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^1H AND ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTRA OF THE LIPIDS IN NORMAL AND SV 40 VIRUS-TRANSFORMED HAMSTER EMBRYO FIBROBLAST MEMBRANES

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SUMMARY

Well resolved ^1H and ^{13}C NMR spectra were obtained with normal and SV 40-transformed cell membranes. Estimation of the ratio of ^{13}C T_2 values of the normal to transformed cell membranes showed an increased intermolecular motion in the transformed cell membranes. The temperature dependence of the $(CH_2)_n$ line in the ^1H spectra in the temperature range 298–343 °K shows an activation energy for the lateral diffusion of the fluid phospholipid regions in the normal cell membranes while the transformed ones show practically no temperature dependence in this temperature range. The fluidity of the phospholipid region in the transformed cell membrane seems to be significantly higher than that observed in the normal cell material. These data support and extend the findings concerning the mobility of the concanavalin A binding/agglutinating sites on the surface of normal and virus-transformed cells and suggest further approaches to the study of the membrane alterations in tumor cells.

INTRODUCTION

Studies of the agglutination of transformed cells by plant lectins and particularly by concanavalin A suggested that the lectins sites have different mobilities on the cell surface of normal and transformed cells [1–5]. These different mobilities are associated with the varying degrees of fluidity of specific membrane components and may be responsible for lectin-induced clustering of surface sites and differences in lectin-mediated agglutination of transformed cells [6].

Considerable differences were reported by Hatanaka et al. [7, 8] between the uptake of sugars by normal and murine-sarcoma-transformed BALB/3T3 cells; although such differences were not found with SV 40 virus-transformed cells, in the former case they were shown to be due to specific alterations in the plasma membrane sugar transport system and not to an increased cellular hexokinase activity [7]. These

and other plasma membrane properties acquired as a result of cell transformation suggest that, among others, the fluidity of the lipids in the cell membrane may change considerably as a result of transformation. ^{1}H and ^{13}C NMR investigations of sarcoplasmic reticulum membranes [9–11] and of intact bovine retinal rod outer segment membranes [12] showed that valuable informations about the intra- and intermolecular mobility of the membrane lipids could be thus obtained. In the present study we report the ^{1}H and natural abundancy ^{13}C NMR measurements of the lipids in normal and SV 40 virus-transformed hamster embryo fibroblasts as well as evidence for considerable differences in the fluidities of the lipids in these membranes.

MATERIALS AND METHODS

(*1*) *Cells*

The cells were normal hamster embryo fibroblasts (HEF) derived from 14-day-old embryos passaged once after primary isolation and SV 40-virus-transformed hamster cells, of the H-50 line [13]. The cells were passaged in Eagle's medium supplemented with 10 % fetal calf serum and antibiotics. Cell layers were washed twice with cold phosphate-buffered saline and scraped into the buffer. The cells were pelleted at $1000 \times g$, resuspended in H_2O and sonicated briefly and gently. The sonication was achieved simultaneously for both types of membranes, with the same type of sonicator, under argon for the same duration and with the same power. The membranes were pelleted by centrifugation at $100\,000 \times g$ for 1 h. The pellet was washed once with H_2O and repelleted at $100\,000 \times g$ for 1 h. The washed pellet was resuspended in water and lyophillized. Prior to the measurements, the lyophyllized material was washed twice with $^{2}H_2O$, pelleted at $100\,000 \times g$ for 1 h and then transferred to the NMR micro cells, under argon. The membrane material in $^{2}H_2O$ was 15 % w/w. Both normal and transformed cell membranes were analyzed by gas chromatography for their fatty acid contents, which did not show significant differences.

