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ELECTRON SPIN RESONANCE STUDIES OF THE MEMBRANES OF THE CELLULAR SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*

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Summary

Doxylstearic acid spin labels are used to study the fluidity of the membranes of the cellular slime mold, *Dictyostelium discoideum*. The τ_0 value of the wild-type cell membrane is close to that of egg lecithin indicating a rather fluid membrane. No detectable change in the fluidity of the bulk lipids at the 16-carbon depth occurs during differentiation of the myxamoebae into stalk and spore cells despite reported changes in the individual lipid components. The results of studies on temperature-sensitive and aggregationless mutants are also presented.

Introduction

The processes resulting in eukaryotic cellular differentiation are not well understood at present. One approach to the problem is to choose an organism which shows a comparatively simple differentiation and study it using a wide variety of biological, biochemical, and chemical techniques. The cellular slime mold *Dictyostelium discoideum* is a simple eukaryote that is well suited for such studies. When the growth medium is removed to starve the cells, the solitary amebae aggregate together and form a pseudoplasmodium consisting of 10^4--10^5 amebae. Subsequently, the amebae differentiate into two cell types: stalk and spore cells. Under suitable conditions each spore germinates releasing a single ameba.

A considerable amount of information on the differentiation of *D. discoideum* has been obtained both from extensive descriptive studies [1] and

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from biochemical and genetic studies [2]. It appears that during differentiation a number of changes occur at the cell surface including an increase in the cyclic AMP phosphodiesterase, an increase in the cyclic-AMP-binding sites which are thought to be involved in chemotaxis, and the appearance of "contact sites" which are thought to be responsible for cell adhesion [3,4]. However, there is no information on changes of the physical characteristics of the membranes such as the fluidity during differentiation.

The membrane fluidity can be examined by using probe or reporter groups and for electron spin resonance (ESR) spectroscopy, the nitroxide group has proved to be an excellent probe [5–9]. Fatty acids having the doxyl group at the 5- and 16-carbon positions can be intercalated into the lipids in a bilayer membrane. Then, information on the polarity of the local environment and the rate and anisotropy of the molecular motion in the neighborhood of the doxyl group can be obtained from the peak heights, peak to peak distances, and hyperfine coupling constants observed in the ESR spectrum.

In the present paper, we examine membrane fluidity of the cell as a function of the time course of differentiation. It has been reported that the lipid composition of *D. discoideum* changes during differentiation [10,11]. The question we deal with is whether the net effect of the changes in one of the cell constituents, e.g. the lipid content [10—12] changes a physical property of the cell, e.g. the fluidity of the membranes, durring the course of the differentiation. This question can be answered by obtaining ESR measurements of the spectrum of a nitroxide spin label which is incorporated into the membranes of *D. discoideum*. We also compare the intrinsic fluidity of the wild and mutant *D. discoideum* membrane lipids to that of model membranes.

Experimental

Materials

All spin labels were purchased from Synvar Associates, Palo Alto, California. The spin labels were I(m,n) stearic acid analogs with the doxyl group located either on the fifth carbon from the OH group (5-doxylstearic acid or I(12,3)-stearic acid) or on the 16th carbon from the OH group (16-doxylstearic acid or I(1,14) stearic acid). The L-α-dipalmitoylphosphatidylcholine used was obtained from Koch-Light and the bovine serum albumin was supplied by Sigma. Doubly distilled water was used in all experiments.

Dictyostelium discoideum amebae (strains AX3 and X2 [13] were grown in axenic medium shaken at 150 cycles per min at 22°C [14] and harvested during mid- to late log phase ($2 \cdot 10^6$—10^7 per ml). The amebae were harvested by centrifugation for 2 min at $120 \times g$, washed twice with distilled water, and resuspended in water at $2 \cdot 10^8$ cells per ml for ESR measurements. Examination of the cells under a microscope indicated that this procedure did not lyse them and viability was greater than 50%. Differentiation of the amebae was induced by depositing 10^8 washed amebae on a 7-cm Whatman No. 50 filter placed on top of a Whatman No. 17 filter; both filters were soaked in LPS (KCl, 1.5 g/$MgCl_2$, 0.5 g/KH_2PO_4, 5.45 g/dihydrostreptomycin sulfate, 0.5 g per liter, pH 6.5 [15]). The amebae were harvested at the various stages of differentiation by removing them from the filter paper with a stream of water

and then centrifuging them as described above. Spin-labeling of the cells was accomplished by placing 10 μl of 1 mM label in 100% ethanol in a glass vial. The ethanol was evaporated with a stream of air and 250 μl of a suspension of $2 \cdot 10^8$ cells per ml was added. This gave 1 mol% label/lipids assuming no biochemical degradation of the lipid occurred.

