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ARTICLE *in* FOLIA MICROBIOLOGICA · FEBRUARY 1990

Impact Factor: 1 · DOI: 10.1007/BF02821406 · Source: PubMed

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Rotational Relaxation Rate of 1,6-Diphenyl-1,3,5-hexatriene in Cytoplasmic Membranes of *Bacillus subtilis*. A New Model of Heterogeneous Rotations

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Received August 9, 1989

ABSTRACT. The temperature dependence of fluorescence anisotropy, lifetime and differential tangent of 1,6-diphenyl-1,3,5-hexatriene (DPH) and its polar trimethylammonium derivative (TMA-DPH) were investigated in cytoplasmic membranes of *Bacillus subtilis*. The fluorescence parameters were compared in the two types of membranes prepared from bacteria cultivated at 20 and 40 °C. Steady-state anisotropy measurements showed that within a broad range of temperatures, membranes cultivated at 20 °C exhibit significantly lower values than those prepared from cells cultivated at 40 °C. The temperature dependence of lifetime and differential tangent measurements (differential polarized phase fluorimetry) were fully consistent with steady-state anisotropy data of both DPH and TMA-DPH. The low anisotropy values in the case of TMA-DPH could be explained by a shorter lifetime and higher temperature-induced decrease as compared with DPH. Surprisingly, the temperature dependence of rotational rate R calculated according to the model of hindered rotations (Lakowicz 1983) gave misleading results. When increasing the temperature from 5 to 25 °C, a marked drop of rotational relaxation rate was observed. The minimum R values were measured between 25 and 30 °C and further increase of temperature (up to 60 °C) was reflected as increase of the R values. Therefore, a new model of "heterogeneous rotations" was developed. This model assumes that even at low temperatures (approaching 0 °C) where the differential tangent reaches zero, a fraction of fast rotating molecules exists. The ratio between fast and slowly rotating molecules may be expressed by this model, the newly calculated rotational rates are fully consistent with anisotropy, lifetime and differential tangent measurements and represent the monotonically increasing function of temperature.

Different fluorescence parameters such as steady-state anisotropy, excited state lifetime, rotational rate and limiting anisotropy were used (Lenz *et al.* 1976a, *b*;

Shinitzki and Barenholz 1974; Shinitzky 1984; Lakowicz *et al.* 1979*a*, *b*) for estimation of temperature induced changes in biological membranes. Majority of such studies was oriented to artificial phospholipid vesicles and/or reconstituted systems with relatively well defined and simple composition of phospholipid membrane matrix (Lakowicz *et al.* 1979*a*, *b*, 1985; Faucon and Lakowicz 1987).

In this work an attempt was made to investigate complex biological membranes, the cytoplasmic membranes of *Bacillus subtilis*. The composition of bacterial cell membrane changes in response to environmental temperature. A decrease of cultivation temperature results in an increased proportion of branched and/or unsaturated fatty acids in the membrane and *vice versa* (Mendoza and Cronan 1983; McElhaney 1967; Butterworth and Bloch 1970; Thompson 1980; Russel 1984; Melchior 1982; Svobodová *et al.* 1988*c*). According to the principle of homeoviscous adaptation formulated by Sinensky (1974) and later modified by Cossins and Sinensky (1984), the composition of bacterial membranes is altered so that "membrane fluidity" remains at constant level. The efficiency or efficacy of this process may vary among various organisms and membrane types (Ray *at al.* 1971; Cossins 1977; Finne and Matches 1976; Yang and Haug 1979; Wakayama and Oshima 1978). Fluorescent probes were used for testing the validity of this principle as well as for the general characterization of the temperature-induced membrane phenomena (Shinitzky 1984). The majority of these studies were limited to steady-state fluorescence spectroscopy which does not permit a detailed description of membrane dynamics.

