# SPIN LABEL TRANSLATIONAL DIFFUSION IN SOLID TRISTEARIN

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ABSTRACT Translational diffusion of the intermediate chain length spin label 7N14 has been detected and studied in a lipid environment which is in the bulk solid state. Under favorable circumstances this can occur at temperatures as much as 50°C below the optical melting point. Translational diffusion allows 7N14 molecules to coalesce into impurity pools of high spin label concentration. Two other spin labels, 2N3 and 14N27, do not show a tendency to form such impurity pools. While 2N3 undergoes rapid tumbling at temperatures far below the melting point of the tristearin matrix, the molecules remain in an isolated state with no evidence of spin exchange. 14N27 is restricted in rotational motion in the solid matrix and also does not form impurity pools.

## INTRODUCTION

The diffusion of nutrients, ions, and various other molecules in the phospholipid bilayer of a cell membrane involves the diffusion of impurity molecules in an ordered hydrocarbon zone. Two basic cases of translational diffusion in membranes can be distinguished: motion parallel to the bilayer surface and entirely within the membrane, and passage into and out of the cytoplasm perpendicular to the surface (Kornberg and McConnell, 1971 *a*, *b*; Scandella et al., 1972). In both cases, it can be reasonably expected that a foreign molecule will disrupt the order of the phospholipid alkyl chains and liquefy the hydrocarbon zone in its immediate vicinity (Mehlhorn et al., 1973). As the molecule proceeds to migrate, it must continually disrupt the new regions it enters while in the volume it vacates, the hydrocarbons are free to reorder. The mechanism and kinetics of the diffusion of impurities in an ordered hydrocarbon environment constitute a problem of fundamental interest in membrane molecular biology.

Because of the complexity of biological membranes, it is profitable to examine model systems whose components can be carefully regulated in order to investigate the diffusion of impurity molecules. The present research was undertaken to study the process of translational diffusion of spin labels in the triglyceride, tristearin, which

is a reasonable analog to the hydrocarbon portion of naturally occurring membrane phospholipids, as is illustrated in Fig. 1. In both phospholipids and tristearin, the alkyl chains are esterified to glycerol. Stearic acid and other fatty acids of similar chain lengths are the usual hydrocarbon constituents of biological membranes. Of course, tristearin has no polar region, while the third carbon position in a phospholipid is usually occupied by a negatively charged phosphate group to which is esterfied a polar group such as choline, ethanolamine, serine, etc. Phospholipid hydrocarbons may include unsatured fatty acid residues and saturated fatty acids of different lengths so in this context tristearin serves as a model system.

Spin-labeled hydrocarbons can be considered as impurity molecules in an otherwise pure tristearin matrix, not only because the simple hydrocarbon chain differs in structure from the triglyceride but also because the paramagnetic group added to the hydrocarbon constitutes a further perturbation to the host matrix. Generally, spin labels serve to report on the properties of their local environment but at the same time may perturb that environment and so influence the properties of the system they are intended to probe. In the present experiments it was precisely this perturbation of the environment by the spin label that was the property of interest. For general spin label reviews see Jost et al. (1971), McConnell and McFarland (1970).

The electron spin resonance (ESR) spectra of spin labels are highly dependent on the orientation and degree of rotational motion of the label, and changes in the motional state may cause substantial alterations of the spectra (Mehlhorn and Keith, 1972; Williams et al., 1971). When a spin label is rigidly fixed and oriented in a matrix whose own orientation with respect to the spectrometer magnetic field can be adjusted, a continuous transition between three distinct spectra can be observed as the matrix is rotated. Each of these corresponds to the alignment of one principal axis of the motionless spin label parallel to the magnetic field $H$. As the molecule is allowed to tumble, the signal obtained without rotating the specimen may show partial time-dependent averaging. If the tumbling becomes sufficiently rapid (rotational correlation time $\tau_0 \lesssim 10^{-10}$ s for nitroxide spin labels) so that many different orientations are assumed by the label during a spin state lifetime, averaging is complete and the spectrum observed consists of three narrow lines of nearly equal height and width. This situation is generally referred to as the fast tumbling or isotropic spectrum.

