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THE STUDY OF ROUS SARCOMA VIRUS-TRANSFORMED BABY HAMSTER KIDNEY CELLS USING FLUORESCENT PROBES

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SUMMARY

The fluorescent probes pyrene, pyrene butyric acid and *N*-phenyl 1-naphthylamine have been used to investigate the changes that accompany in vitro transformation of a baby hamster kidney cell line using Rous sarcoma virus. The fluorescent probes which reside in the membrane were used to compare the changes in microviscosity and polarity of the membranes of normal cells with two transformed cell lines. The spectrofluorimetric data indicate that following transformation the probe *N*-phenyl 1-naphthylamine resides in a more polar environment. However, using the probe pyrene, the yield of excimer indicates decreased mobility of this probe in the membrane of transformed cells. The data also indicate differences between the two transformed cell lines. Laser photolysis was used to study the lifetime of the pyrene probes and the quenching of the pyrene fluorescence in the membrane by several different quenching molecules. The data indicate differences between the three cell lines and suggest that transformation decreases movement within the membrane.

INTRODUCTION

Transformed cells differ from normal cells in cell surface characteristics that are reflected in: (a) differences in cell membrane glycoproteins [1] and glycolipids [2]; (b) agglutination of transformed cells by lectins [3, 5]; (c) presence of specific antigens in transformed cells [6, 7]; (d) loss of density-dependent inhibition of growth upon transformation of normal cells [8, 9] and (e) growth in soft agar by transformed cells [10]. Studies have involved the biochemical characterization of the transformed cells and do not provide information about the structural organization of the cell membrane. Pulsed laser photolysis may provide a method of investigating the organization and interaction of membrane components.

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Fluorescent probes have been used to study physical properties of membranes of malignant cells. Inbar et al. [11] have used the probes 1,6-diphenyl 1,3,5-hexatriene to examine the membrane lipid layer of normal and transformed lymphocytes from humans and rodents. Their results showed an increase in fluidity in the membranes of malignant cells as compared to normal cells. This, they postulated was due to a decrease in the cholesterol level of the lipid layer of the membrane in the vicinity of the probes. In another study [12], the probe, 1-anilinonaphthalene-8-sulfonic acid was used to explore some of the physical properties of rat liver plasma membranes and Morris hepatoma plasma membranes. They concluded that the major difference between the Morris hepatoma membranes and the rat liver membranes was a change in the binding capacity of 1-anilinonaphthalene-8-sulfonic acid. This has also been noted for Rous sarcoma virus-transformed cells using trinitrobenzenesulphonate [13]. Previous work in our laboratory has utilized laser photolysis in the study of micellar systems [14–17] and bacterial membranes [18]. The techniques employed to investigate these systems have now been extended to mammalian cell membranes.

In the present investigation, the baby hamster kidney cell line (BHK-21) and two Rous sarcoma virus-transformed cell lines derived from BHK-21, designated BHK-T-1 and BHK-T-2, have been studied in order to correlate the membrane structure of the transformed cell with that of the normal cell. Laser photolysis is used to excite pyrene and derivatives of pyrene in the cell membranes. The rate constant of various quenchers with these probes is then determined. Normal spectrophotometric techniques are also used to investigate the fluorescence of *N*-phenyl 1-naphthylamine in cell membranes. These different photochemical techniques can yield information about normal cell membranes and their transformed counterparts.

METHOD

Laser-photolysis experiments were carried out with a Korad frequency doubled Q-switched ruby laser. The 347.1 nm laser pulse had a width of 15 ns and an energy output of approx. 200 mJ. Detailed description can be found elsewhere [19]. Fluorescence measurements were performed on an Aminco Bowman spectrofluorimeter. 1-Pyrene butyric acid (Pfaltz and Bauer) and *N*-phenyl 1-naphthylamine (Eastman) were used as supplied. Pyrene (Kodak) was purified by passage through silica gel using cyclohexane solution as a solvent.

