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Lipophilic Interaction between Thylakoid Membranes and Aliphatic Compounds

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Abstract

Spinach chloroplasts pre-incubated at various temperatures, changed their photosynthetic activities (measured at 15° C) as the incubation temperature was raised; these activities showed characteristic activity-temperature profiles as follows:

1. The profiles were shifted to lower temperatures if various aliphatic compounds were present in the incubation mixture.
2. In general, the extent of the shift was proportional to the product of the concentration and the partition coefficient of the compound, i.e. to the concentration of the compound partitioned in the lipophilic chloroplast phase.

The combined effects of heat and the aliphatic compounds tested indicated that the lipophilic groups in these compounds play a determinative role in the heat denaturation of thylakoids. The consequence of such structure alterations is inactivation of photosynthetic functions.

The lipophilic properties of the spinach thylakoid membrane as compared with certain artificial and natural membranes are described.

Introduction

It was reported [1] that the electron-transport activity (at 15° C with ferricyanide as an electron acceptor) in isolated spinach chloroplasts changed after the chloroplasts had been incubated for 5 min at various temperatures. It was slightly depressed by incubation up to about 25° C, then increasingly enhanced up to about 42.5° C and rapidly diminished at higher temperatures and was abolished around 55° C. The maximum level was reached at an incubation temperature, *Tmax*, of about 42.5° C.

In contrast to the above observations, two other characteristics of membrane function diminished monophasically and simultaneously in the temperature range between 25 and 42.5°C and were completely lost around *Tmax* [1, 2]. These two functions were photophosphorylation and the intactness of the thylakoid membrane which was indicated by the light-scattering response and the light-induced pH shift. In addition, incubation at temperatures above *Tmax* appeared to cause some drastic alteration in the membrane structure and the oxygen-evolving system [3, 4] as suggested by a decrease in fluorescence intensity [1, 5] and a fragmentation of chloroplasts [2].

When aliphatic alcohols were included during 5 min incubation at various temperatures, a parallel shift towards lower temperatures was observed in the activity-temperature profiles of both electron-transport and phosphorylation [6, 7]. Neither addition of alcohols after incubation nor transient exposure to alcohol before incubation was effective in shifting the profiles. The change in *Tmax*, $\Delta Tmax$, was used as a measure of effectiveness of these compounds in altering the chloroplast properties. The $\Delta Tmax$ increased as the concentration of the alcohol or the length of the hydrocarbon chain of the alcohols in equimolar concentration increased. The concentration of an alcohol to give a $\Delta Tmax$ of 5°, $C_{5°}$, and the partition coefficient of the alcohol between water and l-octanol, *P*, were shown [6] to obey the relation [8, 9], $\log(1/C_{5°}) = a \log P + b$, where a and b were constants. This suggested that an interaction between the hydrocarbon segments of the alcohols and the lipophilic structure [7] of thylakoids resulted in the changes in photosynthetic activities under the influence of heat.

It was also reported [7] that when alkylalkanoates were mixed into a spinach chloroplast suspension by vigorous shaking at 5°C, these molecules presumably penetrated into the thylakoid membranes and apparently caused uncoupling in the chloroplasts. This also suggested that a modification of the lipophilic structure by lipid solvents could induce a change in photosynthetic activity.

It then became of particular interest to investigate more precisely the effects of lipophilic groups of various kinds of organic compounds on the activities of chloroplasts incubated with them. In this article, aliphatic alcohols, esters, ethers, ketones and amides are investigated to test the validity of the above hypothesis.

Experimental Procedures

Chloroplasts were isolated from market spinach leaves at 4°C according to the procedure described previously [1], and suspended in a

preparation medium at 0° C containing 0.5 M sucrose, 5mM $MgCl_2$ and 10 mM tris(hydroxymethyl)methylglycine buffer (pH 7.8). Chlorophyll, determined by Arnon [10] was present at a concentration of 480 μg/ml. A 0.25 ml aliquot of the suspension was mixed at 0° C with 1 ml of the preparation medium containing a given concentration of an aliphatic compound, then incubated for 5 min in a water bath at a given temperature ranging between 20° C and 55° C (±0.2° C; untreated, at 0° C) under dim green light. The concentration, *C*, of the compound in this incubation mixture was used in the analysis of the results; in some experiments two compounds were added. The incubated mixture was then diluted with 5 ml of a reaction medium composed of 0.1 M sucrose, 5 mM $MgCl_2$, 10 mM tris(hydroxymethyl)methylglycine (pH 8.3), 600 μM potassium ferricyanide, 1 mM ADP and 1 mM orthophosphate. The reaction mixture was illuminated with 5×10^4 lux white light at 15 ± 0.1° C for 5 min and assayed for ferricyanide reduction from the change in absorbance at 420 nm.