ESR measurements

Electron spin resonance spectra were obtained on a JEOL spectrometer operating at about 9.5 GHz and having a cylindrical cavity. Cylindrical quartz tubing of interior diameter 0.8 mm and outer diameter 2.4 mm with a polyethylene cap at one end was used as the sample holder. The usual spectrometer settings were 4—10 mW microwave power, modulation power about 0.5—2.0 G, scanned field 3370 ± 100 G, scanning time 8 min, filter time constant of 0.3—1.0 s.

The nitroxide group can yield information on the correlation time, τ, of the molecular motion because the elements of the g tensor and of the hyperfine coupling constant, A, have different values when the external field (H_0) is applied along the x, y, or z molecular axis of the nitroxide group; i.e. $g_{xx} \neq g_{yy} \neq g_{zz}$ and $A_{xx} \neq A_{yy} \neq A_{zz}$. When the motion of the molecule is isotropic but slow enough that the spectrum obtained along the x, y, and z axes are incompletely averaged, the spin-spin relaxation of the absorption mode of the ESR signal is given by Eqn. 1 where $b = 4\pi\ (A_{zz} - A_{xx})/3$, $\Delta\gamma = -\beta\ (g_{zz} - \frac{1}{2}(g_{xx} + g_{yy}))/h$ and m is the magnetic quantum number.

$$\frac{1}{T(m)} = \tau\{(6 + 5m^2)\,\tfrac{1}{40}b^2 + \tfrac{4}{45}(\Delta\gamma H_0)^2 - \tfrac{4}{15}b\Delta\gamma H_0 m\} \tag{1}$$

Making a ratio of this expression for the $m = -1$, 0, and +1 peaks corresponding to the high, middle, and low field peaks, using the proportionality between this ratio and the ratio of the squares of the peak to peak distances (h_m), and using the sum of the ratios one can obtain the following expression for τ_0 [16—19] since $T_2(0)^{-1} = \sqrt{3} \cdot 2.8 \cdot 10^6 \cdot W_0$ where W_0 is the line width of the central peak in gauss.

$$\tau_0 = \left(\sqrt{\frac{h_0}{h_{-1}}} + \sqrt{\frac{h_0}{h_{+1}}} - 2.0\right) 4\sqrt{3} \cdot \pi \cdot 2.8 \cdot 10^6 W_0/b^2 \tag{2}$$

This expression will give a true correlation time only when $\tau_c < 5 \cdot 10^{-9}$ and isotropic motion is present; however, it is customary to use τ_0 as an empirical measure of τ_c for systems which are nearly approximate under these conditions.

Results and Discussion

Site of labeling

In order to study the membrane lipids of a cell, a probe which preferentially locates in this region must be selected. Previous studies have shown that spin-labeled fatty acids are likely to be a good choice [6,8]. In the case of *D. discoideum*, the ESR spectra shown in Fig. 1 indicate that the probe preferentially attaches to the cells. The slowing of the motion of the label as indicated by the change in the τ_0 value from 0.12 ns in the aqueous medium to 1.9 ns in the