(*2*) *Spectroscopic equipment*

The ^{1}H and ^{13}C NMR spectra were recorded with a WH 90 Bruker spectrometer operating in the Fourier transform mode, at 22.63 MHz for ^{13}C and at 90 MHz for ^{1}H. The spectrometer was equipped with a Nicolet BNC 12 computer, with 8 K data memory. 200 000 accumulations were required for the ^{13}C spectra and 10 000 for the ^{1}H spectra. The ^{1}H spectra were recorded at various temperatures, between 298 °K and 343 °K. All the ^{13}C spectra were recorded at 310 °K.

The spectra of the membrane samples were recorded several times each, under constant conditions. The spectra did not show any significant differences except between the "normal" and "transformed" samples. The compared samples were investigated in Wilmad Inc. microcells, which were contained in 10-mm tubes.

It is well known that a large number of scans induce a line broadening which can be observed with very narrow NMR lines as well (Lockoscillations). Nonetheless, the reliability of the results statistically increases. The possible broadening by the large scan number is less by orders of magnitude than the experimentally observed line width of our lines.

The spectra were simulated with a computer, with regard to their line-shapes

and their signal-to-noise ratios, until an optimal fit was obtained. The synthetic noise function which we used was generated with random numbers. The noise amplitudes, distributed as a function of their frequency, correspond to a Gauss function. The frequency distribution corresponds roughly to the white noise. This noise-function was subjected to numerous tests and the agreement with the actual noise was very satisfactory (Dietrich, W. (1974), in the press).

We observed the same reproducible differences on four samples of HEF and of SV 40 transformed cell membranes (hamster fibroblasts).

RESULTS AND DISCUSSION

^{13}C *Spectra*

The normal and virus-transformed cell-membranes show the resonances of terminal methyl groups, large $-(CH_2)_n-$ resonances, sharp lines corresponding to $N^+(CH_3)_3$ resonance and the $-CH = CH-$ line. The resonances were assigned by comparison with the chemical shifts of dioleyl- and dipalmitoyllecithin. In transformed cell membranes the carbonyl resonance is also observed. Although in both samples the membrane material had the same concentration, the SV 40-transformed H-cell membranes show considerably sharper signals than do the HEF membranes (Fig. 1a and b). The $(CH_2)_n$ resonance in the normal cell membranes is much broader than the corresponding line in the SV 40-transformed H-cell membranes (450 Hz as compared to approx. 200 Hz). The linewidth and T_1 values of the ^{13}C resonance in the phospholipid membranes are an indication of the fluidity of the membrane [14, 15]. The $-CH = CH-$ resonance amplitude shows, at constant linewidth a decrease by a factor of 2 in the normal cell membranes. There is no major difference in the $\Delta\nu_{1/2}$ value of the $N^+(CH_3)_3$ resonance in normal and transformed cells, but the heavy smoothing function applied to the Fourier-transformed spectra to improve the signal-to-noise ratio will tend to obscure any small differences in the linewidths [10]. The CH_3 resonance shows the same linewidth and intensity in the two spectra. The ^{13}C spectra recorded are very similar to those reported by Robinson et al. [10] for sarcoplasmic reticulum membranes and by Millett et al. [12] for bovine retinal rod outer segment membranes. The same resonances appear in the spectrum but, in the transformed cell membranes, the linewidth of the $-(CH_2)_n$ resonance is twice less than in the normal material. Besides, the $-CH = CH-$ resonance is almost twice more intensive in the SV 40- transformed H-cell membranes. The sharp resonances obtained suggest a higher fluidity for the lipids in the membranes investigated and the considerably narrower $-(CH_2)_n-$ line in the ^{13}C NMR spectra of the SV H membranes is evidence for this suggestion. The phospholipid composition of normal and SV 40-transformed cell membranes are very similar (unpublished results) so that the differences in the linewidth can hardly be ascribed to different chemical shifts in the two membranes. Had the membrane compositions been different we would have observed also differences in the number and position of the resonance lines. Another observation concerns the $N^+(CH_3)_3$ resonance. It shows, at constant linewidth, an intensity decrease in the normal cell membranes as compared to the transformed ones of about 30 %.