BHK-21 cells were transformed with Bryan strain of Rous sarcoma virus (obtained from the National Cancer Institute, N.I.H.). BHK-21 cells were grown in Eagle's minimal essential medium with 10 % fetal bovine serum and 5 % tryptose phosphate broth. Rous sarcoma virus was added to 50 % confluent monolayers of BHK-21 cells. Two morphologically different foci were observed, one granular (BHK-T-1) and one smooth (BHK-T-2), and these were isolated by growth in semisolid agar [10]. Cells were passaged by scraping with a sterile rubber policeman. The cells were never exposed to trypsin. Cells were tested for transformation by growth in soft agar and by injection into weanling hamsters. BHK-T-1 and BHK-T-2 but not BHK-21 cells formed large colonies in soft agar after 3 days incubation at 37 °C. BHK-T-1 and BHK-T-2 but not BHK-21 formed tumors in weanling hamsters 2 weeks after subcutaneous inoculation of $1.0 \cdot 10^6$ cells in the paralumbar region.

The transformed state of BHK-T-1 and BHK-T-2 is stable. The cells have been

passaged at least 80 times and the data obtained with pyrene have been identical over this period.

Incorporation of probes into the cells. Stock solutions of each of the three probes were prepared: pyrene in acetone (10^{-2} M), pyrene butyric acid in benzene (10^{-4} M), and *N*-phenyl 1-naphthylamine in acetone (10^{-2} M). Distilled water was purified by distillation of tap water in a Barnstead still, followed by distillation from potassium permanganate in a quartz still.

Pyrene (40 μl) of the stock pyrene solution was added to 10 μl phosphate-buffered saline (pH 7.4). This produces a fine suspension of pyrene in the buffer. A known number of cells ($1 \cdot 10^6$ cells/ml) was added in a ratio of 1 part pyrene/phosphate-buffered saline mixture to 4 parts of cells. This cell suspension was then incubated at 37 °C on a rotator for 1 h. The cells were then centrifuged and the supernatant discarded. The cells were suspended to the original volume in phosphate-buffered saline. Pyrene butyric acid and *N*-phenyl 1-naphthylamine were incorporated into the cells by incubating the cells with the probes in a glass container for 20 min at 37 °C. Known amounts of the probes were coated on the walls of the container. The solvent was evaporated with a stream of N_2 gas before the addition of the probe and cells at the ratio used: 20 μl of pyrene butyric acid/$1 \cdot 10^6$ cells, and 10 μl of *N*-phenyl 1-naphthylamine/$1 \cdot 10^6$ cells.

RESULTS

N-Phenyl 1-naphthylamine. The fluorescence spectra of *N*-phenyl 1-naphthylamine in the three cell types are shown in Fig. 1. All three spectra are significantly

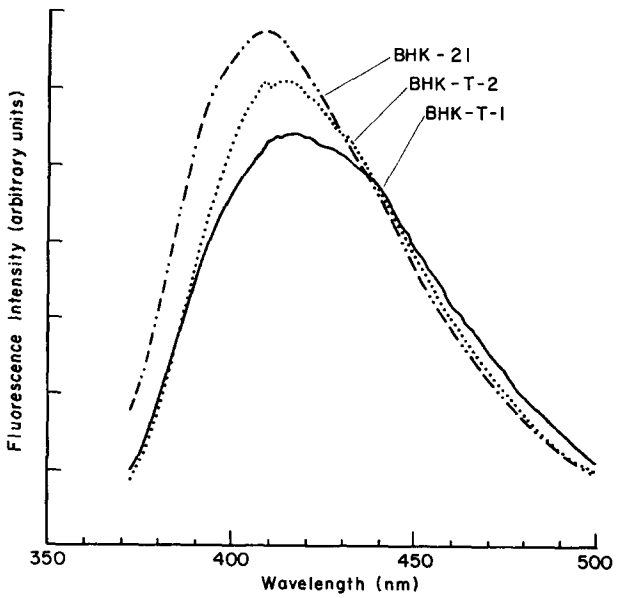

Fig. 1. The fluorescent spectra of *N*-phenyl 1-naphthylamine incorporated into various mammalian cells. Excitation wavelength approx. 310 nm.