At the other extreme of rotational motion for a randomly oriented and randomly dispersed spin label is the case of very slow tumbling. In this situation there is very little time-dependent averaging of the tensor elements and the orientations of the labels are random, in analogy to the physical situation which gives rise to a "powder" pattern in X-ray diffraction. This signal is known as an immobilized, rigid-glass or powder spectrum since it can arise from either a polycrystalline or a glassy sample.

Fig. 2 shows spectra of a freely tumbling spin label and another which is immobilized. States of motion intermediate between these extremes are very sensitive to

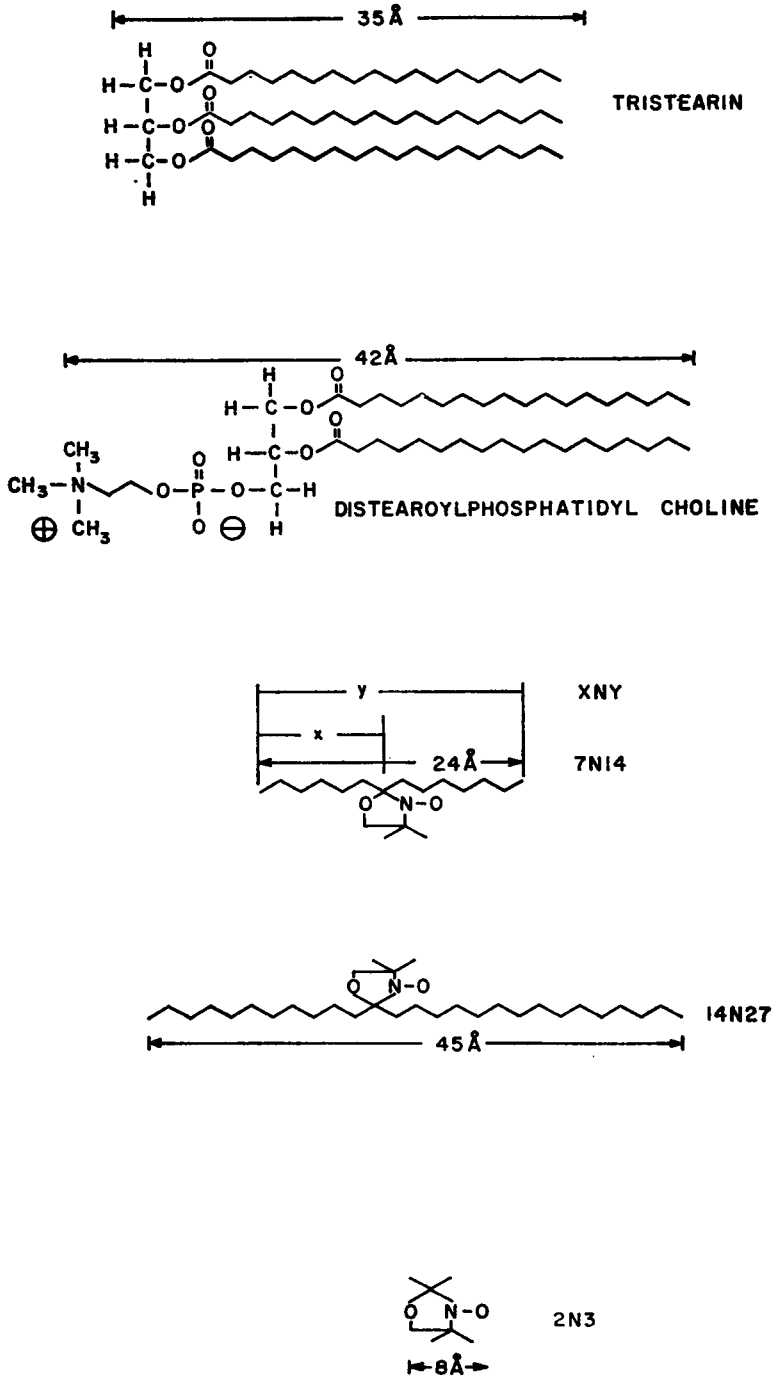


FIGURE 1 Molecular structures of tristearin, distearoylphosphatidylcholine, and the three hydrocarbon spin labels, showing the structural similarities of tristearin to a representative phospholipid and the analogous structures of the spin labels.