Nicolau et al. [18] found, not only in natural membranes but also in phos-

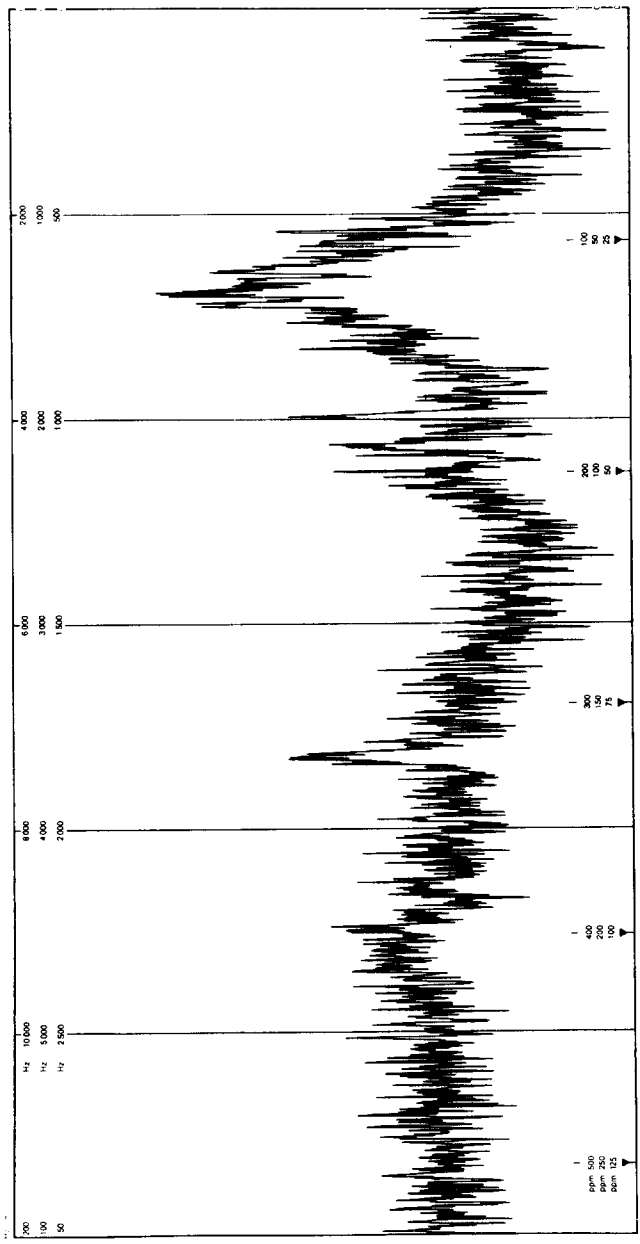


Fig. 1. ^{13}C NMR spectrum of the normal HEF cell membranes in D_2O; 200 000 accumulations; $t = 38\ ^{\circ}C$.