The rates of ferrycyanide reduction were plotted against the incubation temperature to estimate the *Tmax* values, from which $\Delta Tmax$ values were determined. Three $\Delta Tmax$ values obtained with three different concentrations of each compound were plotted against the concentration to evaluate the $C_{5°}$ value. Log $(1/C_{5°})$ was then plotted against log *P*, using the log *P* values previously published [9, 11]. The log *P* value for pentanamide was the average of the values for butanamide and hexanamide [12]; the value for 3-heptanone was taken to be the same (1.79) as that for 2-heptanone [11], and that for 2-octanone was 1.79 + 0.5 = 2.29 [*cf.* 11].

Phosphorylation activity was measured using the same reaction medium as that used for ferricyanide reduction except for the addition of ^{32}Pi. Radioactive ATP was determined by a modification of the method described by Asada *et al.* [13].

The relative intensity of red-band fluorescence was measured by means of a spectrofluorometer at room temperature (approx. 18° C). Chloroplasts were suspended in the preparation medium at a chlorophyll concentration of 16 μg/ml and excited with 437 nm light. Fluorescence was scanned to obtain the maximum intensity near 687 nm (half-band width = 10 nm), without correction for the spectral sensitivity of the apparatus.

Results

In Fig. 1A, the rates of ferricyanide reduction by chloroplasts incubated in the presence of 3-methyl-l-butanol are plotted against incu-

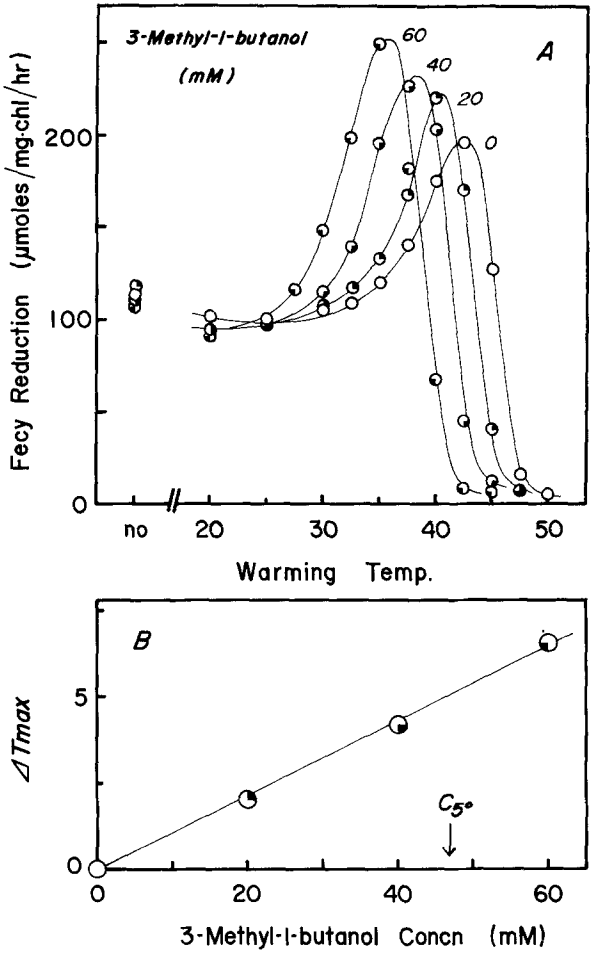

Figure 1. (A) plots showing the shift of the activity profiles for ferricyanide (Fecy) reduction (pH 8.3, 15° C) in isolated spinach chloroplasts in the presence of an aliphatic compound in the incubation mixture. Chloroplasts were incubated for 5 min at temperatures given on the abscissa (no=untreated) in the presence or absence of 3-methyl-l-butanol. (B) Plots of $\Delta Tmax$, the extent of the temperature shift measured at the maximum of each profile in A, against the concentration of 3-methyl-l-butanol.

bation temperature, showing a typical series of results. These activity-temperature profiles, showing a parallel shift of activity are commonly observed in the results obtained with aliphatic compounds. Fig. 1B shows plots of the $\Delta Tmax$ values obtained from the most probable individual *Tmax* values in Fig. 1A, against the alcohol

concentration. The $C_5°$ value for 3-methyl-l-butanol was then estimated to be about 47 mM. Most of the plots obtained with other compounds also showed good linearity although some showed a slight upward curvature.