TABLE I

THE MICROVISCOSITY OF CELLS WITH *N*-PHENYL 1-NAPHTHYLAMINE

Temperature	Microviscosity (cP)		
	BHK-21	BHK-T-1	BHK-T-2
23 °C	298	270	142
37 °C	209	168	147

different with regard to the wavelength position of the maximum for each cell line. The fluorescence of *N*-phenyl 1-naphthylamine in BHK-T-1 and BHK-T-2 shows a red shift with respect to BHK-21.

The microviscosity, η, determined by *N*-phenyl 1-naphthylamine in the different cell lines is shown in Table I. A progressive decrease in η is observed from BHK-21, to BHK-T-1, to BHK-T-2, at both 23 and 37 °C. The fluorescence lifetime of *N*-phenyl 1-naphthylamine in the cells was measured as 7.0 ns while the effective volume (V_0) and P_0 were measured using a viscous hydrocarbon as a solvent and were calculated to be 86 Å^3 and 0.24, respectively. The microviscosity was calculated from the Perrin equation:

$$\frac{(1/P)-1/3}{(1/P_0)-1/3} = 1+\frac{kT\tau}{\eta V_0}$$

where k is the Boltzman constant and T the temperature.

Pyrene. The fluorescence lifetimes of pyrene and pyrene butyric acid in the three cell lines and in simple solvents are shown in Table II. The lifetime (τ) of pyrene butyric acid was similar in all cells and in water. The τ values for pyrene were much lower in the cells compared to solution in cyclohexane or alcohol. The lowest τ of 150 ns was measured in BHK-T-1. The lifetimes were all measured at low pyrene concentrations under conditions where no excimers of pyrene formed.

Excimer fluorescence was observed at high pyrene concentrations and ratios of excimer to monomer intensities (I_E/I_M) are given for the three cell lines at 23 and 37 °C (Table III). At 37 °C the ratios I_E/I_M were similar for BHK-21 and BHK-T-2 and greater than those for BHK-T-1. The corresponding data at 23 °C were not identical to those at 37 °C. This may reflect changes in the rigidity of the membranes with temperature.

Quenching of pyrene fluorescence. The fluorescence of the probes, pyrene and

TABLE II

THE LIFETIMES (ns) OF VARIOUS PROBES IN MAMMALIAN CELLS, WATER AND CYCLOHEXANE AT 37 °C

	BHK-21	BHK-T-1	BHK-T-2	Water
Pyrene	173	150	193	450*
Pyrene butyric acid	127	128	122	140

* In cyclohexane

TABLE III

THE $I_{E_{472\ nm}}/I_{M_{390\ nm}}$ RATIO OF CELLS

Cell line	Temperature	
	23 °C	37 °C
BHK-21		
($8 \cdot 10^{-6}$ M)*	0.20	0.20
($4 \cdot 10^{-6}$ M)	0.12	0.19
BHK-T-1		
($8 \cdot 10^{-6}$ M)	0.14	0.14
($4 \cdot 10^{-6}$ M)	0.08	0.10
BHK-T-2		
($8 \cdot 10^{-6}$ M)	0.08	0.18
($4 \cdot 10^{-6}$ M)	0.08	0.135

* Concentration of pyrene.

pyrene butyric acid, in the cells was quenched by O_2, NaI, $TlNO_3$ and nitromethane. Table IV shows quenching data at 37 °C. The quenching rate constants were calculated from the rates of fluorescent decay in the presence of the quencher. Fig. 2 shows typical data for the rate of decay of fluorescence versus quencher concentration. As the quencher concentration is much larger than that of the fluorescing molecules, the slopes of the lines in Fig. 2 give the quenching rate constants.