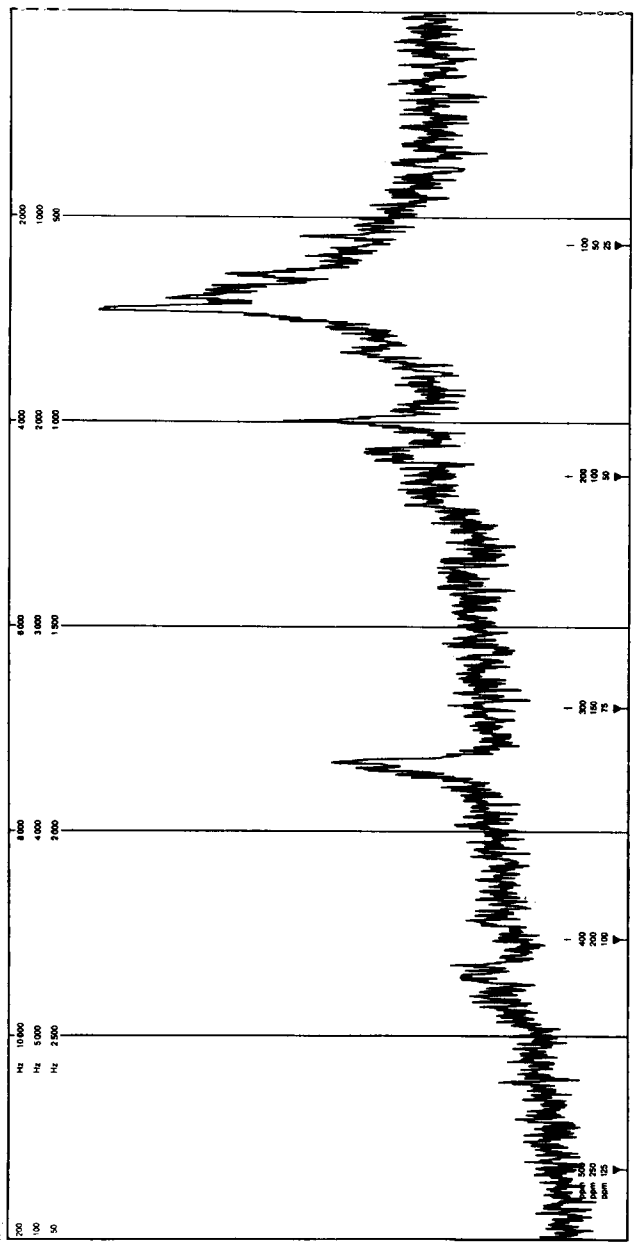
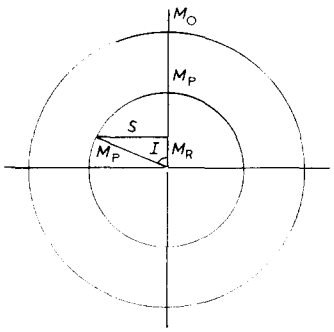


Fig. 2. ^{13}C NMR spectrum of the SV 40-transformed H-cell membranes in $^{2}H_2O$; 200 000 accumulations; $t = 38$ °C.

pholipid vesicles, that the addition of certain small peptides reduces the value of the ^{13}C T_1 of the choline carbons by 35–40 %, in agreement with Robinson et al. [10] who showed that the ^{13}C T_1 value for the $N^+(CH_3)_3$ group decreases in membranes, as compared to phospholipid vesicles, by some 35 %. The differences in the ^{13}C T_1 values of the choline group observed between the normal and the transformed cell membranes are not large, but they are beyond the experimental error.

It should be pointed out that, compared with the ^{13}C NMR spectra of the sonicated phospholipid vesicles (dioleyllecithin and dipalmitoyllecithin), the linewidth of the membrane lipids are significantly higher, a fact which supports our conclusions.

Although, for experimental reasons, we cannot yet give the ^{13}C T_1 values of the various carbon atoms in our membrane phospholipids, we can show that there are, between the two membrane types, appreciable differences in the T_2 values. This, in turn allows some comments regarding the relative fluidities of the two materials investigated. If we consider:



$$\sin I = \frac{S}{M_P} \qquad M_P = \frac{S}{\sin I} \tag{1}$$

$$\cos I = \frac{M_R}{M_P} \qquad M_R = S\frac{\cos I}{\sin I}$$

where

S = signal amplitude
I = pulse torsional angle
M_P = magnetization formed during the pulse
M_R = rest magnetization
M_0 = limit magnetization

we can write

$$M_P = M_0\left[1 - \left(1 - \frac{M_R}{M_0}\right)e^{-t/T_1}\right] \tag{2}$$

when

$$\begin{aligned} t &= 0 \qquad M = M_R \\ t &= t_p, \qquad M = M_P \end{aligned} \tag{3}$$

where t_p = pulse rate.
Cf. (1) we can write

$$\frac{S}{\sin I} = M_0 \left[1 - \left(1 - \frac{S}{M_0} \frac{\cos I}{\sin I}\right) e^{-t/T_1}\right] \tag{4}$$