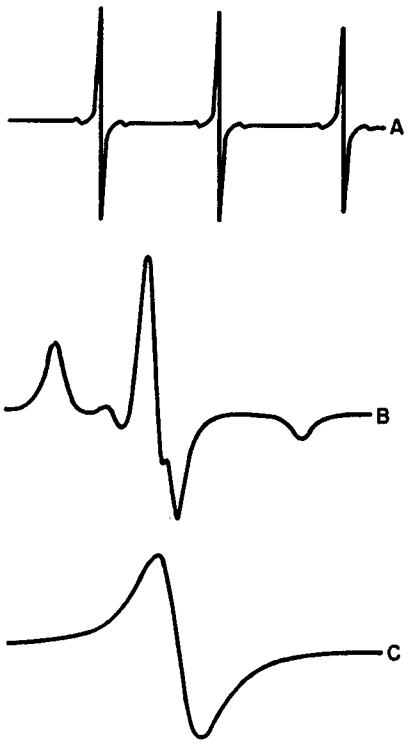


FIGURE 2 Nitroxide spin label spectra illustrating motional and concentration effects. In all three spectra *H* increases from left to right; *A* and *C* are 50 G scans, and *B* is a 100 G scan. (*A*) Free tumbling of 2,2,6,6-tetramethylpiperidone-*N*-oxyl (TEMPONE) in water. (*B*) Immobilized spectrum of 12-nitroxide stearate bound to bovine serum albumin. (*C*) Exchange signal of TEMPONE in water.

viscosity changes, and the state of rotational motion can be determined from spectral measurements.

For present purposes it is necessary to distinguish a third type of spectrum which reflects local spin label concentration. Spin label molecules in close proximity to each other undergo electron dipole-dipole interactions and electron exchange interactions. The effects of these processes appear as broadening of the three lines of an isotropic signal as the local concentration increases; eventually, the spectrum shifts entirely into one line centered at the same position as the three-line isotropic signal (Fig. 2 *C*).

## EXPERIMENTAL

Tristearin of purity greater than 99% (Hormel Institute, Austin, Minn., and Nu Chek Scientific, Elysian, Minn.) was used as the triglyceride matrix with no further preparation. Hydrocarbon spin labels of the oxazolidine type were synthesized according to the Keana procedures (Williams et al., 1971) and were purified by thin-

layer chromatography. For those spin labels stored in organic solvents, the solvent was driven off by steam heating before the tristearin solutions were prepared. The three spin labels used consisted of dimethyl substituted oxazolidines attached to the hydrocarbon at the midpoint of the chain, so that the labels were all similar in structure and differed only in overall length. Fig. 1 presents the structures of the spin labels used.

Mixtures of tristearin andspin label were heated in a hot water bath until the solvent melted and the spin label was well dispersed. Aliquots of the solution were drawn into specially prepared thin-walled capillaries (inside diameter approximately 0.1 mm) which were then torch sealed. The samples were slowly heated until a liquid state was achieved, and were then mechanically accelerated into Freon-12 at −160°C or a dry ice-acetone bath at −78°C. The rapid cooling achieved by these procedures was essential for trapping spin labels randomly as isolated molecules in the solid tristearin matrix. Spectra were observed primarily on a Japan Electron Optics Laboratory Model JES ME-1X ESR spectrometer equipped with a variable temperature controller. Several spectra were also taken on a Varian Model 4502 EPR spectrometer (Varian Associates, Palo Alto, Calif.) equipped with a Model V-4540 variable temperature controller. The cavity temperature was monitored continuously during the runs.

Proton magnetic resonance experiments were carried out with a Varian A60-A spectrometer equipped with a Varian variable temperature controller.

Tristearin can occur in three different forms in the crystalline state. The lowest of the three melting points is 55°C for the $\alpha_L$-form, which is the form observed in tristearin cooled from the liquid state. It is therefore expected that the tristearin in these studies was in the $\alpha_L$-form below 55°C. Solutions referred to as being liquid at a particular temperature could be observed optically to have melted.