The quenching rate constants "k_q" were lower in all cell lines compared to simple solution such as the probe and quencher in methanol or water. The difference k_q between cells and solution was not large for O_2. However, an order of magnitude difference in k_q was noted for cells compared to simple solvents for NaI, Tl^+ and nitromethane. It is noteworthy that for the last two quenchers, k_q is lower for BHK-T-1 than for cells BHK-21 or BHK-T-2. The quenching of pyrene fluorescence by I^- in BHK-T-2 appears to be anomalously high. No immediate explanation of this fact

TABLE IV

QUENCHING RATE CONSTANT ($10^9\ m^{-1} \cdot s^{-1}$) OF PYRENE PROBES AT 37 °C

		BHK-21	BHK-T-1	BHK-T-2	Methanol
Pyrene	O_2 (0–1.1 mM)	8.40	13.5	9.03	20.0
	NaI (0–20 mM)	0.24	0.17	1.01	4.5
	Nitromethane (0–3 mM)	0.46	0.26	0.41	20.0
	$TlNO_3$ (0–3 mM)	0.59	0.21	0.24	5.0
		BHK-21	BHK-T-1	BHK-T-2	Water
Pyrene butyric acid	O_2 (0–1.1 mM)	9.42	8.85	8.60	9.3
	NaI (0–20 mM)	0.68	0.23	0.40	5.2
	Nitromethane (0–3 mM)	0.77	0.31	0.62	26.0
	$TlNO_3$ (0–3 mM)	0.54	0.39	0.36	9.3

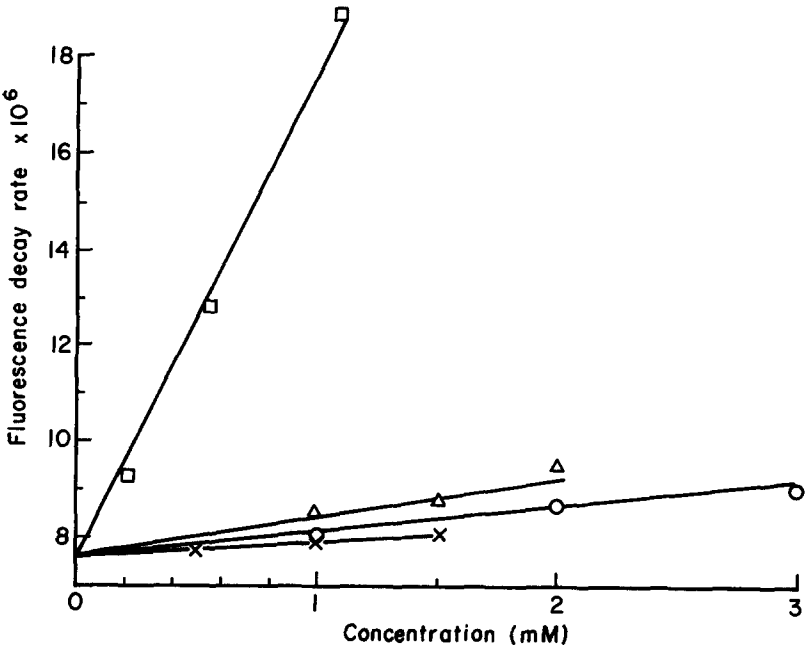

Fig. 2. Quenching rate plots for pyrene butyric acid-incorporated BHK-T-2 cells. □, oxygen; ×, thallium nitrate; △, nitromethane; ○, sodium iodide.

is available, the unusual effect may be due to the negative charge of the I^-. The experimental finding does illustrate further the differences in the cell membranes.

