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Determination of Microviscosity and Location of 1,3-Di(1-pyrenyl)propane in Brain Membranes

Jung-Sook Kang[1], In-Goo Kang[2] and Il Yun[2]

Departments of [1]Oral Biochemistry and Molecular Biology and [2]Dental Pharmacology and Biophysics, College of Dentistry and Research Institute for Oral Biotechnology, Pusan National University, Pusan 602-739, Korea

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We determined the microviscosity of synaptosomal plasma membrane vesicles (SPMV) isolated from bovine cerebral cortex and liposomes of total lipids (SPMTL) and phospholipids (SPMPL) extracted from SPMV. Changes in the microviscosity induced by the range and rate of lateral diffusion were measured by the intramolecular excimerization of 1,3-di(1-pyrenyl)-propane (Py-3-Py). The microviscosity values of the direct probe environment in SPMV, SPMTL and SPMPL were 38.17, 31.11 and 27.64 cP, respectively, at 37°C and the activation energies (E_a) of the excimer formation of Py-3-Py in SPMV, SPMTL and SPMPL were 8.236, 7.448 amd 7.025 kcal/mol, respectively. Probe location was measured by polarity and polarizability parameters of the probe Py-3-Py and probe analogues, pyrene, 1-pyrenenonanol and 1-pyrenemethyl-3β-hydroxy-22,23-bisnor-5-cholenate (PMC), incorporated into membranes or solubilized in reference solvents. There existed a good linear relationship between the first absorption peak of the ^1L_a band and the polarizability parameter $(n^2-1)/(2n^2+1)$. The calculated refractive index values for SPMV, SPMTL and SPMPL were close to 1.50, which is higher than that of liquid paraffin (n=1.475). The probe location was also determined by using a polarity parameter (f-1/2f'). Here $f=(\varepsilon-1)/(2\varepsilon+1)$ is the dielectric constant function and $f'=(n^2-1)/(2n^2+1)$ is the refractive index function. A correlation existed between the monomer fluorescence intensity ratio and the solvent polarity parameter. The probes incorporated in SPMV, SPMTL, and SPMPL report a polarity value close to that of 1-hexanol (ε=13.29). In conclusion, Py-3-Py is located completely inside the membrane, not in the very hydrophobic core, but displaced toward the polar head groups of phospholipid molecules, e.g., central methylene region of aliphatic chains of phospholipid molecules.

Key words : Microviscosities, Activation energies, Fluorescent probe technique, Brain membranes, Probe location, Polarity parameter, Polarizability parameter

INTRODUCTION

The intramolecular excimerization of 1,3-di(1-pyrenyl)-propane (Py-3-Py) has been successfully used to quantitate membrane fluidity induced by the range and rate of lateral diffusion within native and model membranes (Kang *et al.*, 1996; Kang and Yun, 1994; Yun *et al.*, 1994; Chung *et al.*, 1993; Kang *et al.*, 1992; Schachter, 1984; Melnick *et al.*, 1981; Zachariasse *et al.*, 1980). Using this probe, one monitors emission of both the monomer (I) and the excimer (I') components so that a ratio can be derived and used as a measure of membrane fluidity. As probe mobility within membranes increases, emission from the excimer predominates since formation of the intramolecular excimer is dependent upon lateral movement of its two components. The excimer fluorescence technique of Py-3-Py has an advantage over its counterpart based on intermolecular excimerization because very small probe concentrations can be employed ($<5\times10^{-7}$ M) and perturbations of the membrane by the probe molecule are minimized. However, the fluorescence spectroscopic method is an indirect way of measuring membrane fluidity. Although the localization of the probe molecule should be clearly established, the exact location of Py-3-Py has not yet been known.

In this paper, we determined the location of Py-3-Py in synaptosomal plasma membrane vesicles (SPMV) isolated from bovine cerebral cortex and liposomes of total lipids (SPMTL) and phospholipids (SPMPL) extracted from SPMV. We also measured the microviscosity and the activation energy of excimer formation of Py-3-Py in the same membranes. Changes in the microviscosity were measured by the

Correspondence to: Jung-Sook Kang, Department of Oral Biochemistry and Molecular Biology, College of Dentistry, Pusan National University, Pusan 602-739, Korea

intramolecular excimer formation of Py-3-Py.