### *Impuirity Pool Formation*

Above the optical melting point of tristearin, the ESR spectrum of 7N14 in a tristearin matrix is typical of that for a freely tumbling, dispersed nitroxide. Fig. 3 shows a tracing taken at 60°C with a concentration of $10^{-2}$ M 7N14. If the sample is

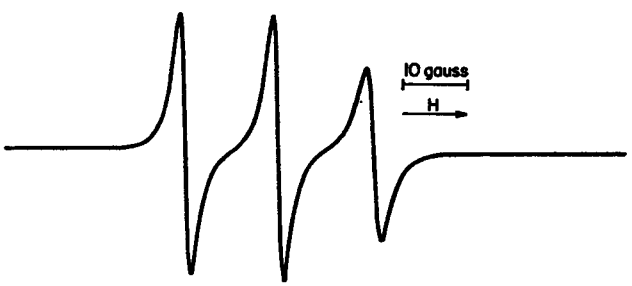


FIGURE 3 7N14 in tristearin at 60°C. The spin label concentration was $10^{-2}$ M.

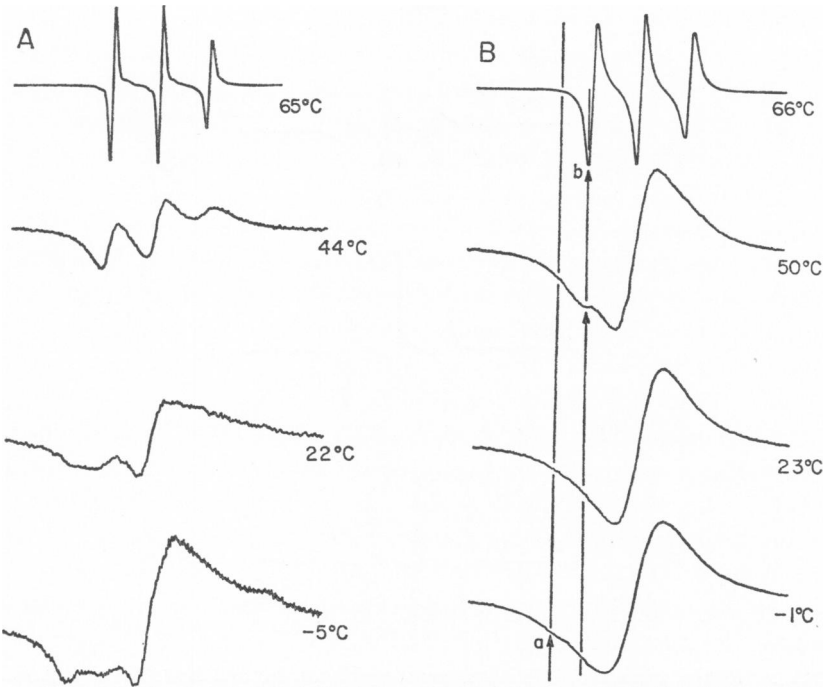


FIGURE 4 (*A*) $10^{-5}$ M solution of 7N14 in tristearin cooled slowly, with spectra recorded at the temperatures indicated. In this and all other spectral series the spectrometer sensitivity was adjusted to give similar peak-to-peak amplitudes for all the spectra. (*B*) $10^{-2}$ M solution of 7N14. Details as for Fig. 4 *A*.

slowly cooled to temperatures below the melting point, the spectrum observed shows characteristics of strong electron exchange. This indicates that the 7N14 molecules are no longer randomly dispersed, but have coalesced to form impurity pools of very high local spin label concentration. The appearance of this exchange pattern is dependent upon the concentration of 7N14, as is seen in Fig. 4. However, it seems likely that impurity pools are formed even at the lower concentrations of 7N14, but that these pools contain other impurities in amounts greater than that of 7N14 (Keith et al., 1973). This in effect would dilute the 7N14 molecules, preventing electron exchange, but would still allow the 7N14 molecules to tumble more freely than if they were randomly dispersed in tristearin at these temperatures. Data presented later confirm that isolated 7N14 molecules in tristearin can be immobilized much more effectively than the molecules giving the spectra of Fig. 4 A.