DISCUSSION

The probe *N*-phenyl 1-naphthylamine has been used to investigate membrane systems [20]. It is suggested that *N*-phenyl 1-naphthylamine resides in the hydrophobic region of the lipids. However, the polar nitrogen groups probably cause this probe to lie at an interface of the polar/non-polar region, with the hydrophobic aromatic naphthalene groups directed toward the non-polar region. A non-polar probe such as pyrene should lie further into the hydrophobic region of the lipid phase. The position of the fluorescence spectrum and the degree of polarization of the fluorescence give information about the environment of the *N*-phenyl 1-naphthylamine probe. The spectra in Fig. 1 shows that the fluorescence of *N*-phenyl 1-naphthylamine in BHK-T-1 and BHK-T-2 is red shifted with respect to the BHK-21 control cell line. This is indicative of a more polar environment of the probe in the transformed cells leading to increased solvation of the probe excited state causing a red shift of the fluorescence. The microviscosities experienced by the probe show that the environment of BHK-T-1 is less rigid than BHK-21, while BHK-T-2 has an even lower microviscosity than BHK-T-1. Other factors can influence the fluorescence spectrum of *N*-phenyl 1-naphthylamine. In particular, a change in the rigidity of the environment could lead to a spectral shift in the fluorescence. However, the fluorescence spectra are independent of temperature, but the rate of solvent relaxation

should vary with temperature. It is concluded that the relaxation of the *N*-phenyl 1-naphthylamine environment is not an important factor influencing the spectra in Fig. 1. The data show that the environment of *N*-phenyl 1-naphthylamine in each cell is different, the transformed cells, BHK-T-1 and BHK-T-2 being less rigid than BHK-21.

Pyrene can also give information on the degree of rigidity of the membrane. However, the pyrene environment is expected to be less polar than that of the *N*-phenyl 1-naphthylamine. The membrane will influence pyrene in a different way to *N*-phenyl 1-naphthylamine. The latter has a lifetime of 7 ns and little gross diffusion of the probe occurs during its lifetime. The lifetime of pyrene is longer (> 100 ns). Hence the pyrene may diffuse some considerable distance in the membrane structure during the experimental measurements. A summary of the rigidity of the environment visited by the excited pyrene is thus obtained with this probe.

The excited state of pyrene is quenched by many chemical groups and in particular by amino groups that exist in a membrane [18]. The fluorescence lifetime of pyrene thus depends on the environment, the availability of quenching groups, and on the ease of movement or rigidity of the membrane. Pyrene diffuses in the membrane and is eventually quenched. Hence, as shown in Table II, the τ of pyrene is much smaller in membranes compared to a simple solution. The fluorescence lifetime of pyrene has been successfully used to study the nature of membranes and micelles [14–18]. The probe pyrene butyric acid is less mobile in the membrane system due to the attachment of the -COOH groups to charged groups in the membrane. The τ values in simple solution and membranes are similar.

A trend is noted for the τ of pyrene in Table II which is also followed in Tables III and IV for the kinetic properties of pyrene. The BHK-T-1 cells have a lower τ than either BHK-21 or BHK-T-2. This indicates either a less rigid environment for the pyrene in BHK-T-1 leading to more diffusion and quenching, or to an increased presence of quenching groups in the vicinity of the pyrene.

At high concentrations, the pyrene τ is shortened by the interaction of the excited pyrene with ground state pyrene to form excimers. The degree of formation of excimers may be used with caution to get an estimate of the relative mobility of pyrene in the membrane [21–23]. If the pyrene concentration is low or diffusion restricted, then excimers are not readily formed. In Table III at 37 °C the relative excimer to monomer yield, I_E/I_M, is smallest for BHK-T-1 cells, indicating a more rigid environment for the pyrene in these cells. This does not contradict the *N*-phenyl 1-naphthylamine microviscosity data of Table I, which only measured the immediate *N*-phenyl 1-naphthylamine probe environment and no gross displacement of *N*-phenyl 1-naphthylamine need take place. The pyrene data monitors a large section of the membrane as the pyrene diffuses within the membrane.

The decreased mobility of pyrene in BHK-T-1 compared to BHK-21 and BHK-T-2 is also observed in the quenching data of Table IV. The quenchers, nitromethane, Tl^+ and I^-, move into the membrane to quench the pyrene singlet excited state. The lower k_q in the cells compared to simple solutions reflects the hinderance encountered by the quenchers due to the membrane. A lower k_q is indicative of a more restricted motion of quencher and probe. The lowest k_q is also found in BHK-T-1, indicating an increased restriction of movement in the membrane.