MATERIALS AND METHODS

Materials

Py-3-Py, pyrene, 1-pyrenenonanol and 1-pyrenemethyl-3β-hydroxy-22,23-bisnor-5-cholenate (PMC) were purchased from Molecular Probes (Junction City, OR, USA) and liquid paraffin was obtained from Shinyo Pure Chemicals Co., Ltd. (Osaka, Japan). Liqiud paraffin was purified chromatographically over Al_2O_3 as a (1:1) mixture with *n*-hexane. The *n*-hexane was removed from the mixture by distillation. The liquid paraffin was subsequently distilled under high vacuum. All solvents and other reagents were of the highest quality and water was deionized.

Membrane preparations

The SPMV were isolated from fresh bovine cerebral cortex and characterized by the formerly reported method in our laboratory (Yun *et al.*, 1990). The purity of SPMV was determined by enzymatic and morphological standards. Lipids were extracted from the SPMV as described earlier (Yun and Kang, 1990). Phospholipids were quantitated by measuring the amounts of inorganic phosphate (Bartlett, 1959) after hydrolysis at 180°C in 70% $HClO_4$ (Madeira and Antunes-Madeira, 1976). The liposomes were prepared and separated by the procedure of Melnick *et al.* (1981). The membranes were suspended in 0.1 M KCl/10 mM Tris-HCl (pH 7.4) to a concentration of 0.70 mg of phospholipids/ml.

Viscosity determinations

The kinematic viscosity of liquid paraffin has been determined with Physica-Rheometer (Physica). Dynamic viscosities of liquid paraffin were calculated by multiplying kinematic viscosity by density and densities were measured with Westphal balance.

Fluorescence measurements

The incorporatrion of Py-3-Py was carried out by adding aliquots of a stock solution of 5×10^{-5} M in absolute ethanol to the membranes, so that the final probe concentration was less than 5×10^{-7} M. The mixtures were initially vigorously vortexed for 10s at room temperature and then incubated at 4°C for 18 hr under gentle stirring. After incorporation of the probe, the membrane suspension was placed in cuvettes. Blanks, prepared under identical conditions without Py-3-Py, served as controls for the fluorometric measurements. The measurements were carried out with an SPF-500C spectrofluorometer (SLM Aminco Instruments, Urbana, IL, USA). The excitation wavelength was 330 nm. The excimer to monomer fluorescence intensity ratio (I'/I) was calculated by the ratio of signals at 480 nm to signals at 379 nm.

Determination of activation energies (E_a)

Activation energies (E_a) were determined from the slopes of Arrhenius plots as follows:

$$\log\frac{I'}{I}=\frac{E_a}{R\times2.303\times1000}\quad\frac{1000}{T}+\log A$$

where R is gas constant, T is absolute temperature, A is a constant for excimerization reaction and I'/I is the excimer to monomer fluorescence intensity ratio of Py-3-Py.

Determination of the location of Py-3-Py

Probe location was measured by polarity and polarizability parameters of the probe Py-3-Py and probe analogues, pyrene, 1-pyrenenonanol and PMC, incorporated into membranes or solubilized in reference solvents. The following solvents were used: methanol, acetone, ethanol, diisopropylether, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, ethyleneglycol, toluene, pyridine, acetonitrile, *n*-hexane, di(*n*-butyl) ether, methylcyclohexane, 1,4-dioxane, *n*-hexadecane, 1,3-propanediol, dimethylsulfoxide, liquid paraffin, chlorobenzene, benzonitrile and glycerol.

RESULTS AND DISCUSSION

Temperature dependence of microviscosity in membranes

Py-3-Py is well suited for intramolecular excimer studies such as here in biomembranes, because of its large quantum yield of excimer fluorescence and the fact that no photochemical reactions have been found to occur upon excimer formation (Kang *et al.*, 1996; Kang and Yun, 1994; Yun *et al.*, 1994; Chung *et al.*, 1993; Kang *et al.*, 1992; Schachter, 1984; Melnick *et al.*, 1981; Zachariasse *et al.*, 1980). Although we realized that an isotropic solvent is not equivalent to the anisotropic medium of membranes, we studied intramolecular excimer formation of Py-3-Py in liquid paraffin as a function of temperature. The present methodology could be used since the fluorescence spectra of Py-3-Py in membranes are identical to those in liquid paraffin. The excimer to monomer fluorescence intensity ratio of Py-3-Py in liquid paraffin, I'/I, has been plotted as a function of T/η (°K/cP). As shown in Fig. 1, a linear relationship exists between I'/I and T/η. This line served as a calibration curve in determining the microviscosity of the membranes.