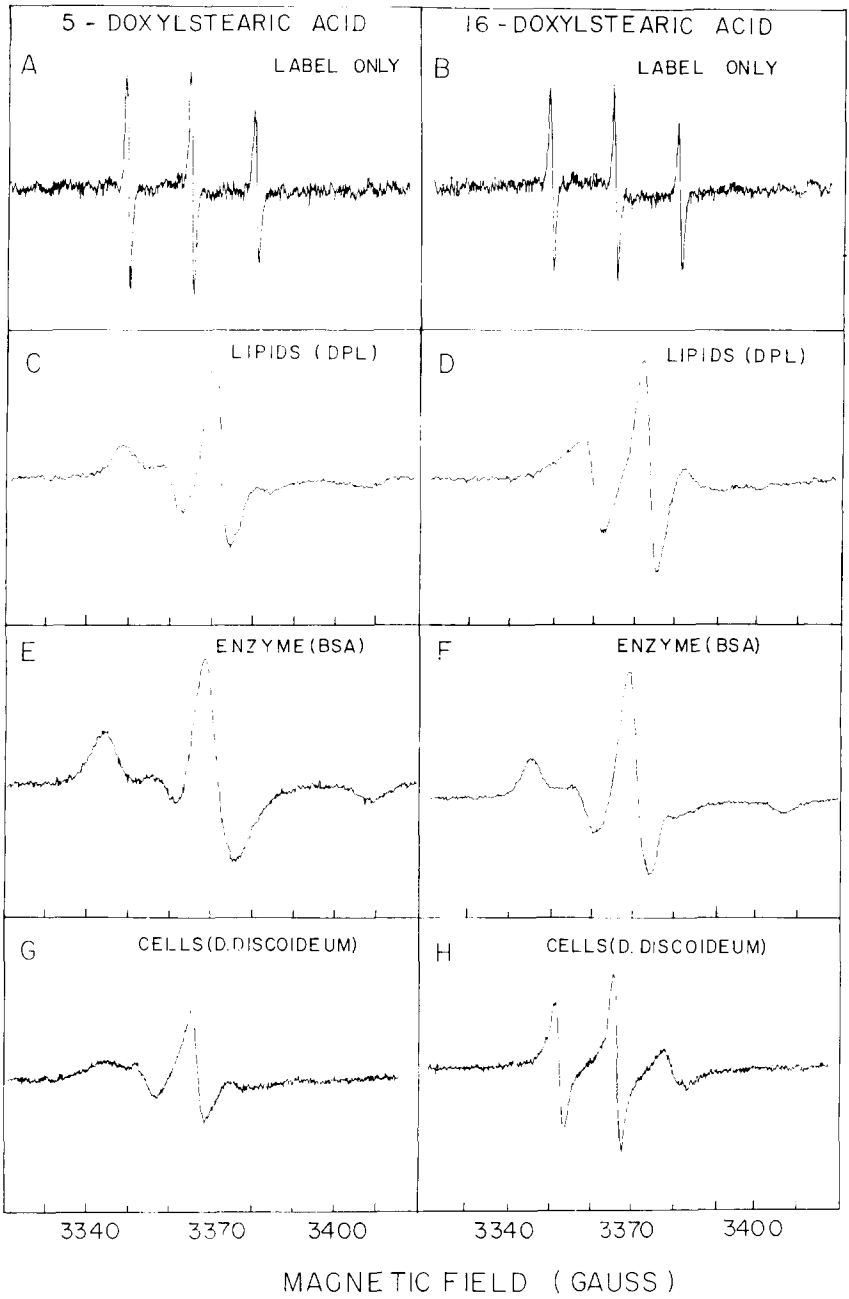

Fig. 1. The ESR spectrum of 5-doxylstearic acid (A) in aqueous solution, (C) in dipalmitoylphosphatidylcholine (DPL) vesicles, (E) in bovine serum albumin (BSA), and (G) in *D. discoideum* amebae, and of 16-doxylstearic acid in the same solutions (B, D, F and H, respectively).

cell suspension indicates that the label has bound to the cells. The doxylstearic acid is not likely to be located in the cytoplasm since the ESR spectrum of spin-labeled cytoplasm [20] indicates that cytoplasm is a far more fluid environment than the region of *D. discoideum* being labeled. Addition of ascorbic

acid (which reduces the doxyl group) produces no change in the ESR spectrum of labeled cells; hence, we can conclude that (1) the bulk of the spin label is bound to a part of the cells which is inaccessible to the aqueous medium and that (2) the fatty acid labels are not rapidly exchanging between the solution and the cells. The change in A value from 15.4 G in aqueous medium to 13.8 G in the cells indicates that on binding to the cells the label locates in a hydrophobic environment such as that in the interior of a membrane [17,18].