and

$$S = \sin I M_0 - \sin I M_0 \left(e^{-t/T_1} - \frac{S}{M_0} \frac{\cos I}{\sin I} e^{-t/T_1}\right) \tag{5}$$

which may be written as

$$S = M_0 \sin I - M_0 \sin I e^{-t/T_1} + S \cos I e^{-t/T_1} \tag{6}$$

hence

$$M_0 \sin I(1 - e^{-t/T_1}) = S(1 - \cos I e^{-t/T_1}) \tag{7}$$

Now, considering the amplitude of any resonance in the ^{13}C spectrum of the normal (N) and transformed material (T), if

$$S_N = S_T$$

we can write

$$\frac{M_{0N}}{T_{2N}} = \frac{M_{0T}}{T_{2T}}$$

which gives

$$M_{0N} = M_{0T} \frac{T_{2N}}{T_{2T}} \tag{8}$$

then

$$\frac{M_{0T} \sin I(1 - e^{-t/T_{1T}})}{M_{0N} \frac{T_{2N}}{T_{2T}} \sin I(1 - e^{-t/T_{1N}})} = \frac{S_T(1 - \cos I e^{-t/T_{1T}})}{S_N(1 - \cos I e^{-t/T_{1N}})}$$

which gives

$$(1 - e^{-t/T_{1T}})(1 - \cos I e^{-t/T_{1N}}) = P \frac{T_{2B}}{T_{2T}} (1 - \cos I e^{-t/T_{1T}})(1 - e^{-t/T_{1N}}) \tag{9}$$

P = proportionality factor
taking

$E_N = e^{-t_p/T_{1N}}$

$E_T = e^{-t_p/T_{1T}}$

$W = \cos I$

where t_p = pulse rate
we obtain

$$1-E_T-WE_N+WE_TE_N = P\frac{T_{2N}}{T_{2T}}[1-WE_T-E_N+WE_TE_N] \tag{10}$$

and

$$E_T\left[WE_N-1+P\frac{T_{2N}}{T_{2T}}W-P\frac{T_{2N}}{F_{2T}}WE_N\right] = WE_N-1+P\frac{T_{2N}}{T_{2T}}-P\frac{T_{2N}}{T_{2T}}E_N \tag{11}$$

it results

$$E_T = \frac{E_N\left(W-P\frac{T_{2N}}{T_{2T}}\right)-1+P\frac{T_{2N}}{T_{2T}}}{E_NW\left(1-P\frac{T_{2N}}{T_{2T}}\right)-1+P\frac{T_{2N}}{T_{2T}}} \tag{12}$$

which yields

$$T_1 = t_p/\ln\frac{e^{-t_p/T_{1N}}\cos I\left(1-P\frac{T_{2N}}{T_{2T}}\right)+P\frac{T_{2N}}{T_{2T}}\cos I-1}{e^{-t_p/T_{1N}}\left(\cos I-P\frac{T_{2N}}{T_{2T}}\right)+P\frac{T_{2N}}{T_{2T}}-1} \tag{13}$$

Based on this reasoning we calculated the values of T_{2N}/T_{2T} for three resonances. The data are listed in Table I.
The values for the ratio T_{2N}/T_{2T} show clearly that the ^{13}C T_2 of the $(CH_2)_n$ line is longer in the transformed cells by a factor of 2. This supports the evidence of the linewidth and further indicates [11] a higher fluidity of the phospholipids in the latter

TABLE I

Group	T_{1N} s*	$\frac{T_{2N}}{T_{2T}}$	T_{1T} s**
$-CH_3$	3.1 ±0.6	1	2.82
		0.964	3.1
$-(CH_2)_n-$	0.42±0.03	0.500	0.42
		0.516	0.133
$N^+(CH_3)_3$	0.36±0.03	0.666	0.367
		0.682	0.128

* Taken from [10].
** Lower and upper limits.