Fig. 2 shows the temperature dependence of the microviscosity of the direct environment of Py-3-Py in

SPMV, SPMTL and SPMPL. In all membrane systems, the bilayer fluidity in terms of excimer to monomer fluorescence intensity ratio strongly increased with increasing temperature. Over the entire temperature range from 4 to 45°C, the value of I'/I was larger in SPMTL than in SPMV. Further, the I'/I value of the probe in SPMPL is again larger than in the two other membrane systems. In SPMV, the I'/I values ranged from 0.079 (575.99 cP) to 0.539 (29.06 cP) over the temperature range from 4 to 45°C. The value changed from 0.093 (346.57 cP) at 4°C to 0.631 (24.40 cP) at 45°C in SPMTL and it changed from 0.128 (173.65 cP) at 4°C to 0.699 (21.88 cP) at 46°C in SPMPL. At the physiological temperature (37°C), the I'/I values of Py-3-Py in SPMV, SPMTL and SPMPL were 0.415 (38.17 cP), 0.496 (31.11 cP) and 0.551 (27.64 cP), respectively.

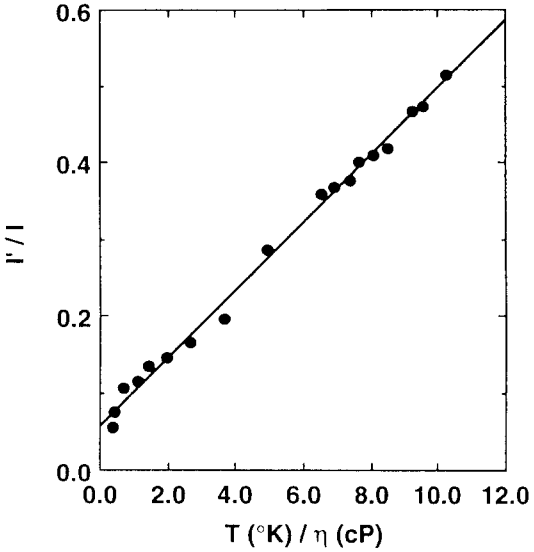

Fig. 1. Excimer to monomer fluorescence intensity ratio (I'/I) of 1,3-di(1-pyrenyl)propane (Py-3-Py) in liquid paraffin as a function of T/η. This line served as a standard curve in determining the microviscosity. Each point represents the mean of 5 determinations.

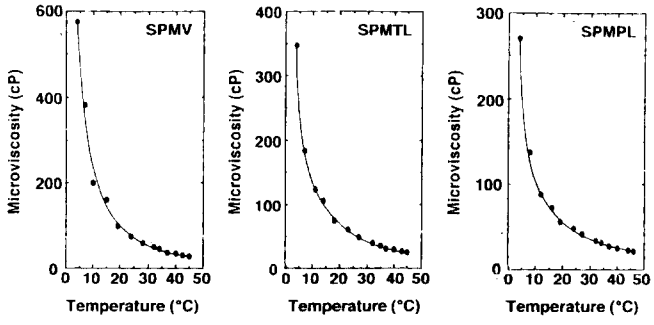

Fig. 2. The temperature dependence of the microviscosity of the direct environment of 1,3-di(1-pyrenyl)propane (Py-3-Py) in synaptosomal plasma membrane vesicles (SPMV) and liposomes of total lipids (SPMTL) and phospholipids (SPMPL) extracted from SPMV. Membranes were suspended in 0.1 M KCl/10 mM Tris-HCl (pH 7.4) and microviscosities were calculated from the standard curve. Each point represents the mean of 5 determinations.

Activation energies (Ea) of intramolecular excimerization of Py-3-Py

As shown in Fig. 3, the logarithms of the excimer to monomer fluorescence intensity ratio, I'/I, of Py-3-Py in liquid paraffin, SPMV, SPMTL and SPMPL were plotted as a function of the reciprocal absolute temperature. The activation energies (E_a) of the intramolecular excimer formation, determined from the slopes of the lines of Fig. 3, are given in Table I. Among the three membranes, E_a were the highest in SPMV and the lowest in SPMPL.