The doxylstearic acids which are located in the membrane can be present either in the lipid bilayer portion of the membrane or bound to an enzyme [21]. These two cases can be distinguished by examining the difference in mobility along the hydrocarbon chain. The ESR spectrum of a spin label in a lipid bilayer shows a more fluid environment at the 16-carbon depth than at the 5-carbon depth (Fig. 1C and D) while the ESR spectra of a spin-labeled lipid bound to an enzyme such as bovine serum albumin show that the mobility is about the same at both the 5- and 16-carbon positions (Fig. 1E and F). The mobility pattern for the doxylstearic acids in the *D. discoideum* cells matches that of vesicles as opposed to that of a lipid-enzyme complex indicating that the spin label is located in the lipid bilayer part of the cell membrane. Studies on mouse L cells spin-labeled with stearic acid suggest that the label is located in the plasma membrane and does not penetrate further into the cell [20]. If the same penetration pattern characterizes the *D. discoideum* amebae, then the membrane site labeled is the plasma membrane.

Intrinsic mobility of the lipids in the membranes

Since the physical state of the lipids in the bilayer plays an important role in determining the physical properties of the membrane such as the lateral diffusion and the rigidity, the active and passive transport through the membrane, and the functioning of membrane bound enzymes, it is important to obtain a measure of the bilayer fluidity. In the introduction and experimental section of this paper, two parameters, τ and S, were defined which can be used to estimate the lipid mobility at the 16 and 5 carbon depth of the bilayer, respectively. ESR spectra of the doxylstearic acids in *D. discoideum* amebae were obtained and are shown in Fig. 2 along with the spectra of the same spin labels in dipalmitoylphospatidylcholine vesicles (Figs. 2A and B) and also egg lecithin vesicles (Figs. 2E and F) for comparison. The quantities in the spectrum which are measured and used with the equations given in the experimental section to obtain τ and S are shown in Figs. 2B and E, respectively. The τ value for *D. discoideum* is 1.90 ns compared to a value of 0.89 ns for egg lecithin and 5.6 ns for dipalmitoylphosphatidylcholine, while the S values for *D. discoideum*, egg lecithin, and dipalmitoylphosphatidylcholine are 0.71, 0.54, and 0.61, respectively. These values indicate that the mobility of the cellular lipid is intermediate between that of egg lecithin and dipalmitoylphosphatidylcholine.

It is interesting to consider the lipid composition of *D. discoideum* membranes to determine the molecular origin of the observed fluidity. There are three aspects of the lipid composition which will affect the membrane fluidity: (1) the presence or absence of sterols, (2) the nature of the polar head-group and (3) the nature of the alkyl groups. Data on all three of these factors is