The aim of the present study was to apply the methodology of differential phase polarized fluorimetry (Weber 1977; Lakowicz and Prendergast 1978) for the study of temperature adaptation of *Bacillus subtilis* cell membranes. Two fluorescent probes 1,6-diphenyl-1,3,5-hexatriene, DPH (Shinitzky and Barenholz 1974, 1978) and its polar trimethylammonium derivative, TMA-DPH (Prendergast *et al.* 1981; Genz and Holzwarth 1985) were used to monitor two different membrane microenvironments – the inner, hydrophobic region of the hydrocarbon aliphatic chains (DPH) and the lipid–water interphase (TMA-DPH). It was found that the model of hindered rotations which is usually used for analysis of time dependent spectroscopy data is not applicable to this complex membrane system. A new model of "heterogeneous rotations" was therefore developed.

MATERIALS AND METHODS

Cultivation of bacteria and isolation of plasma membranes. Bacillus subtilis 168 *trp* cells were grown aerobically at 20 or 40 °C in complex medium containing (in g/L): Bacto beef extract (Difco) 1.5, Yeast extract (Difco) 1.5, NaCl 3.5, KH_2PO_4 1.32, Bacto peptone 5, glucose 5 (pH 7.0). Cultivation was terminated in mid-exponential phase and cells were harvested by rapid filtration through a Millipore filter

(pore size 0.6 μm, flow rate 40 mL/min). The material left on the filter was washed twice and suspended in phosphate buffer (0.1 mol/L, pH 7.0). Cytoplasmic membranes were prepared from cells cultivated at 20 or 40 °C according to Bohin *et al.* (1976). The final membrane suspension was suspended in phosphate buffer (0.1 mol/L) at 15–20 mg protein per mL and stored at −70 °C. Once-thawed membrane preparations only were used for fluorescence measurements. Protein was determined according to Lowry method.

Fluorescence measurements. The cytoplasmic membranes were labelled with DPH and TMA-PDH essentially in the same manner as described by Shinitzki and Barenholz (1974, 1978). A few microliters of DPH (1 mmol/L) in acetone or of TMA-DPH (1 mmol/L) in ethanol were added to a rapidly stirred membrane suspension in phosphate buffer (0.1 mol/L, pH 7.4, 50–100 μg protein per mL) to the final concentration 3 μmol/L (DPH) or 0.3 μmol/L (TMA-DPH). The incubation of membranes with the fluorescent probe was continued for 30 min at 40 °C or for 60 min at 20 °C.

Differential tangent, fluorescence lifetime and steady-state anisotropy were measured and calculated according to Lakowicz *et al.* (1979) using an SLM 4800S subnanosecond spectrofluorimeter equipped with Hewlett-Packard 85 computer. POPOP was used instead of glycogen as reference solution. For differential polarized phase and lifetime measurements, emission monochromator and cut-off filter were removed to obtain signals of maximum intensity. A constant 18 MHz modulation frequency was used throughout. Background fluorescence or scattered light of the control membrane suspensions (without fluorescent probe) did not exceed 2 % of the DPH or TMA-DPH labelled samples.

RESULTS AND DISCUSSION

The steady-state anisotropy r of DPH and TMA-DPH incorporated into cytoplasmic membranes (cultivated at 20 or 40 °C) was measured between 5 and 60 °C. Fig. 1 shows, that within the whole range of temperatures (T_m), membranes prepared from "20 °C cells" exhibit significantly lower r values than those from "40 °C cells". This finding applies to DPH as well as to TMA-DPH.

It is also shown in Fig. 1 that membranes prepared from "20 °C cells" exhibit at 20 °C significantly higher anisotropy values than those cultivated and measured at 40 °C. Thus, comparison of the two types of membranes at T_m identical with the temperature of cultivation T_c ($T_m = T_c = 20$ °C *versus* $T_m = T_c = 40$ °C) shows that efficiency or efficacy of compensatory processes such as homeoviscous adaptation (Cossins and Sinensky 1984) is rather low in *B. subtilis*.