Two other nitroxides, 2N3 and 14N27, do not show this tendency to form impurity pools of high spin concentration in tristearin. The small spin label 2N3 tumbles freely over a wide range of temperatures at which tristearin is a solid but shows no electron exchange effects (Fig. 5). This was found to be the case for 2N3 concentrations of $10^{-5}$ M and $10^{-2}$ M, and was not dependent upon the rate at

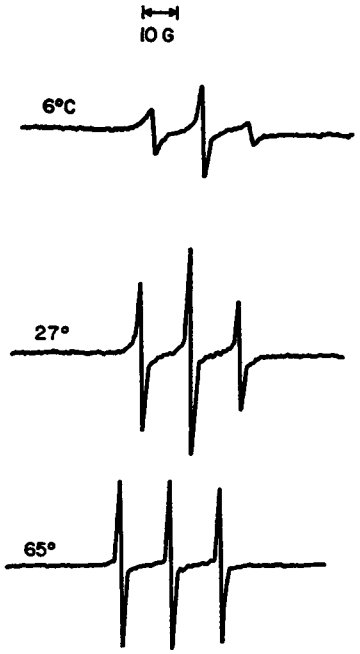


FIGURE 5 $10^{-5}$ M solution of 2N3 in tristearin rapidly cooled and heated slowly, showing spectra taken at three of the temperatures. The spectra for slow cooling from the liquid state were virtually identical to those at corresponding temperatures for slow heating.

which the samples were cooled. For all conditions used, the 2N3 molecules were sufficiently far apart to prevent significant electron exchange.

The long hydrocarbon spin label 14N27 likewise remained dispersed under all our experimental conditions. In contrast to 2N3, however, this spin label was strongly immobilized in solid tristearin (Fig. 6). This immobilization is to be expected because of the large size of 14N27 and the correspondingly greater opportunity for hydrophobic interactions between its hydrocarbon chains and the fatty acid residues of tristearin.

### *Translational Diffusion in Solid Tristearin*

The tendency of 7N14 to exist in pools of high local spin concentration offers the possibility of detecting the translational diffusion of this molecule in the solid tristearin matrix, provided that the 7N14 molecules can be initially trapped in a dispersed state. This trapping was achieved by extremely rapid cooling of the sample from the liquid state to −78 or −160°C (see Experimental section). The upper spectrum of Fig. 7 exhibits the characteristic outer lines (*a*) and central line of an immobilized molecule. Not all the 7N14 molecules are trapped as immobilized, isolated molecules, and other features of the spectrum indicate the presence of

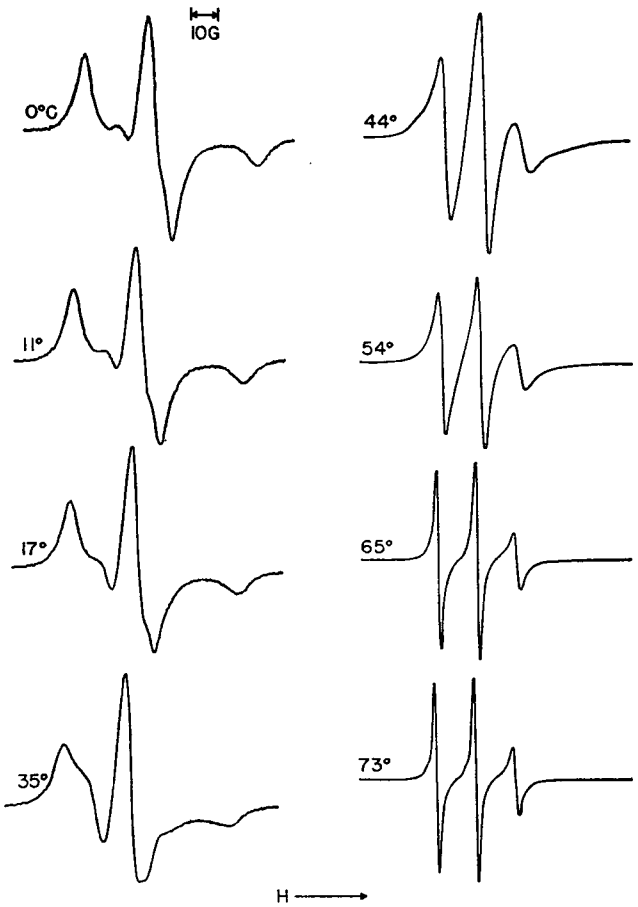


FIGURE 6 $10^{-2}$ M solution of 14N27 in tristearin rapidly cooled and heated slowly, with spectra taken at 0°C, 11°C, 17°C, 35°C, 44°C, 54°C, 65°C, and 73°C.