The Location of Py-3-Py in membranes: polarizability and polarity parameters

In order to determine the probe location in the

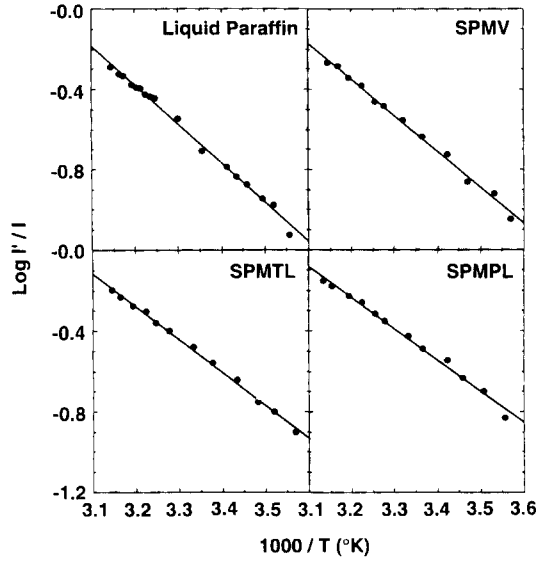

Fig. 3. A plot of I'/I against 1000/T in liquid paraffin, synaptosomal plasma membrane vesicles (SPMV) and liposomes of total lipids (SPMTL) and phospholipids (SPMPL) extracted from SPMV. Each point represents the mean of 5 determinations.

Table I. Slopes of Arrhenius plots and activation energies (E_a) for excimer formation of 1,3-di(1-pyrenyl)propane in liquid paraffin, synaptosomal plasma membrane vesicles (SPMV) and liposomes of total lipids (SPMTL) and phospholipids (SPMPL) extracted from SPMV

	Slope	E_a (kcal/mol)
Liquid paraffin	1.9443	8.897
SPMV	1.7999	8.236
SPMTL	1.6275	7.448
SPMPL	1.5352	7.025

Activation energies (E_a) were calculated from the slopes of the lines of Fig. 3. Values are represented as the mean of 5 determinations.

membranes, we measured spectral parameters of the probe Py-3-Py and probe analogues, pyrene, 1-pyrenenonanol and PMC, incorporated into membranes or solubilized in several reference solvents. It has been known that the spectral position of the first strong absorption peak (^1L_a band) (Fig. 4) responds mainly to the polarizability of the medium, i.e., to the refractive index, n (Platt, 1949). So, the energies of the first vibrational peak of the ^1L_a band in the absorption spectrum of Py-3-Py and its analogues, pyrene, 1-pyrenenonanol and PMC were plotted as a function of the polarizability parameter, $(n^2-1)/(2n^2+1)$ (Fig. 5). As shown in Fig. 5, there exists a good linear relationship between the first absorption peak of the ^1L_a band and the polarizability parameter. Although there was a little difference among each probes, the apparent polarizability, *i.e.*, the calculated refractive index values for SPMV, SPMTL and SPMPL, was about 1.50 (Table II), which was much higher than the values for methanol (1.327, No. 1), 1-hexanol (1.415, No. 8) and n-hexadecane (1.432, No. 17) and even liquid paraffin (1.475, No. 20). The high value for the apparent refractive index, n, in the membrane systems can result from the fact that the local density of the probe in the membrane is much higher than that encountered in appropriate homogeneous solvents. This would lead to an increase in the polarizability by increasing the electron density. Thus, it is suggested that the probes are located well inside the membrane, away from the aqueous interphase.