The temperature-induced decrease of DPH anisotropy is much greater than that of TMA-DPH. At low temperatures (5–15 °C), the absolute value of DPH

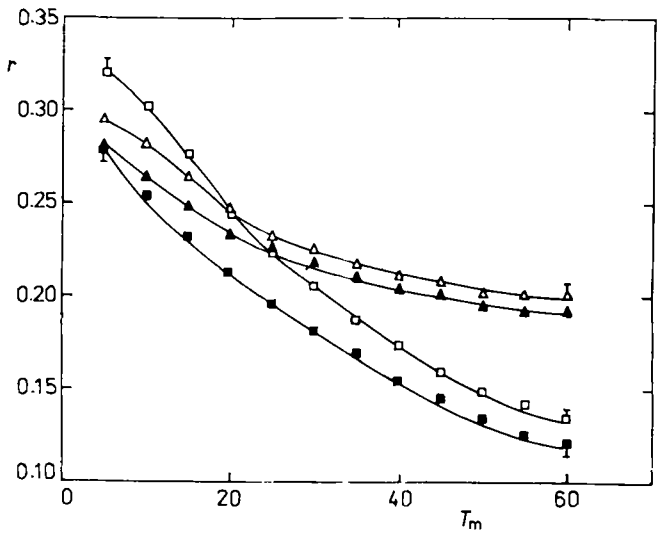

Fig. 1. Steady-state anisotropy r of DPH and TMA-DPH. Cytoplasmic membranes prepared from cells cultivated at T_c = 20 °C (*closed symbols*) or 40 °C (*open symbols*) were labeled with fluorescent probes (DPH = *squares*, TMA-DPH = *triangles*). Steady-state anisotropy r was measured as function of temperature of measurement T_m (°C) in 5 independent membrane preparations. The error bars show the uncertainty in r resulting from ±0.01 in r. This error is relatively constant across the r temperature profile.

anisotropy is slightly higher than that of TMA-DPH. When increasing the temperature above 20 °C, DPH anisotropy sharply decreases while TMA-DPH remains almost at a constant level. Thus, the ratio between the two probes is reversed at about 20 °C and when increasing T_m further, TMA-DPH anisotropy is much greater than that of DPH.

An unequivocal interpretation of these data cannot be offered because the steady-state anisotropies of DPH and TMA-DPH depend on both fluorescence lifetime and rotational rate. Thus, the different topological orientation of these two probes within the membrane (Prendergast *et al.* 1981; Genz and Holzwarth 1985) can not be considered and discussed. Therefore, in the next part of our work, the DPH and TMA-DPH fluorescence lifetimes and rotational rates were measured and calculated with the help of differential polarized phase fluorimetry.

Fluorescence lifetime measurements were carried out, similarly as in the previous section, in cytoplasmic membranes isolated from *B. subtilis* cells cultivated at 20 or 40 °C (see Fig. 2). The two types of membranes (T_c = 20 °C *versus* T_c = 40 °C) appeared identical when the lifetimes of a given probe (DPH or TMA-DPH) were compared over a wide range of T_m. Some minor difference was only found for TMA-DPH between 5 and 20 °C. In this case, membranes from "40 °C cells" exhibited slightly higher values.

