamine	20.5	8.2	0.049	5	0.24	0.036	0.3
ATP	15.0	6.0	0.012	4	0.048	$7.2 \cdot 10^{-3}$	1.9
Chromogranin	27.0	10.8	0.14				
(i) <i>lys, arg, his</i>			10^{-3} ^c	407 ^c	0.055	$8.2 \cdot 10^{-3}$	1.9
(ii) amide and other labile sidechain H				865 ^c	0.12	$18 \cdot 10^{-3}$	0.3
H ₂ O	—	60	3.34	2	6.7	1.0	0.005

values to exchangeable protons in each of these sites as would be required to obtain a unique fit to the data. Our intention here is simply to demonstrate that the number of exchangeable protons associated with the ATP site is sufficient to produce the observed T_1 dispersion. Thus we make the simplifying assumption that the average dipolar couplings experienced by all exchangeable protons are equal, and that differences in T_1 reflect only differences in the spectral density factor (i.e. the factor in brackets in Eqn. 1). These assumptions produce a satisfactory fit, which is described by the dashed line in Fig. 1. A small adjustment of the geometric factors produces a fit that accurately reproduces the data (the solid line in Fig. 1). The frequency range of the T_1 dispersion is determined in a sensitive manner by the ATP correlation time. τ_r values of less than about 1.2 ns cannot be reconciled to the data, even under extreme assumptions concerning bound fractions and dipolar coupling constants.

Temperature dependence of T_1

The spin-lattice relaxation time of the composite signal due to $^1\text{H}_2\text{O}$ and other exchangeable protons has been measured over a range of temperatures from -3 to 25°C . The results are shown in Fig. 2. It has been demonstrated above that magnetic relaxation of this peak is dominated by protons on ATP, and that ATP has substantially lower reorientational mobility than do other soluble components in the aqueous phase. Presumably this lower rotational mobility results from the participation of ATP as a structural element in the storage complex. Thus there is reason to expect that the T_1 of the peak due to labile protons may provide a more sensitive probe of structural changes in the storage complex than do T_1 values of other resonances.

Variable temperature measurements were conducted in the following sequence. (1) A fresh sample prepared at 3°C was warmed to 25°C over the course of 2 h, during which T_1 measurements were obtained (Fig. 2, open circles). (2) The sample was then lowered to -3°C (closed circles) and frozen at -9°C . (3) The sample was thawed (no change in the T_1 value at -3°C was observed after freezing) and allowed to warm to 25°C overnight. (4) Measurements were obtained after 24 h at both 25°C and 3°C (circles with centers).

Measured values of T_1 are remarkably insensitive to temperature variations. T_1 increases slowly as temperature is raised, as is expected from the shortening

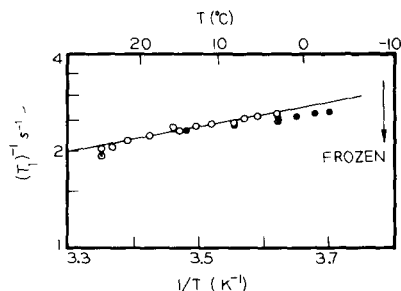


Fig. 2. Spin-lattice relaxation rate against temperature for the composite NMR peak due to $^1\text{H}_2\text{O}$ and other labile protons at 54.117 MHz in sedimented bovine chromaffin granules. The sequence in which measurements were taken is described in the text. \circ , warmed sample; \bullet , cooled sample; \odot , after 24 h, 3°C and 25°C .

of the rotational correlation times. The apparent activation energy of T_1^{-1} is not equal to that for τ_c , since measurements were made in the vicinity of the magnetic field dispersion. T_1 shows no anomalous behavior that would indicate structural breakdown of the complex with rising temperature. Even after 24 h at 25°C, T_1^{-1} shows a decrease of less than ten percent, indicating that the complex is remarkably stable in sedimented pastes of the granules. Our previous ^{13}C and proton high resolution nmr data showed a similar insensitivity to temperature variation.

Previous measurements of epinephrine efflux from chromaffin granules have shown that efflux is fairly rapid in aqueous suspensions, and that it is biphasic at 37°C. Approx. 25% of the endogenous epinephrine leaves with a half-life of 4.7 min, while the remaining 7% has a half-life of 117 min [9]. The rate of efflux is greatly retarded at lower temperatures, and catecholamine storage appears to be stable indefinitely at 3°C [10,11]. It seems quite likely that the relatively high stability of catecholamine storage observed in the present experiments results in part from the fact that packed sediments of chromaffin granules were used rather than aqueous suspensions. The extraventricular space in sedimented pellets is small compared to the total sample volume, and efflux is necessarily inhibited. The apparent insensitivity of the aqueous storage complex to temperature variations is nevertheless remarkable.