The probe location was also assessed by using a polarity-polarizability parameter, (f-1/2f'), where f=(ε-1)/(2ε+1) is the dielectric constant function and f'=$(n^2-1)/(2n^2+1)$ is the refractive index function. This study is

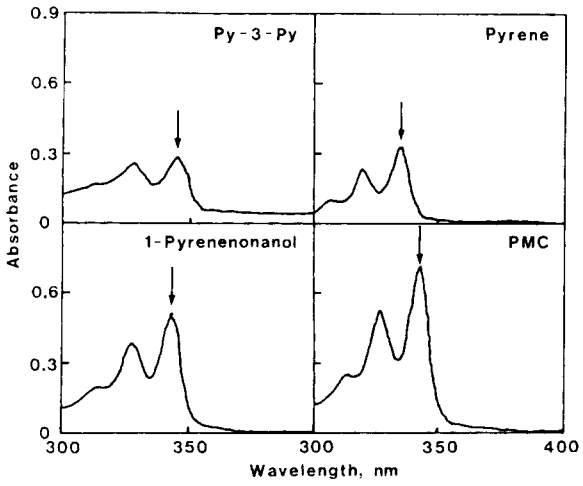

Fig. 4. Typical absorption spectra of 1,3-di(1-pyrenyl)propane (Py-3-Py), pyrene, 1-pyrenenonanol and 1-pyrenemethyl-3β-hydroxy-22,23-bisnor-5-cholenate (PMC) in di(n-butyl) ether.

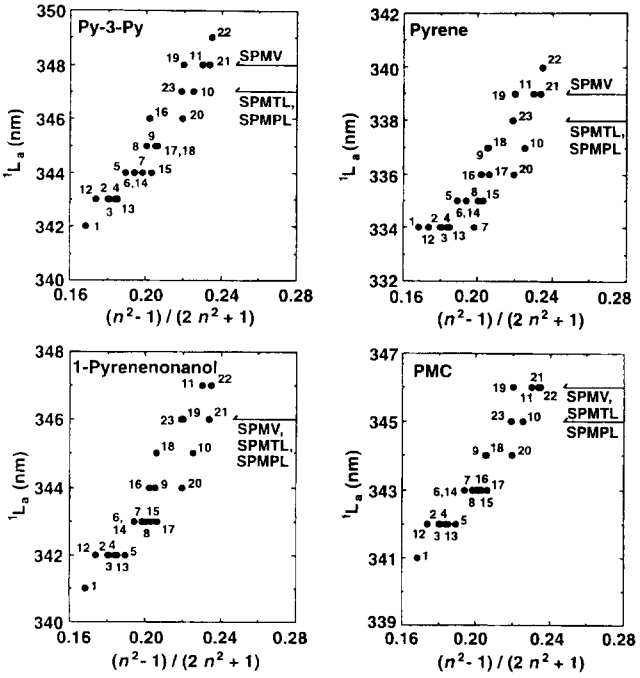

Fig. 5. The energy (in nm) of the first absorption peak of the ^1L_a transition in the spectrum of pyrene, 1-pyrenenonanol and 1-pyrenemethyl-3β-hydroxy-22,23-bisnor-5-cholenate (PMC) as a function of the solvent polarizability parameter f' =$(n^2-1)/(2n^2+1)$. The transition energies for the probe molecules incorporated into synaptosomal plasma membrane vesicles (SPMV) and liposomes of total lipids (SPMTL) and phospholipids (SPMPL) are indicated by line segments. Solvent symbols: 1, methanol; 2, acetone; 3, ethanol; 4, diisopropylether; 5, 1-propanol; 6, 1-butanol; 7, 1-pentanol; 8, 1-hexanol; 9, ethyleneglycol; 10, toluene; 11, pyridine; 12, acetonitrile; 13, n-hexane; 14, di(n-butyl)ether; 15, methylcyclohexane; 16, 1,4-dioxane; 17, n-hexadecane; 18, 1,3-propanediol; 19, dimethylsulfoxide; 20, liquid paraffin; 21, chlorobenzene; 22, benzonitrile; 23, glycerol. Each point represents the mean of 5 determinations.

Table II. Polarizability parameters and calculated refractive indexes of synaptosomal plasma membrane vesicles (SPMV) and liposomes of total lipids (SPMTL) and phospholipids (SPMPL) extracted from SPMV

Membrane	Polarizability parameter				Refractive index (n)			
	Py-3-Py	Pyrene	1-Pyrenenonanol	PMC	Py-3-Py	Pyrene	1-Pyrenenonanol	PMC
SPMV	0.232	0.235	0.229	0.235	1.517	1.528	1.506	1.528
SPMTL	0.222	0.224	0.229	0.235	1.483	1.490	1.506	1.528
SPMPL	0.222	0.224	0.229	0.222	1.483	1.490	1.506	1.483

Polarizability parameters were calculated from Fig. 5 and refractive indexes were calculated from the calculated polarizability parameters. Values are represented as the mean of 5 determinations.