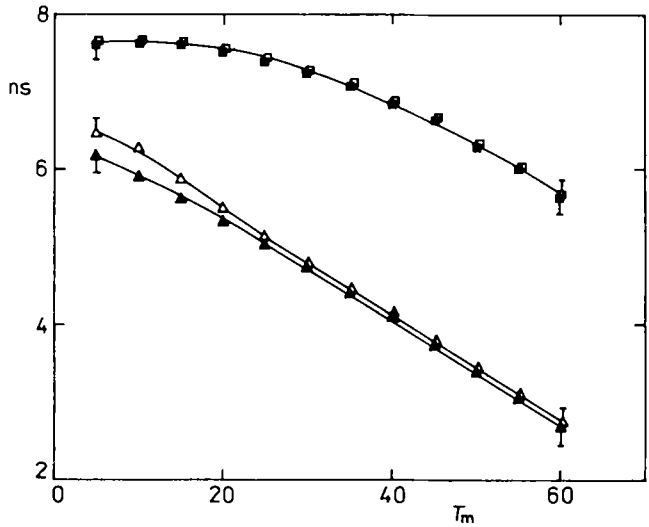

Fig. 2. Fluorescence lifetime (ns) as a function of temperature of measurement T_m (°C) for cytoplasmic membranes. DPH = *squares*, TMA-DPH = *triangles*, *open* (*closed*) *symbols* represent cells cultivated at 40 (20) °C. Lifetime measurements were carried out on the SLM 4800S spectrofluorimeter. The data represent the average of 4 independent experiments, the accuracy of measurements was ±0.1 ns.

At all temperatures of measurement, TMA-DPH had much shorter lifetime than DPH and its temperature-induced decrease (when increasing T_m) was two-fold larger than that of DPH. This finding may account for the difference in steady-state anisotropy between the two probes (see Fig. 1).

Similarly as in earlier literature data on phospholipid membrane systems (Lakowicz 1983), the DPH as well as TMA-DPH lifetimes determined by amplitude modulation were not equal with those determined by phase fluorimetry. Such findings could be expected and indicated lifetime heterogeneity in the given sample. The data shown in Fig. 2 represent therefore average lifetime determined by phase or modulation fluorimetry.

The cytoplasmic membranes were further analyzed by differential phase polarized measurements (Fig. 3). It was found that differential tangents of DPH and TMA-DPH fluorescence signals were lower in membranes from "40 °C cells" than in membranes prepared from cells cultivated at 20 °C. As the lifetime values for the 20 and 40 °C samples are the same for a given probe, it is logical to suppose that different rotational rates exist in these two types of membranes – *i.e.* higher in T_c = 20 °C membranes. Such result would confirm the steady-state anisotropy data obtained before (Fig. 1).

It can also be seen in Fig. 3, that the differential tangent reaches a maximum within the measured temperature range at about 30 °C. In the case of free rotation of the

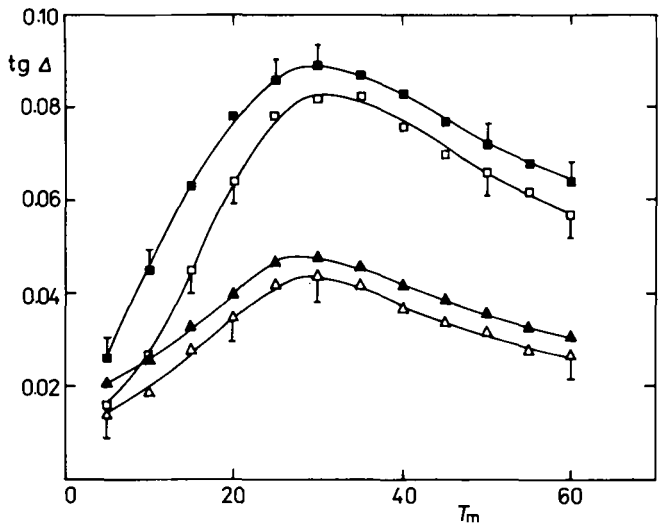

Fig. 3. Differential tangent ($tg\Delta$) for DPH and TMA-DPH fluorescence as function of temperature T_m (°C). The symbols and legend are otherwise identical with those of Figs 1 and 2, the values of differential tangent were precise to ±0.005 at 18 MHz.

probe, this maximum $tg\ \Delta$ may be expressed according to Lakowicz (1983) as the function of lifetime τ, modulation frequency ω and anisotropy r_0:

$$tg_{max}\Delta = 3\omega\tau r_0/\{2 + r_0 + 2[(1 + 2r_0)\,(1 - r_0)\,(1 + \omega^2\tau^2)]^{1/2}\} \qquad (1)$$

The maximum values of $tg\ \Delta$ obtained in our experiments (Fig. 3) were much lower than those calculated according to eq. (1) for a given lifetime and given modulation frequency. The value of the frozen solution anisotropy $r_0 = 0.4$ is known for both probes (Lakowicz *et al.* 1979; Prendergast *et al.* 1981). Under such conditions it is usual to apply the model of hindered rotations (Weber 1977; Lakowicz 1979*a*, *b*) for the expression of $tg\ \Delta$ and calculation of rotational rates.