In Fig. 2A, a typical result is shown for the effect of two compounds simultaneously present in the incubation mixture. Chloroplasts were incubated in the presence of 48 mM cyclohexanol and various concentrations of pentanamide. The $\Delta Tmax$ values obtained from these profiles are plotted in Fig. 2B (upper line) against pentanamide concentration. The $\Delta Tmax$ values for pentanamide in the absence of cyclohexanol were determined separately with a different preparation of chloroplasts and also plotted in Fig. 2B (lower line). In each case, the $C_5°$ value was found to be 35 mM. The temperature difference between the two parallel lines is 6.6°C. Similar parallelism has been found consistently for other combinations, such as cyclohexanol/ethylbutanoate, cyclopentanol/ethylpropanoate and l-butanol/l-hexanol/l-octanol.

Fig. 3 shows the activity-temperature profiles of (A) phosphorylation efficiency (P/2*e*), (B) ferricyanide reduction, and (C) relative fluorescence intensity at the maximum, obtained in the presence or absence of 50 mM 4-methyl-2-pentanone. Each curve for the relative fluorescence showed two sigmoidal segments, the second of which appeared at temperatures higher than 55°C (not shown in Fig. 3C).

In Fig. 4, log $(1/C_5°)$ values of various aliphatic compounds are plotted against the log P values of each compound. A reference line, passing through the majority of alcohols, has a slope of 1 and an intercept of 0.1.

Discussion

As shown in Fig. 1, the plots of the most probable $\Delta Tmax$ values manifest a linear relationship between $\Delta Tmax$ and C for 3-methyl-l-butanol. The same relationship was found for most of the compounds examined in the present experiments. This linear relationship, combined with the parallel shift of the profiles, will predict the concentration of a compound necessary to inactivate 50% of phosphorylation at an arbitrary temperature, regardless of the solubility of the compound at that temperature.

It should be noted that ethers were far less effective than expected. Their high volatility together with low solubility might reduce their practical concentration in the incubation mixture.

It has been verified that the effects of various compounds are additive (Fig. 2). In a separate experiment, a $C_5°$ value of 40 mM

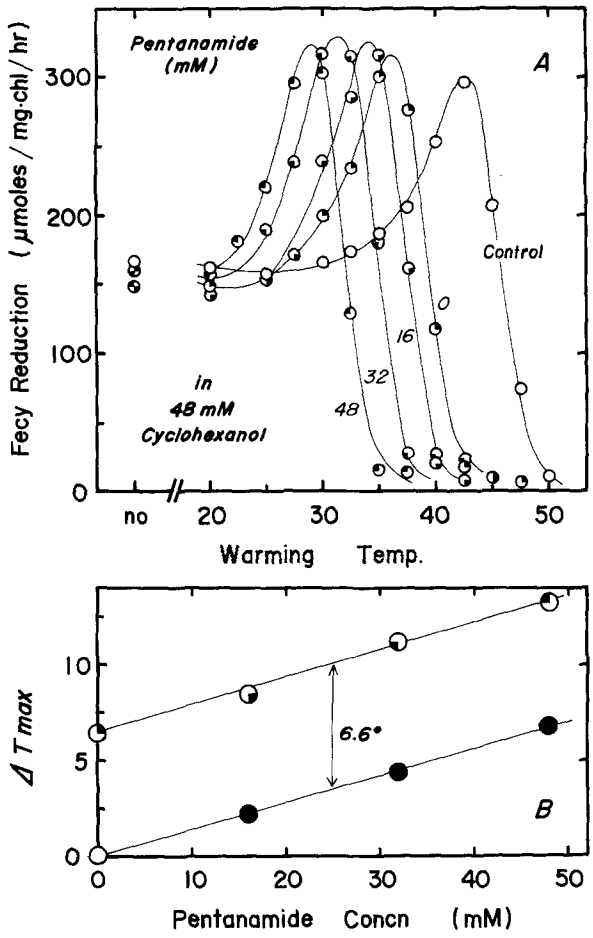

Figure 2. (A) Activity profiles for ferricyanide (Fecy) reduction (pH 8.3, 15°C) in isolated chloroplasts after incubation in the simultaneous presence of two aliphatic compounds. Chloroplasts were warmed for 5 min at temperatures given on the abscissa (no=untreated) in the presence of 48 mM cyclohexanol and various amounts of pentanamide (control=incubated in the absence of both). (B) Plots of $\Delta Tmax$ obtained from the profiles in A (upper line, in the presence of 48 mM cyclohexanol also) and those obtained in the presence of pentanamide only (the lower line represents a separate experimental result) against pentanamide concentration.