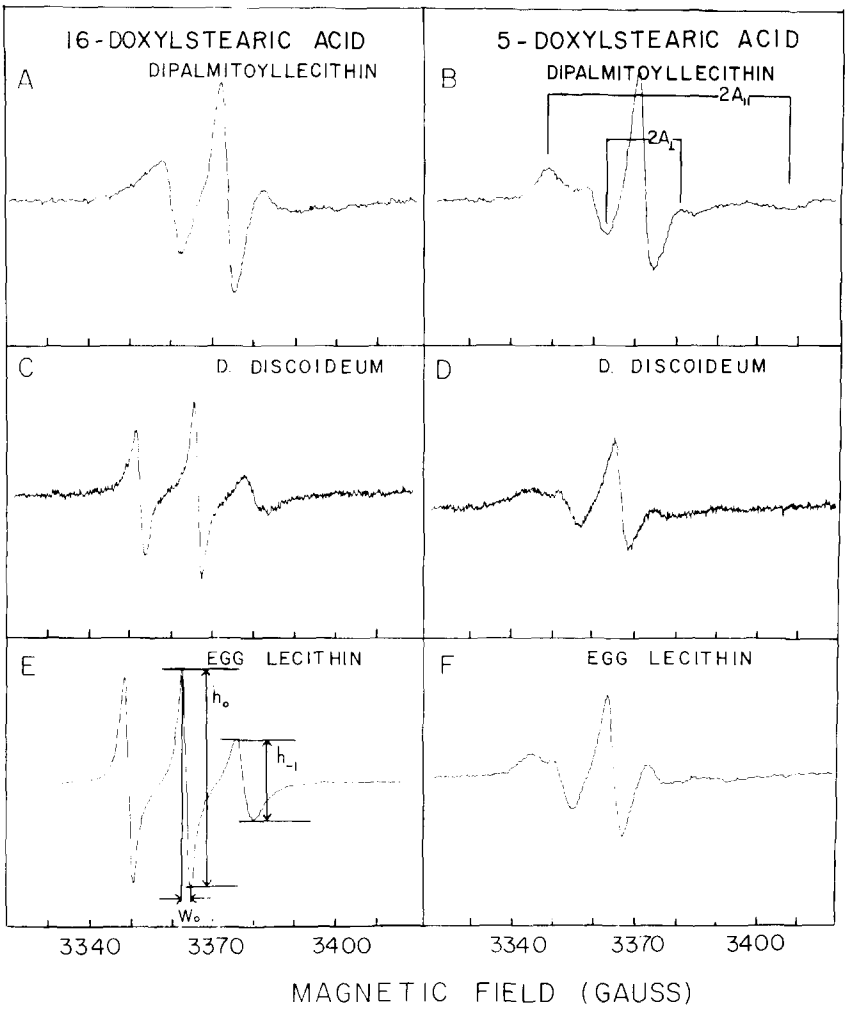

Fig. 2. The ESR spectrum of 16-doxylstearic acid (A) in dipalmitoylphosphatidylcholine, (C) in *D. discoideum* cells, and (E) in egg lecithin and of 5-doxylstearic acid in the same solutions (B, D and E, respectively).

available for *D. discoideum* amebae from the studies of the constituent lipids which have been done by Long and Coe [11], Davidoff and Korn [12], and Ellington [10]. With regard to the first factor i.e. the presence of sterols, about 21% of the lipid content of *D. discoideum* was found to be neutral lipids, principally the sterol stigmastenol [11]. The introduction of a sterol into a lipid bilayer of egg lecithin produces a larger order parameter indicating a more rigid membrane [22,23]. Hence, for a membrane above its T_{tr} the introduction of sterols would be expected to produce a more ordered membrane. Therefore, this factor would tend to produce a more rigid membrane. With regard to the second factor i.e. the nature of the head-group, studies of the phase transition temperature of model membrane bilayers indicate that for the same alkyl group phospholipids with an ethanolamine head group will have a transition tem-

perature higher than phospholipids with a choline head-group [24]. Therefore, at many temperatures the phosphatidylethanolamine bilayers will be in the rigid gel state while phosphatidyl choline bilayers are fluid. The data of Ellington [10] on the polar lipids of *D. discoideum* indicates that they are principally phospholipids with phosphatidylethanolamine comprising the major (40–44 mol%) portion of these. Thus, this factor would tend to produce a more rigid membrane. The third factor influencing the membrane fluidity is the nature of the alkyl group. The presence of a double bond is known to introduce a disorder into the fatty acid bilayer which lowers T_{tr} producing a more fluid membrane. Unsaturated fatty acids are present in the lipids of *D. discoideum* in an unusually high percentage, being about 90% of the fatty acids of the phospolipids and about 50% of the fatty acids of the neutral lipids [12]. In relating the observed fluidity parameter (τ_0) to the factors influencing the membrane mobility, it is important to remember that the doxyl fatty acid probe does not partition between the lipid and the aqueous region in the same way for all bilayers and therefore in a heterogeneous membrane not all regions will be equally labeled by this technique. In particular, the partition coefficient, K, for spin labels going into steroid containing regions is very much less than that for nonsteroid containing regions. Further, the precise value of K will vary somewhat with the nature of the headgroup and the sidechain. The existance of a fluid portion of the membrane indicates that the effect of the unsaturated alkyl groups is dominant in those portions of the membrane accessable to the spin label.