Using the equations of the hindered rotations model, the temperature behavior of the rotational rate R and of limiting anisotropy r were evaluated from original experimental data (measured anisotropy, lifetime and $r_0 = 0.4$). The results of such computations are shown in Fig. 4. It was found that as the temperature of the sample T_m decreases from 60 to 25 °C, the calculated values of R are also decreased. However, a further decrease of temperature from 25 to 0 °C is reflected in a marked increase of R. Such a behavior of calculated R values cannot be accepted and has to be considered as artificial because under no conditions can the mobility of a probe (membrane fluidity) increase with decreasing temperature.

The reason why this is so may be arrived at by the following argument. Using the hindered rotation model at temperatures approaching 0 °C, the differential tangent must approach zero while the steady-state anisotropy r should be close to $r_0 = 0.4$. In our experiments, however, r never exceeded 0.33. It was carefully checked that

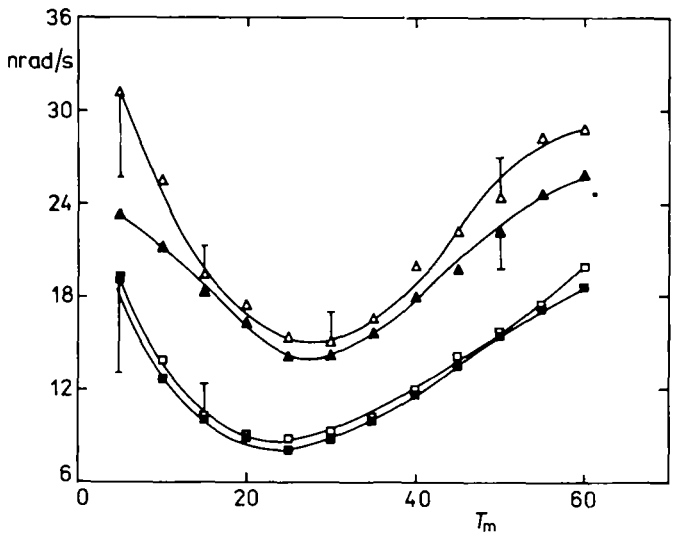

Fig. 4. Rotational rates (nrad/s) of DPH and TMA-DPH calculated according to the model of hindered rotations. Cytoplasmic membranes isolated from cells grown at 40 °C (*open symbols*) or 20 °C (*closed symbols*) were labeled with fluorescent probes. The error bars of the calculated values of *R* result mainly from errors of the differential tangent (±0.005) and, to a lesser extent, from the variation of lifetimes (±0.1 ns). The coefficient of variation is 10 % between 15 and 60 °C, at lower temperatures it may be as high as 30 %.

depolarization due to light scattering in a membrane suspension nor the energy--transfer were responsible for this decrease of *r*.

Thus, the unusually low *r* at temperatures close to 0 °C has to be regarded as a correct value and the theory of hindered rotations itself must be changed to correspond to the measured data. Faucon and Lakowicz (1987) faced similar problems when measuring the effect of mellitin on rotational rates and lifetimes of DPH incorporated in phospholipid vesicles by multifrequency phase and modulation fluorimetry. They found that rotational rates could differ markedly in a sample at a given temperature and the hindered rotation model was inadequate for the calculation of *R*.