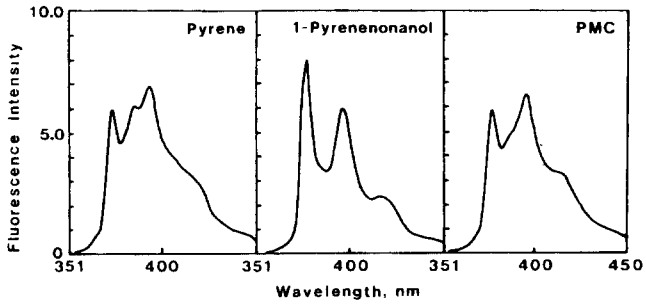

Fig. 6. Typical fluorescence spectra of pyrene, 1-pyrenenonanol and 1-pyrenemethyl-3β-hydroxy-22,23-bisnor-5-cholenate (PMC) in chlorobenzene.

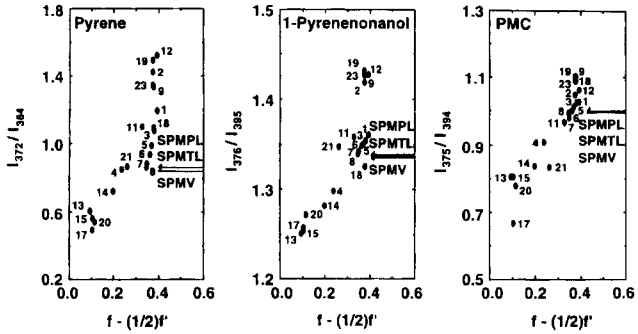

Fig. 7. The ratios of the fluorescence intensities of pyrene, 1-pyrenenonanol and 1-pyrenemethyl-3β-hydroxy-22,23-bisnor-5-cholenate (PMC) at 30°C as a function of the solvent polarity parameter. Here, f=(ε-1)/(2ε+1) is the dielectric constant function and f'=(n^2-1)/(2n^2+1) is the refractive index function. The ratios for the probe molecule incorporated into synaptosomal plasma membrane vesicles (SPMV) and liposomes of total lipids (SPMTL) and phospholipids (SPMPL) are indicated by line segments. Solvent symbols are as described in the legend to Fig. 5. Each point represents the mean of 5 determinations.

based on the dependence of the vibrational structure of the monomer fluorescence spectrum of pyrene and its analogues on the polarity of the surrounding medium (Kalyanasundaram and Thomas, 1977; Nakajima, 1973). Here we employed probe analogues such as pyrene, 1-pyrenenonanol, and PMC because Py-3-Py is almost insensitive to polarity. Typical fluorescence spectra of probe analogues in one of solvents are shown in Fig. 6. The ratios of the monomer fluorescence intensities of them are plotted versus solvent polarity parameter (Fig. 7). As illustrated in Fig. 7, a correlation existed between the monomer fluorescence intensity ratio and the polarity parameter in reference solvents. The probes incorporated in SPMV, SPMTL, and SPMPL report a polarity value close to that of 1-hexanol (ε=13.29, No. 8), which is more nonpolar than methanol (ε=32.61, No. 1) or ethanol (ε=24.29, No. 3) but much more polar than liquid paraffin (ε=2.21, No. 20). This suggests that the probe is located away from the aqueous phase in regions different from pure paraffinic environment. As compared with these probes, the dependence of the monomer fluorescence spectra on the solvent polarity, *i.e.*, the Ham effect (Kalyanasundaram and Thomas, 1977; Nakajima, 1973), is barely detectable with Py-3-Py; this fact makes this probe, Py-3-Py, more suitable for fluidity measurements, since I'/I ratios are not affected by the possible differences in polarity of different membrane systems.

In conclusion, from the absorption spectra as well as from the vibrational structure of the monomer fluorescence spectra, Py-3-Py is located completely inside the membrane, not in the very hydrophobic core, but displaced toward the polar head groups of phospholipid molecules, e.g., central methylene region of aliphatic chains of phospholipid molecules.

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