Developmental studies

When *D. discoideum* amebae are starved on an air/water interface, the cells aggregate and undergo a differentiation process whose purpose is to produce spore cells. The steps in the differentiation process are shown in Fig. 3 begining with the plated solitary cells at 0 h followed by the aggregation commencing at 6–8 h to form a clump of amebae at 12 h and subsequently the formation of the fruiting body which has stalk and spore cells at about 28 h in the axenic strain studied. Also shown in Fig. 3 are the changes in lipid composition that occur during the developmental process as measured by Long and Coe [11]. They found that the polar lipids decreased during differentiation from 95 mg/g dry cells to 75 mg/g while the neutral lipids and sterols increased from 25 mg/g to 40 mg/g.

The question arises as to whether the changes in the constituent lipids result in net changes in the physical properties of the membranes which may be related to changes in the biological behavior of the cells. To answer this question, we have spin-labeled cells at various stages of differentiation and have obtained ESR measurements of the treated cells so any differences in lipid mobility can be noted. After about 9 h of differentiation, a change in the character of the cell surface occurs such that the spin label will no longer be taken up completely by the cell. The portion of the label which remains in solution giving a narrow line spectrum which can be removed by ascorbic acid so ESR spectra of the readily labeled lipids are obtained. The ESR spectra obtained at various stages of the differentiation process are shown in Fig. 4 for the 5-doxyl and 16-doxylstearic acids. As can be seen, the membrane bound

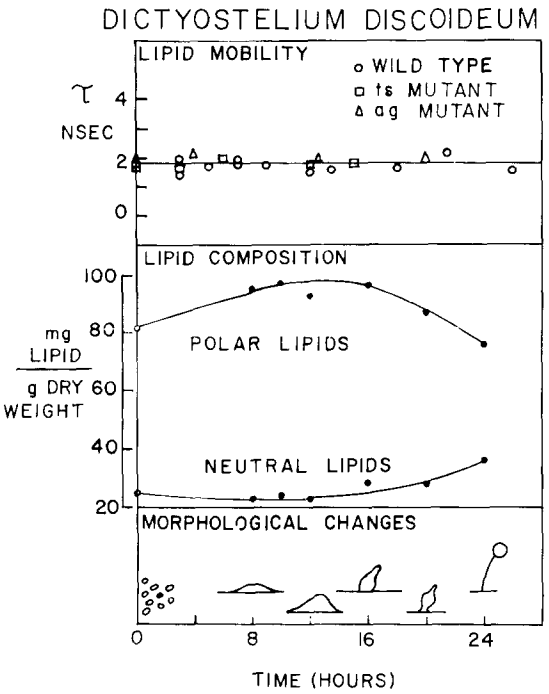

Fig. 3. The changes in *D. discoideum* during the time course of the differentiation: the morphological changes, the changes in lipid composition, and the changes in lipid mobility.

lipids maintain essentially the same fluidity as the cells change from amebae to stalk and spore cells. The τ values remain constant at about 1.9 ns (Fig. 3); hence, the mobility of the fluid portion of the membrane lipids does not change during differentiation even though there are changes in the amounts and proportions of the individual lipids present. A similar lack of change of τ over the time period of differentiation was also found for a ts mutant and an aggregationless mutant of *D. discoideum*.

Genetic mutants

The D. discoideum cells have been mutated to develop strains other than the wild type which have different biological behavior. The chromosomal location of the various mutations can be mapped and mutants in the same gene can be grouped together. While many mutants affect metabolic pathways, some will affect the membrane fluidity and thereby the functioning of the membrane. The mutation may alter the intrinsic mobility of the membrane lipids or it may alter the transition temperature of the phospholipids. We have studied two kinds of mutants of *D. discoideum:* (1) a temperature-sensitive (ts) mutant, AX_2, which will grow at 22°C but not at 27°C and (2) an aggregationless mutant (ag) which will grow at 22°C but which when plated out fails to complete the first step of the developmental processes, i.e. aggregation towards a fixed point to form the pseudoplasmodium.

The intrinsic mobility of the membrane lipids of the mutants has been followed during the course of differentiation and the results are shown in Fig.