In trying to formulate a new model for the behavior of DPH or TMA-DPH in complex membrane systems, let us suppose that even at low temperatures, some (minor) fraction of fast rotating molecules exists. Simultaneously, the major part of the probe molecules exhibit some mean rotation rate which continuously increases with temperature. The behavior of this second, mean population explains why the differential tangent has a maximum at about 30 °C. The second, alternative assumption is that the "highly mobile component" at low temperatures is represented by a limited (small) angle where all molecules can exhibit fast rotation. Under such conditions all probe molecules would behave in an identical manner when the temperature is decreased.

Thus, the the measured data may be better interpreted by the model of "heterogeneous rotations" which allows for at least two different values for rotation rates – the fast $R_f \gg 1/6\ \tau$, and the medium R.

The anisotropy response to δ-excitation will be:

$$r(t) = r_0[(1 - Y) \exp(-6R_f t) + Y \exp(-6Rt)] \tag{2}$$

where R_f and R are the fast and medium rotation rates introduced by heterogenous rotations model. The parameter Y ($0 < Y < 1$) determines the proportion between slow and fast rotations. If some fraction of the probe molecules rotate fast, then the Y is simply the number of slowly rotating molecules divided by the total number of the probe molecules.

In the second interpretation where every probe molecule can rotate fast (R_f) but only within a narrow angle around some axis which, simultaneously, can also rotate at a low rotation rate R, then $Y = r_{f\infty}/r_0$, where $r_{f\infty}$ is the limiting anisotropy of this fast hindered rotation. These two ideas represent two opposite situations.

In the first situation, the $(1 - Y)$ part of the molecules have $r_{f\infty} = 0$ and remaining Y part have $r_{f\infty} = r_0$.

In the second situation, all the molecules have the same $r_{f\infty} = Yr_0$. What may exist in biological membranes *in situ* is probably some intermediate situation when all probe molecules exhibit some continuous spectrum of $r_{f\infty}$ from 0 to r_0, where Y is the average $r_{f\infty}$ divided by r_0.

The model of heterogeneous rotations has three unknown parameters – Y, R_f and R. To evaluate all three, the results of at least three independent measurements related to the probe rotation are needed. Unfortunately, only two such parameters could be measured here, the steady-state anisotropy r and the differential tangent $tg\ \Delta$ determined at a single modulation frequency of $f = 18$ MHz. It should be mentioned in this context that repetition of the measurements at different frequencies available with the SLM 4800S spectrofluorimeter (*viz.* 6 or 30 MHz) would not improve the situation because a much higher frequency would be needed for the evaluation of the high-rotation rate component R_f. Therefore, to be able to evaluate at least two parameters, namely Y and R from the experimental data, it is necessary to take $R_f = \infty$. This is the main difference from the hindered rotation model (Lakowicz 1979*a*, *b*) mentioned above, which takes $R = 0$.

The measured values of r and $tg\ \Delta$ in the case of infinite R_f are functions of the unknown parameters R and Y:

$$r = Yr_0/(1 + 6R\tau) \tag{3}$$

$$tg\Delta = 3\omega\tau \cdot 6R\tau \cdot Yr_0/[(1 + 2Yr_0)(1 - Yr_0)(1 + \omega^2\tau^2) + (2 + Yr_0)\,6R\tau + (6R\tau)^2] \tag{4}$$

Eqs (3) and (4) can be solved with respect to the unknown parameters R and Y.

The simplest way to do this is to solve the equivalent square equation:

$$AX^2 + BX + C = 0 \tag{5}$$

where

$$A = [1 + r - 2r^2(1 + \omega^2\tau^2)]\ tg\Delta - 3\omega\tau r \tag{6}$$

$$B = [2 + r + r(1 - 4r)\ (1 + \omega^2\tau^2)]\ tg\Delta - 3\omega\tau r \tag{7}$$

$$C = (1 + 2r)\ (1 - r)\ (1 + \omega^2\tau^2)\ tg\Delta \tag{8}$$

Then R and Y may be determined as:

$$R = X/6\tau \qquad \text{and} \qquad Y = r/r_0 \cdot (1 + X) \tag{9}$$

Eq. (5) yields two different solutions. To select the correct solution it is necessary to determine the temperature dependence of R and Y for each solution. In the case of the correct solution the R values must decrease with decreasing temperature and Y will remain between 0 and 1. In the case of the false solution both R and Y values increase with decreasing temperature and at low temperatures (approaching 0 °C) the Y will exceed 1.