was obtained for cyclohexanol. At 48 mM, the $\Delta Tmax$ expected would be 6.0° C, in good agreement to the $\Delta Tmax$ of 6.6° C determined experimentally. The additivity suggests that each of the compounds which are simultaneously present in the incubation mixture

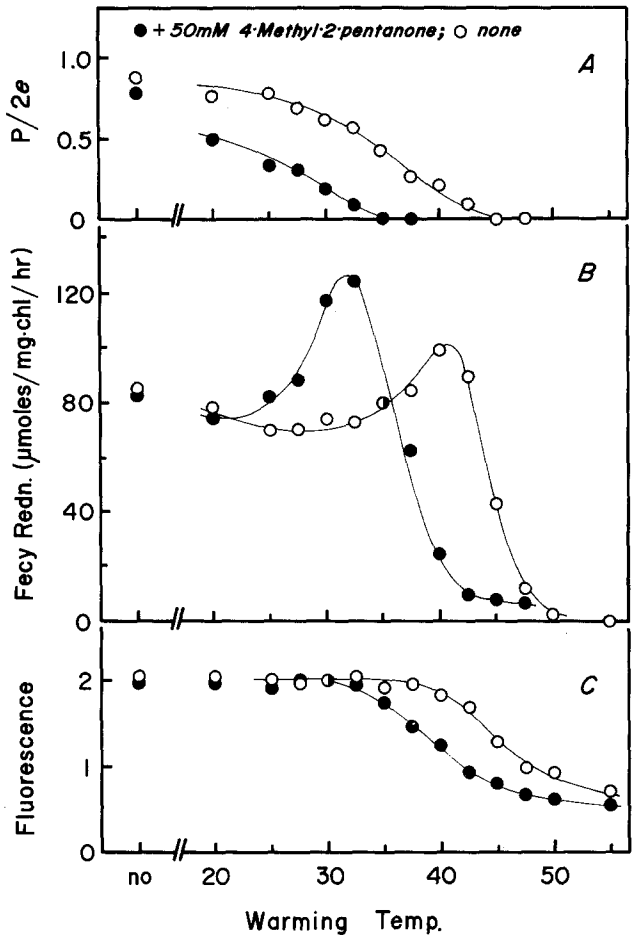

Figure 3. Activity profiles of the P/2*e* ratio (A), ferricyanide (Fecy) reduction (B) at pH 8.3 and 15°C, and the relative intensity of red-band fluorescence (C) at pH 7.8 and 18°C, for chloroplasts incubated for 5 min at temperatures given on the abscissa (no=untreated) in the presence (closed circles) or absence (open circles) of the 50 mM 4-methyl-2-pentanone.

acts independently and probably non-specifically in altering the observed activity.

The combined effects of warming and presence of various compounds on P/2*e*, ferricyanide reduction and the fluorescence intensity depicted in Fig. 3 were those generally observed. The P/2*e* ratio decreases as ferricyanide reduction increases (so-called uncoupling), in parallel with a decrease in the intactness of thylakoid membranes

[2, 7], and is abolished near $\Delta Tmax$; the fluorescence intensity starts to decrease at $\Delta Tmax$ and reaches its lowest value (of the first sigmoid) at the same point where the ferricyanide reduction stops. Therefore, it seems that these lipophilic compounds can penetrate the membrane, altering its structure. This alteration results in lowering of the incubation temperature necessary to cause the same degree of heat denaturation of the functional membrane [6, 7].