cell membranes. This finding agrees with those by Rosenblith et al. [5] who reported that both transformation and proteolysis may exert some effect(s) on the membrane, permitting greater mobility of lectin binding sites than is present in normal cells. This mobility might account for the increased agglutinability of transformed and protease-treated cells. Evidence from ferritin-conjugated concanavalin A experiments [16] points also to the formation of concanavalin A binding sites (clusters, on transformed cells only, which could also be due to the increased fluidity of the transformed cell membranes). Recent investigations have shown that HEF cells grcwn in medium supplemented with low levels of 2-deoxy-D-glucose could be agglutinated by concanavalin A to a significantly greater degree than control cells, an effect which could be reversed by subsequent growth in medium free of this sugar (17). Alterations of the membrane glycoprotein appeared simultaneously so that the question emerges whether alterations in the glycoproteins or changes of the fluidity of the membrane are responsible for the enhanced agglutinability of transformed cells. Our experimental evidence in this communication is that a significant increase in the fluidity of the lipids is observed in transformed cell membranes. Similar results have been observed by Barnett et al. with different virus-transformed cells and methylcholanthrene-transformed cells, by using the spin label technique [19].

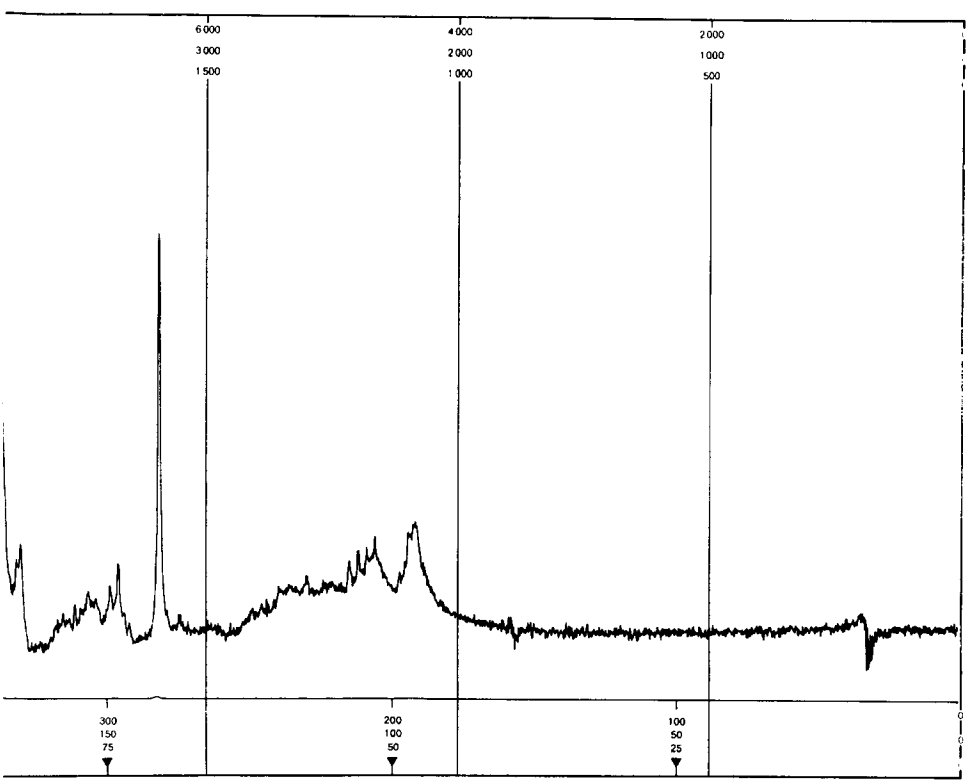


Fig. 3. ^1H NMR spectrum of the SV 40-transformed H-cell membranes in ^2H_2O; 10 000 accumulations; $t = 38$ °C.