molecules with exchange effects and others that are isolated but not immobilized (*b*). When the temperature was raised to 44°C, 10°C below the melting point of tristearin, the spectrum underwent a rapid and very pronounced transition in which the outermost lines of the immobilized molecules disappeared in less than 10 min, broad inner lines grew, and were themselves replaced by a single broad line which reached a stable state in about 100 min (Fig. 7 E).

We offer the following interpretation of these data. As a consequence of the rapid cooling, a portion of the 7N14 molecules were trapped in an unaggregated, immobilized state in the tristearin matrix. At higher temperatures, but below the bulk melting point, these molecules were able to liquefy their immediate crystalline surroundings to form a local fluid zone. The spin label impurity could then migrate by liquefying the matrix along a continuous path until two such paths intersect, in which case the pools coalesce into a single pool containing both molecules. Con-

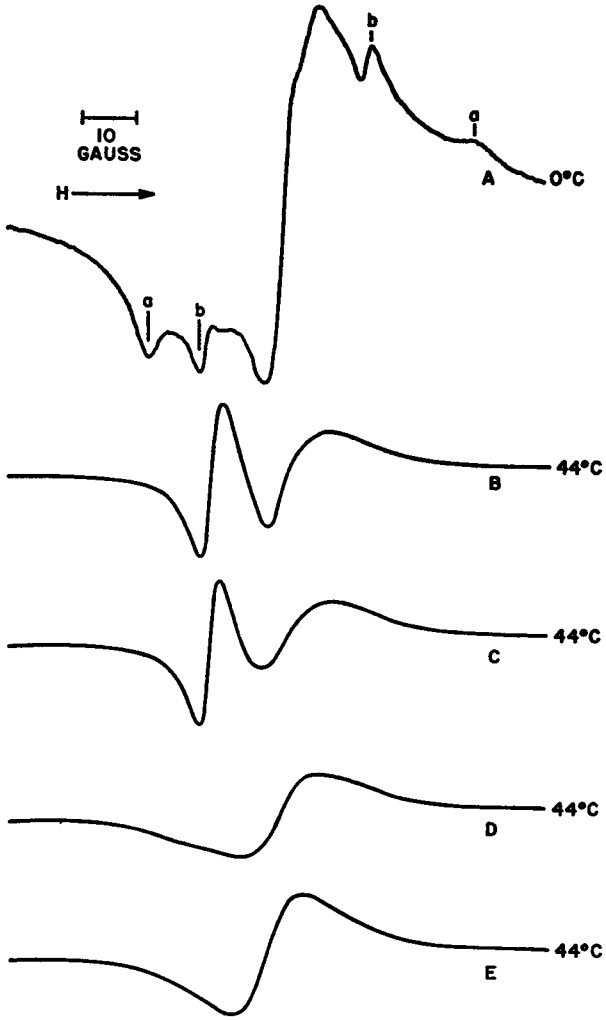


FIGURE 7 $10^{-2}$ solution of 7N14 in tristearin rapidly cooled, reheated to 44°C, and allowed to equilibrate for total times increasing from zero in *A* to 100 min in *E*.

tinuation of this process resulted in pools with very high local spin label concentration and the appearance of an ESR signal characteristic of exchange.