Most of the plots of log $(1/C_5{}^\circ)$ values of each homologous series in Fig. 4 can be interpreted to have slopes of 1 in parallel with the reference line. This shows that regardless of the functional group in the compound, the stepwise increase in hydrocarbon-chain unit (CH_2, in other words, log P) results in an arithmetic increase in log $(1/C_5{}^\circ)$. McCarty and Coleman [14] reported slopes of 0.76 and 1.05 (after adapting to Fig. 4) for 50% uncoupling by fatty amines at 18-21°C in spinach chloroplasts and digitonin-fragmented chloroplasts, respectively. In the present experiments, most of the aliphatic compounds obey the relation log $(1/C_5{}^\circ) = a \log P + b$. For the alcohols lying on the line drawn in Fig. 4, $a = 1$ and $b = 0.1$ so that $C_5{}^\circ P = 0.8$ (M). This value would then represent the concentration of any of the alcohols in the lamellar phase (a lipophilic phase in chloroplasts) necessary to induce a $\Delta Tmax = 5^\circ$ during a 5 min incubation. Assuming the volume of the lamellar phase per chlorophyll molecule to be approximately 2.5 nm^2 [15, 16] x 3.5 nm [17], the volume becomes 8.75×10^{-24} liter. At 0.8 M, this volume would contain 7×10^{-24} mole of alcohol during incubation. The molecular ratio of alcohol to chlorophyll is then 4:1* for $\Delta Tmax = 5^\circ$.

It is also apparent from Fig. 4 that each homologous series of compounds has its own intercept, b, but appears to have a slope of 1.0. This would indicate that each functional group in each homologue, *e.g.*, $-CONH_2$ in amides, will have a group-specific effectiveness. The degree of effectiveness, if the (real or imaginary) compounds with identical log P values are compared, will differ depending on the electronic and/or volumetric and/or geometrical features of each individual compound. An amide, for example, has a larger volume and dipole moment† due to $CONH_2$ than an (imaginary) alcohol of the same log P value. Therefore, each class of compounds would have a unique ratio of compound to chlorophyll to give a $\Delta Tmax = 5^\circ$.

* This figure will be more accurate than that reported in the previous article [6] in which the volume of whole chloroplasts was used as the lipophilic phase.

† The averages of dipole moments are found around 3.5, 2.8, 1.7 and 1.7 D for amides, ketones, alcohols and esters, respectively. Parachors of the real and imaginary compounds having log $P = 0.29$, for example, are approx. 245, 176, 167 and 181 for pentanamide, 2-butanone, $C_{2.9}H_{6.8}OH$ and $CH_3COOC_{1.1}H_{3.2}$, respectively.

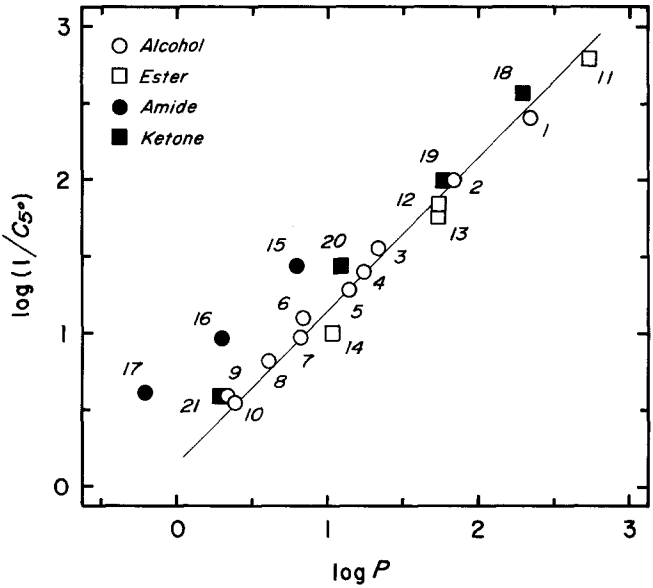

Figure 4. Plots of log (1/*C*) *vs.* log *P*. For details, see text. The compounds used are: 1, l-heptanol; 2, l-hexanol; 3, l-pentanol; 4, cyclohexanol; 5, 3-methyl-l-butanol; 6, l-butanol; 7, cyclopentanol; 8, 2-butanol; 9, l-propanol; 10, 2-methyl-2-propanol; 11, ethylhexanoate; 12, butylethanoate; 13, ethylbutanoate; 14, isopropylethanoate; 15, hexanamide; 16, pentanamide; 17, butanamide; 18, 2-octanone; 19, 3-heptanone; 20, 4-methyl-2-pentanone; 21, 2-butanone.

When the linear $\Delta Tmax$–C relation and the additivity of the effects are considered and combined with the above relation, a generalized relationship can be written in the form, $\Delta Tmax = \Sigma K_i P_{ij} C_{ij}$, where K_i, P_{ij} and C_{ij} are the constant, the partition coefficient and the concentration, of the compound j in the series i, present in the incubation mixture, respectively.