The *N*-phenyl 1-naphthylamine and pyrene data indicates that the membranes of BHK-T-1, BHK-T-2 and BHK-21 are different. The movement of molecules in the

membrane of BHK-T-1 is more restricted than in BHK-21 or BHK-T-2. This situation leads to lower quenching rates of pyrene excited states in BHK-T-1 compared to BHK-21 and BHK-T-2 as shown in Tables III and IV. The environment of the probes is quite different in all three cells. The *N*-phenyl 1-naphthylamine fluorescence indicates a more polar environment for the probe in BHK-T-1. The lower τ for pyrene also indicates the presence of increasing quenching groups, which could also lead to a more polar environment.

The quencher O_2 behaves in a similar fashion in all three cells. The k_q values are only slightly lower in cells compared to a simple solution. This is taken to indicate an easy access of O_2 into the cell membrane in the region of the probe. This may be understood as O_2 is a metabolite of the cell. However, the k_q values with the other quenchers for both pyrene and pyrene butyric acid are nearly always lower in BHK-T-1 and BHK-T-2 than in BHK-21, the exception being the iodide quenching of pyrene in BHK-T-2. Apparently, the access of these probes to the cell membrane is decreased after transformation.

It is instructive to consider the physical changes that have been reported to take place in the membranes of transformed cells. Fluorescence depolarization studies [24] suggest that the membranes of tumor cells are less rigid than those of their "normal" counterparts. The present *N*-phenyl 1-naphthylamine fluorescence depolarization studies agree with this concept. However, our data show that the long range mobility of molecules such as pyrene in membranes of transformed BHK-T-1 cells is significantly decreased. This may suggest that the membranes of transformed cells are organized differently than those of normal cells. A molecule is thus able to rotate more freely in the transformed cell membrane structure. However, movement within the membrane may be restricted due to an increased rigidity of the lipid chains and proteins. The environment within the membrane must then change upon transformation. The *N*-phenyl 1-naphthylamine fluorescence spectra in Fig. 1 indicate differences in the membranes of each cell.

A significant difference between membranes of normal and transformed cells is the increased mobility of the surface components [25]. Such an alteration may lead to a change in the degree of penetration of molecules into the cell membrane. Such changes are noted in the quenching data in Table IV for I^-, nitromethane and Tl^+ but not for the O_2 data. The data shows a decreased penetration into the transformed cell BHK-T-1 compared to BHK-T-2 and BHK-21. The cell BHK-T-2 is similar in kinetic properties to BHK-21. It is not possible from the data to correlate the observed changes with surface effects. However, external changes of the membrane as observed with pyrene τ, I_E/I_M ratio and *N*-phenyl 1-naphthylamine are in accord with the quenching data.

The fluidity of lipids was reported to be similar [26] in normal and transformed cells using spin-labelled fatty acids. This may indicate that the spin-labelled fatty acids investigate a different region of the membrane than the probe pyrene.

The *N*-phenyl 1-naphthylamine microviscosity measurements indicate a more fluid surface in cells that were transformed with Rous sarcoma virus compared to normal cells. Inasmuch as *N*-phenyl 1-naphthylamine measures microviscosity only in the immediate area of the probe, this increased fluidity may or may not be related to the mobility of glycoproteins of transformed cells when agglutinated with lectins. This increased fluidity may be related to the conformation of proteins and cell surface

related to biological differences of normal and transformed cells.

It is worth pointing out that both transformed cells BHK-T-1 and BHK-T-2 are equally adept at producing tumors in weanling hamsters. However, physical measurements show differences between the two transformed cell lines. It is thus with caution that a single physical measurement of cell membranes should be applied to objectively assess the tumorigenicity of cells. This may be particularly true when cells transformed in vitro are used.

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