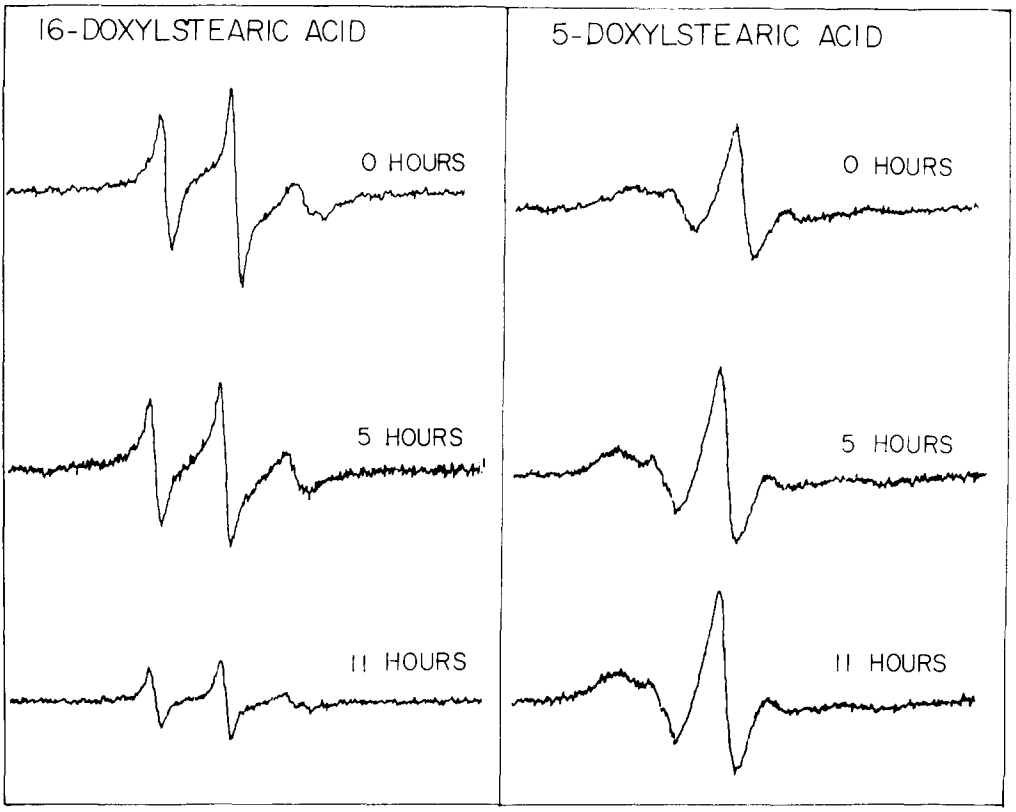

Fig. 4. The ESR spectra of spin-labeled *D. discoideum* amebae harvested at various times during the differentiation process.

3. As can be seen, there is no substantial change in the intrinsic mobility of the membrane lipids of either mutant during the course of development. This means that the genetic defect in the aggregationless mutant does not produce amebae that cannot aggregate because their membranes are too rigid.

The ts mutant was examined to determine whether a change in the physical state of the lipids in the bilayer occurred between the restricted-growth temperature, 27°C, and the permitted-growth temperature, 22°C. Such a change has been recorded for the membrane-bound ATPase of lamb kidney outer medulla and is manifested in a break in the temperature dependence of the fluidity of the membrane lipids [25]. The AX_2 *D. discoideum* cells in the vegetative stage were cropped and treated with 16-doxylstearic acid. The mobility parameter, τ_0, was measured over the temperature range of 1—46°C and the data are shown in Fig. 5. No break occurs in the Arrhenius plot which would indicate a change in the activation energy accompanying a phase transition was observed for any temperature in this range. The signal to noise ratio in these experiments was rather small since relatively few cells were in the resonance cavity. The results of these studies indicate that the nature of the ts mutation in AX_2 is not one which affects a phase transition in the lipid bilayer.