The temperature behavior of R and Y evaluated from the experimental data and calculated according to the correct solution of eq. (5) are shown in Figs 5 and 6.

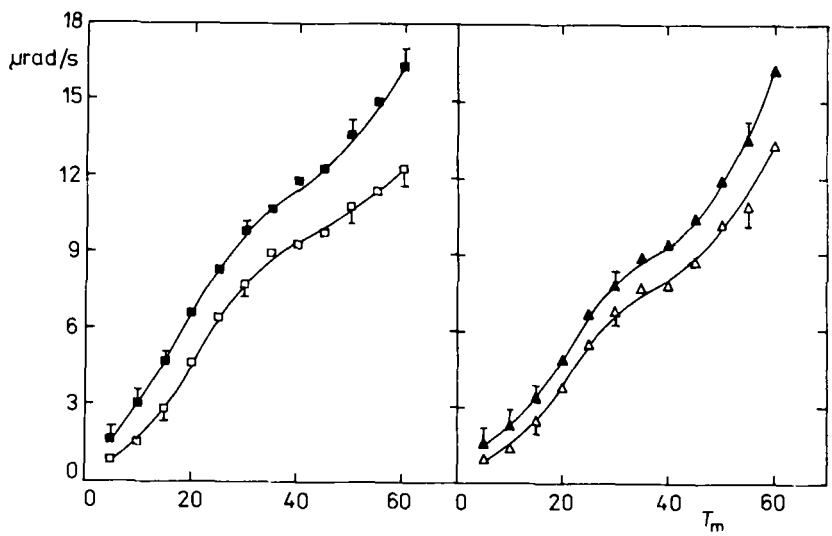

Fig. 5. Rotational rate (μrad/s) calculated according to the model of heterogeneous rotations. The newly formulated model was used for analysis of the DPH (*squares*) and TMA-DPH (*triangles*) fluorescence signals. The data were expressed as a function of increasing T_m (°C) and compared with R determined by the hindered rotation model. The variation coefficient of R is 5 % at T_m = 15–60 °C, and 10 % at T_m = 5–10 °C. The main source of error results from the error of the differential tangent ±0.005.

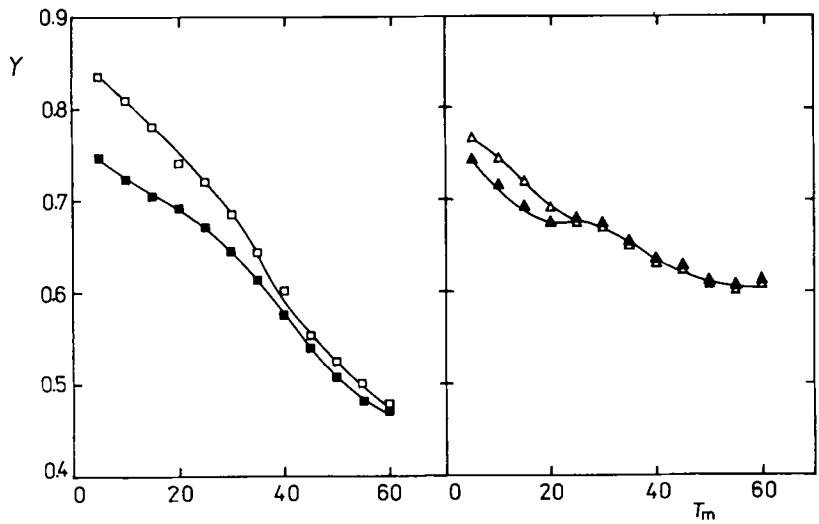

Fig. 6. The *Y* parameter as a function of temperature T_m (°C). The theory of heterogeneous rotations formulated in this work was applied for the analysis of DPH (*squares*) and TMA-DPH (*triangles*) fluorescence. The *Y* values are relatively insensitive to random errors of steady-state anisotropy *r*, lifetime τ and differential tangent $tg\Delta$, the coefficient of variation was always less than 1.5 %.