In this interpretation we have stressed the perturbing effect of the spin label impurity in liquefying an otherwise ordered, solid matrix. To substantiate the vast difference in molecular motion of the host tristearin molecules in the liquid vs. solid states, some nuclear magnetic resonance spectra were taken above and below its melting point. At 65°C, well resolved proton magnetic resonance absorptions typical of the liquid state were observed. At 45°C, no absorption could be detected at a sensitivity 10,000 times that used at 65°C. This extreme line broadening indi-

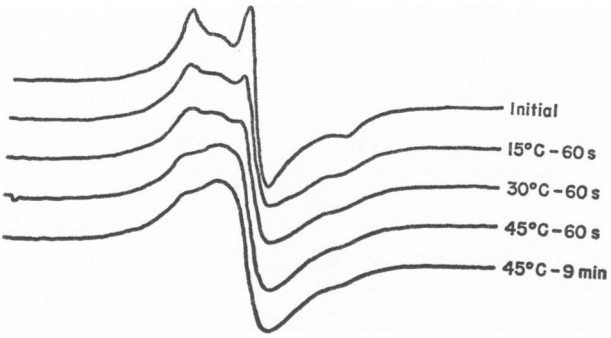


FIGURE 8 Pulsed heating of a $10^{-3}$ M solution of 7N14 in tristearin. The initial spectrum at −190°C followed rapid cooling; later spectra were taken after one minute pulses of heat at 15°C, 30°C, and 45°C, and after an additional 9 min at 45°C. All spectra were taken at −190°C after the indicated heating pulse.

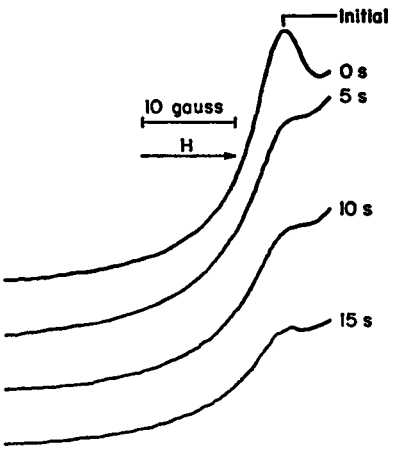


FIGURE 9 $10^{-3}$ M solution of 7N14 in tristearin given short exposures to 0°C. Total exposures to the 0°C bath were 0, 5, 10, and 15 s. There was no noticeable change for two further 5 s heating periods. All spectra were taken at −190°C.

cates that the host matrix is indeed quite solid below its optical melting point and little if any translational motion of the host molecules would be expected in the pure material.

Additional experiments were carried out to study more carefully the short term characteristics of translational diffusion and to study this process at lower temperatures. In Figs. 8–10, the spectra were all recorded at the same temperature (−190°C) and various heat treatments were applied for translational diffusion to occur. These three figures show the same general phenomenon under different conditions and give the qualitative aspects of translation; no quantitative description of the process was attempted. In summary, the migration of 7N14 through a solid tristearin

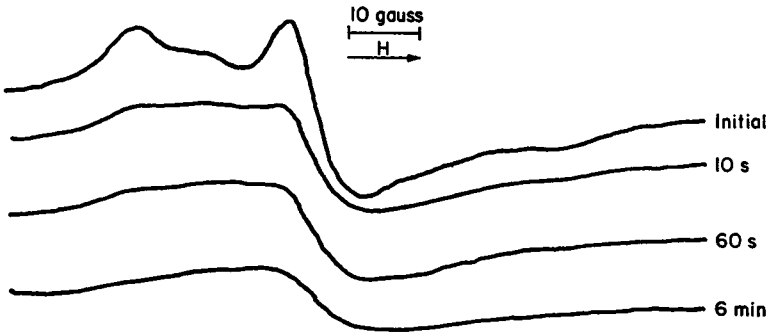


FIGURE 10 Pulsed heating at 45°C of a $10^{-3}$ M solution of 7N14 in tristearin: the initial immobilized signal and spectra taken for a total heating time (sum of all preceding pulses) of 10 s, 60 s, and 6 min.

matrix can be detected in a time interval as short as 5 s at a temperature as low as 55°C below the bulk melting point.

These data illustrate that molecular translational diffusion can occur in a solid tristearin matrix. Since such molecular events take place in a tristearin matrix, we believe that similar events may be import in the transport of a variety of molecules within or across biological membranes. Locally liquefying a solid matrix, or a relatively rigid bilayer zone, confers fluid properties which may enhance translational diffusion transport while maintaining the structural stability characteristic of a solid matrix or a relatively rigid bilayer.

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