Effects of paramagnetic metal ions

Borowitz, Fuwa and Weiner [4] analyzed chromaffin granules for divalent metals and found that among transition metal ions, iron is present in the highest concentration (3.9 $\mu\text{mol/g}$ protein). Trace quantities of Mn and other transition metals, such as Cu(II), which occurs in dopamine β -hydroxylase at a level of (0.37 $\mu\text{g/ml}$) in the purified enzyme [5], are also present. Undoubtedly, most if not all of the transition metals are associated with enzymes in the insoluble fraction of chromaffin granules and may well be isolated from the bulk solvent of the aqueous phase. In view of the fact that paramagnetic metal ions can in general produce magnetic field dependence in the T_1 values of solvent protons however, it is necessary to consider whether the observed T_1 dispersion could be a paramagnetic effect without invoking assumptions concerning the structural organization of the granules. Relaxation enhancements produced by dopamine β -hydroxylase have been studied previously [12] however and are found to be far too small to account for the T_1 dispersion.

Magnetic relaxation in paramagnetic solutions has been discussed systematically by Dwek [13]. It is readily shown from the Solomon-Bloembergen equations that an interpretation of the T_1 dispersion in terms of paramagnetic ions can be consistent with the qualitative temperature and magnetic field dependence of the proton T_1 data only if the following three conditions are met:

(1) The correlation time for the paramagnetic dipolar interaction equals 2 ns at 3°C;

(2) The dominant paramagnetic correlation time must be interpreted physically either as a reorientational correlation time, τ_R , or as the mean lifetime, τ_M , of water molecules in the first hydration sphere of the metal ion.

(3) The longitudinal spin relaxation time of the paramagnetic ion must satisfy the inequality, $\tau_{s1}^{-1} \ll \tau_R^{-1} + \tau_M^{-1}$.

If paramagnetic ions are present in a site of extremely high reorientational mobility in the aqueous phase, as would be implied by a τ_R value of 2 ns, then the binding site would of necessity be an anionic group on one of the soluble components. The phosphate esters of ATP, which bear four negative charges at pH 7, would presumably be the preferred binding site. Thus this interpretation requires that a significant portion of the ATP in the aqueous phase be present as a transition metal complex. The presence of such complexes would be evident through large perturbations in the ^{31}P spectra of the α -, β - and γ -phosphorus atoms, the resonances of which are extremely sensitive to small concentrations of paramagnetic ions [13]. Since these spectral perturbations would arise solely from interactions among soluble components, they could not be reduced substantially by lysis of the granules and removal of the membrane vesicle fragments. The β - and γ - ^{31}P resonances are indeed chemically shifted and are fairly broad in intact chromaffin granules, but these effects largely disappear when the granules are disrupted and the insoluble fraction removed [8]. Such behavior is consistent with broadening that is produced by residual anisotropy in the internal aqueous phase, rather than by complexation with paramagnetic ions. Furthermore it is very unlikely that the restriction on τ_{s1} ($\tau_{s1} \gg 2 \cdot 10^{-9}$ s) would be met for soluble complexes of any of the transition metal ions present in chromaffin granules [14].

If, on the other hand, the paramagnetic ion were bound to an ordered phase but exposed to the solvent of the aqueous phase, then τ_R would be very great, and the dominant correlation time would be τ_M . A value of $\tau_M = 2$ ns appears to be at least an order of magnitude too short to describe solvent exchange with the hydration sphere of Fe(II), Fe(III), Mn(II), and most other transition metal ions. NMR measurements of τ_M for the hexaquo complexes in nearly all cases give values greater than 10^{-8} s at 3°C ; for example, for Mn(II), $\tau_M = 74$ ns [13]; for Fe(II), $\tau_M \approx 0.3 \mu\text{s}$ [15] and for Fe(III), $\tau_M \approx 0.5$ s [15].

Consequently there appears to be no satisfactory physical basis for explaining the observed correlation time of 2 ns in terms of one of the molecular processes that modulate the paramagnetic interaction. While we cannot dismiss categorically the possibility that transition metal ions contribute to the proton relaxation rate, we consider it very unlikely that the T_1 dispersion is a paramagnetic effect.

Summary

The frequency dependence of the T_1 data demonstrate the existence of a site of exchangeable protons for which $\tau_r \approx 2.0$ ns at 3°C . This correlation time is about six times longer than the values measured directly from ^{13}C data for epinephrine and the chromogranin backbone. It agrees well, however, with the value inferred previously for ATP, and is consistent with the anomalous temperature and nuclear Overhauser enhancement dependence of the ATP proton resonances [1,2]. The relatively long correlation time of ATP has been explained elsewhere in term of a specific model of the catecholamine storage complex (Sharp, R.R. and Sen, R. unpublished). In this model, ATP acts as the basic structural element of a ternary chromogranin · epinephrine · ATP complex by cross-linking cationic side-chains of the protein.

The storage complex is quite stable with respect to temperature changes between -3 and 25°C . Remarkably little evidence of structural deterioration is observed over a period of 24 h in packed sediments of chromaffin granules.

Acknowledgements

Assistance by E.P. Richards in the preparation of chromaffin granules is gratefully acknowledged, as are helpful discussions with Mr. Richards and Dr. Edward Domino. This work was supported in part by Institutional Research Grant No. IN-40P to the University of Michigan from the American Cancer Society.

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