Similarly as in previous Figs 1–3, the DPH and TMA–DPH fluorescence was analyzed in membranes from cells cultivated at 20 and 40 °C.

Before describing the results demonstrated in Figs 5 and 6 it is important to understand which property of the membrane is represented by each of these parameters. A decrease of *Y* means a higher number of fast rotating molecules or that a wider angle within the fluorescent probe molecule can rotate fast. Low *Y* values could be also described as a low density of packing of membrane components (low order?).

The *R* parameter represents the rotation rate of the slowly rotating component or (more complicated interpretation) the slow rotation of the axes around which the probe can rotate fast within a limited (small) angle. It is not known whether these axes rotate because the neighboring phospholipid molecules rotate together with the fluorescent probe or whether the probe moves in cavities formed in the surrounding lipid molecules. In either case, however, the faster the phospholipid motion the higher the *R* values that result. In other words, the *R* parameter corresponds to "membrane fluidity" and must increase with increasing temperature.

The results shown in Figs 5 and 6 are fully compatible with these qualitative characteristics of *Y* and *R* parameters. The *Y* values calculated for DPH and TMA-DPH monotonically decrease as temperature T_m increases (Fig. 5). Such a finding may be interpreted as an increase of the number of fast rotating molecules or a decrease in the density of packing of membrane components. Simultaneously,

the rate of the slowly rotating population of DPH or TMA-DPH molecules, parameter R, is increased when T_m increases from 5 to 60 °C (Fig. 6).

Therefore, the "new" rotational relaxation rate calculated according to the model of heterogeneous rotations is fully comparible with the general rules of temperature behavior of biological membranes.

It can also be unequivocally concluded from the results presented in Figs 5 and 6 that membranes of cells cultivated at T_c = 20 °C and T_c = 40 °C are not the same. Small but significant differences exist between these membranes within the whole range of T_m studied. The question why the Y parameter is higher in membranes of cells cultivated at 40 °C than in T_c = 20 °C membranes, while the opposite situation is observed in the case of the R parameter, is difficult to answer. It may be suggested that membranes of cells cultivated at 20 °C exhibit higher R values simply because some homeoviscous adaptation of low efficiency takes place. *B. subtilis* cells try to compensate the physical rigidization of their membranes, induced by a decrease of environmental temperature T_c, by increasing the intrinsic fluidity. Such an interpretation would support our previous data using steady-state fluorescence anisotropy of DPH (Svobodová 1988*b*, *c*).

Membranes of cells cultivated at 20 °C contain phospholipids with predominantly branched fatty acids (Butterworth and Bloch 1970; Svobodová *et al.* 1988*c*). The increased proportion of branched and/or unsaturated aliphatic chains will decrease the order (density of packing) of membrane molecules, which may be sensed, in turn, by the fluorescent probes. Thus, the number of fast rotating DPH and TMA-DPH molecules (Y parameter, see Fig. 5) in such "disordered" membranes is higher than in membranes of cells cultivated at 40 °C. It is also shown in Figs 5 and 6, that the difference between T_c = 40 °C and T_c = 20 °C membranes is much higher for DPH than for TMA-DPH. This finding supports the above interpretation because DPH molecules dissolve mainly in the hydrophobic core of the membrane (Shinitzky 1984) where aliphatic fatty acid chains are present.

It may be therefore concluded that the new model of heterogeneous rotations which was developed here for analysis of differential polarized phase fluorimetry of DPH and TMA-DPH in complex biological membranes is consistent with the temperature behavior of cytoplasmic membranes of *B. subtilis*.

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