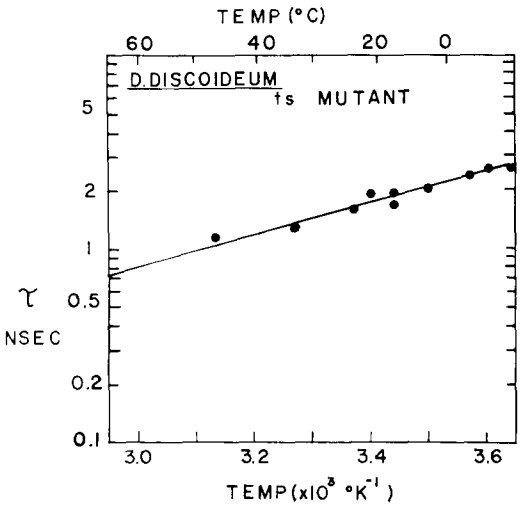

Fig. 5. An Arrhenius plot of the correlation time versus the inverse temperature for spin labels located in the membranes of a temperature sensitive (ts) mutant of *D. discoideum*.

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References

1 Bonner, J.T. (1967) The Cellular Slime Molds, Princeton University Press, Princeton, New Jersey
2 Doomis, W.F. (1975) *Dictyostelium Discoideum* -- a Developmental System, Academic Press, New York
3 Gerisch, G., Malchow, D. and Hess, B. (1974) in Mosbacher Colloquim der Gesellschaft für Biologie Chemie (Jaenicke, L., ed.), Vol. 25, p. 279
4 Newell, P.C. (1976) in Microbiology (Dworkin, M. and Shapiro, I., eds.), American Society for Microbiology Publication, in the press.
5 Libertini, L.J. and Griffith, O.H. (1970) J. Chem. Phys. 53, 1359
6 Jost, P., Libertini, L.J., Hebert, V.C. and Griffith, O.H. (1971) J. Mol. Biol. 59, 77
7 Birrell, G., Van, S.P. and Griffith, O.H. (1973) J. Am. Chem. Soc. 95, 2451
8 Hubbell, W.L. and McConnell, H.M. (1971) J. Am. Chem. Soc. 93, 314
9 Gaffney, B.J. (1974) Methods Enzymol. 32, 161
10 Ellington, J.S. (1974) Biochim. Biophys. Acta 337, 60
11 Long, B.H. and Coe, E.L. (1974) J. Biol. Chem. 249, 521
12 Davidoff, F. and Korn, E.D. (1963) J. Biol. Chem. 238, 3199
13 Williams, K.L., Kessin, R.H. and Newell, P.C. (1974) J. Gen. Microbiol. 84, 59
14 Watts, D.J. and Ashworth, J.M. (1970) Biochem. J. 119, 171
15 Sussman, M. (1966) Methods Cell Physiol. 2, 397
16 Stone, T.J., Buckman, T., Nordio, P.L. and McConnell, H.M. (1965) Proc. Natl. Acad. Sci. U.S. 54 1010
17 Waggoner, A.S., Griffith, O.H. and Christensen, C.R. (1967) Proc. Natl. Acad. Sci. U.S. 57, 1198
18 Hamilton, C.L. and McConnell, H.M. (1968) in Structural Chemistry and Molecular Biology (Rich, A and Davidson, N., eds.), p. 115, W.H. Freeman Co., San Francisco

19 Smith, I.C.P. (1972) in Biological Applications of ESR (Swartz, H.M., Bolton, J.R. and Borg, D.C. eds.) Wiley-Interscience, New York
20 Kaplan, J. Canonico, P.G. and Caspary, W.J. (1973) Proc. Natl. Acad. Sci. U.S. 70, 66
21 Jost, P.C., Griffith, O.H., Capaldi, R.A. and Vanderkooi, G. (1973) Proc. Natl. Acad. Sci. U.S. 70, 480
22 Marsh, D., Phillips, A.D., Watts, A. and Knowles, P.F. (1972) Biochim. Biophys. Acta 49, 641
23 Shimshick, E.J. and McConnell, H.M. (1972) Biochem. Biophys. Res. Commun., 53, 446
24 Shimshick, E.J. and McConnell, H.M. (1973) Biochem. 12, 2351
25 Grisham, C.M. and Barnett, R.E. (1973) Biochemistry 12, 2635