The effects of concentration and chain length of alcohols on various membrane systems have been the subject of many investigations. Blocking of conduction in frog sciatic nerves was studied by Skou [18], the stabilization of human erythrocytes from hypotonic hemolysis by Seeman [19], the electrical resistance of black lipid membranes prepared from sheep erythrocyte lipids by Gutknecht and Tosteson [20] and disordering of spin labels in lipid multi-bilayers by Paterson *et al.* [21]. The log (1/*C*) values corresponding to the concentrations of the alcohols required to completely block nerve conduction, for 50% inhibition of hemolysis, to give a membrane resistance of 1×10^6 Ω-cm^2 and for a 50% increase in spin label mobility are plotted against log *P* in Fig. 5. The line represents the alcohol data obtained in the present study, as depicted in Fig. 4.

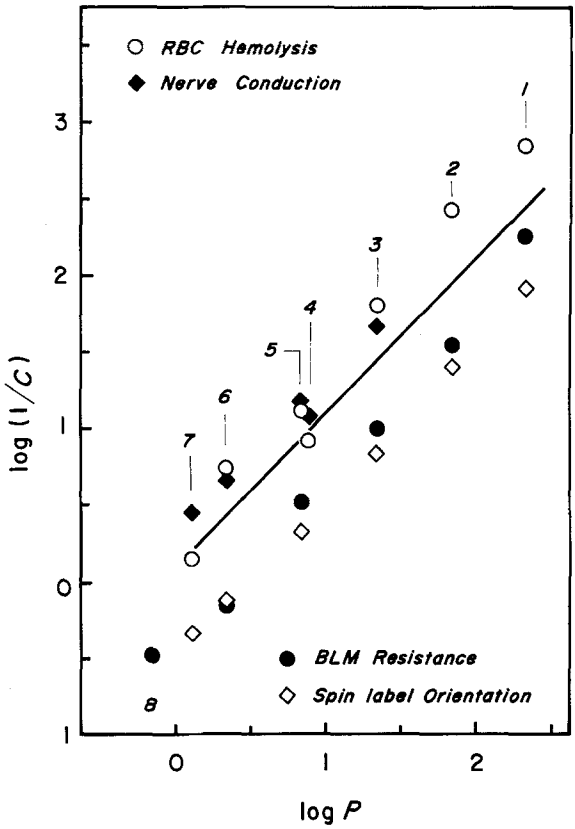

Figure 5. Plots showing the general log $(1/C)$ log P relation in various membrane systems of biological origin. The line is copied from Fig. 4. For details, see text. The compounds used are: 1, 1-heptanol; 2, 1-hexanol; 3, 1-pentanol; 4, 2-methyl-2-butanol; 5, 1-butanol; 6, 1-propanol; 7, 2-propanol; 8, ethanol.

In molecule-molecule interaction studies [9, 12, 22] most of the coefficients of log P, a, have been found to be different from one. The results in Fig. 5, however, show good parallelism with our data for several compounds, in which $a = 1.0$. This value, therefore, represents a general feature of the non-specific interaction of small lipophilic molecules with membrane structures of biological origin. The molecules, regardless of their functional groups, will partition into the lipophilic structure of biological membrane, loosening the structure and consequently inhibit membrane function. This idea is well supported by the results of spin label studies shown in Fig. 5, in which similar effects are seen using isolated lipid multi-bilayers.

The effect of alcohols (and other compounds) on natural and artificial membranes appears to be similar, although its degree is different depending on the sensitivity of the measured parameter. This implies that the lipophilic structure discussed above consists mainly of lipids (probably lipid bilayers) and that lipid structures in various membrane systems resemble each other in their role of maintaining biological function, regardless of their chemical composition [23] or of proposed membrane models [24, 25].

It may be possible, however, to interpret the results in terms other than *P*. Even if *P* is simply replaced by surface tension (*cf.* Traube's rule) etc., the characterization of the action of added compounds will be somewhat altered.

It should be noted that the activity-temperature profiles of ferricyanide reduction by chloroplasts incubated in the presence of aromatic compounds or tetraalkylammonium compounds differed, in most cases, from those influenced by aliphatic compounds, as will be reported elsewhere.

Isolated poke-weed chloroplasts exhibited patterns of photosynthetic activity identical to those reported so far for spinach chloroplasts upon incubation in the presence of lipophilic compounds.

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