^1H spectra

The ^1H NMR spectra show the same lines as those observed by Davis and Inesi [9] and by Robinson et al. [10] but with some differences. At −0.90 ppm we observe the CH_3 resonance, which is narrower than the resonance at −1.20 ppm, which is attributed to the $-(CH_2)_n-$ groups. The $-(CH_2)_n-$ resonance is the envelope of many resonances and, in both normal and transformed cell-membranes, it is quite broad. We observe at −3.3 ppm the choline resonance; under the same experimental conditions its relative intensity is 2.5 higher in the transformed cell membranes than in the normal. Its appearance, we believe, might be due to the brief sonication used in preparing the samples. It cannot be ruled out that in hamster fibroblast membranes such a signal can be observed, but until we are able to measure completely unsonicated samples we cannot state definitely the reason for observing the choline signal. It should be added that the ^1H NMR spectra were obtained at 38 °C. At this temperature Davis and Inesi [9] detect the $N^+(CH_3)_3$ resonance, with a linewidth of 20 Hz. Our choline signal has a line width of approx. 5 Hz, which supports our suspicion that it is mainly due to sonication. Even so, the difference in the line intensity, at constant linewidth, between the normal and the transformed cell membranes parallels the observations made in the ^{13}C spectra, namely a considerably shorter relaxation time T_1 for this group in the transformed cell membranes. It is difficult to draw any conclusions about this signal, as long as we cannot rule out that sonication is responsible for its appearance.

The temperature dependence of the peak amplitude of the line at −1.20 ppm was measured in the temperature range 298–343 °K. We measured the peak height since the measurement of $\Delta\nu_{1/2}$ is very uncertain. The difficulty arises from the fact that this line is actually the envelope of a number of resonances and slight temperature shifts in any of these resonances can induce large errors in our estimation of linewidth.

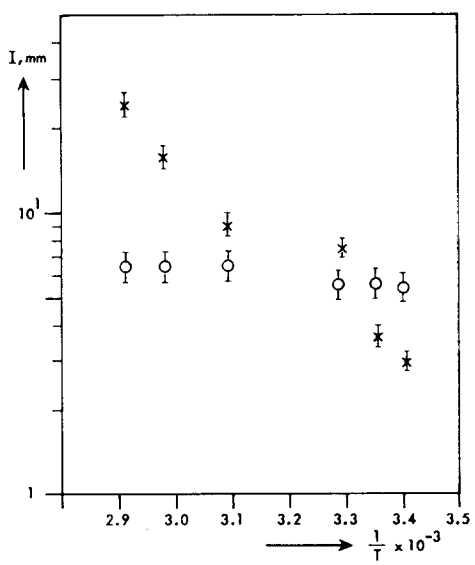


Fig. 4. Temperature dependence of the $-(CH_2)_n-$ peak amplitude in the ^1H NMR spectra of normal and transformed cell membranes. (●) Normal cells, (○) SV 40 virus-transformed cells.

We are well aware that measuring the peak amplitude may be just a qualitative indication of the line behaviour and therefore we don't give a value for the activation energy, but just show the temperature dependence of the $-(CH_2)_n-$ peak amplitude (Fig. 4).

In the temperature range used, the normal cell membranes show a strong temperature dependence of this amplitude, while the SV 40-transformed H-cell membranes show practically none. This is additional support for the significantly increased fluidity of the phospholipid in the latter. The reason for this difference is being currently investigated in our laboratory.

A word of caution must be added. There are a number of considerable experimental difficulties in achieving a satisfactory standardization of the samples on one side, and on the other side there are quite a number of difficulties in comparing NMR spectra obtained after large numbers of scans. The results reported above were nevertheless reproducible on a number of samples and even in unsonicated material the results are similar (Nicolau, Cl., Hildenbrand, K., Steiner, M. R. and Steiner, S. (1974) in preparation). It is difficult to assess the precise biological significance of the fluidity differences which we found between the normal and transformed cell membranes but it is conceivable that it may influence a considerable number of surface properties.

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