M. (1973), *Biochemistry 12,* 2645.
Edelhoch, H. (1967), *Biochemistry 6,* 1948.
Fisher, W. R., Granade, M. E., and Mauldin, J. L. (1971), *Biochemistry 10,* 1622.
Fless, G. M. (1971), Ph.D. Thesis, University of Illinois, Urbana-Champaign.
Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226,* 497.
Forte, G. M., Nichols, A. V., and Glaeser, R. M. (1968), *Chem. Phys. Lipids 2,* 396.
Harris, C. E., Kobes, R. D., Teller, D. C., and Rutter, W. J. (1969), *Biochemistry 8,* 2442.
Hatch, F. K., and Lees, R. S. (1968), *Adv. Lipid Res. 6,* 1.
Lee, D. M., and Alaupovic, P. (1974), *Biochem. J. 137,* 155.
Lee, J. A., and Morris, M. D. (1974), *Biochem. Med. 10,* 245.
Lindgren, F. T., Jensen, L. C., Wills, R. D., and Freeman, N. K. (1969), *Lipids 4,* 337.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Moore, S. (1963), *J. Biol. Chem. 238,* 235.
Nelson, C. A., Lee, J. A., Brewster, M., and Morris, M. D. (1974), *Anal. Biochem. 58,* 69.
Nichols, A. V. (1967), *Adv. Biol. Med. Phys. 11,* 109.
Oncley, J. L., Walton, P. W., and Cornwell, D. G. (1957), *J. Am. Chem. Soc. 79,* 4666.
Pollard, H., Scanu, A. M., and Taylor, E. W. (1969), *Proc. Natl. Acad. Sci. U.S.A. 64,* 304.
Rush, R. L., Leon, L., and Turrel, J. (1971), *in* Advances in Automated Analysis, Technicon International Congress 1970, Vol. 1, Thurman Assoc., Miami, Fla., p 503.
Scanu, A. M., Edelstein, C., Vitello, L., Jones, R., and Wissler, R. (1973), *J. Biol. Chem. 248,* 7648.
Scanu, A. M., Pollard, H., and Reader, W. (1968), *J. Lipid Res. 9,* 342.
Scanu, A. M., Vitello, L., and Deganello, S. (1974), *Crit. Rev. Biochem. 2,* 175.
Schachman, H. K. (1957), *Methods Enzymol. 4,* 32.
Schumaker, V. N. (1973), *Acc. Chem. Res. 6,* 398.
Shore, B., and Shore, V. (1962), *J. Atheroscler. Res. 2,* 104.
Smith, R., Dawson, J. R., and Tanford, C. (1972), *J. Biol. Chem. 247,* 3376.
Tanford, C. (1961), *in* Physical Chemistry of Macromolecules, New York, N.Y., Wiley.
Teller, D. C. (1973), *Methods Enzymol. 27,* 346.
Toro-Goyco, E. (1958), Ph.D. Thesis, Harvard University, Cambridge, Mass.
Van Holde, K. E., and Sun, S. F. (1962), *J. Am. Chem. Soc. 84,* 66.
Wong, J. P.-W. (1972), M.A. Thesis, University of California, Los Angeles, Calif.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.

Fusion of Fatty Acid Containing Lecithin Vesicles†

Howard L. Kantor and James H. Prestegard*

ABSTRACT: The rate and temperature at which fusion of purified homogeneous lecithin vesicles containing several per cent fatty acid occurs have been determined by analysis of proton nuclear magnetic resonance (nmr) spectra. Dimyristoyllecithin with myristic acid as 2% of the lipid was found to fuse rapidly at temperatures between 17 and 20°, and dimyristoyllecithin with 4% lauric acid was found to fuse rapidly at temperatures between 11 and 15°, while dimyristoyllecithin with 4% palmitic acid did not fuse at an appreciable rate anywhere in the range 17–37°. These results, along with data on dipalmitoyl- and dilauroyllecithin, are discussed in terms of a possible dependence on separation of a fatty acid rich phase especially conducive to fusion.

Membrane fusion plays an important part in biological functions, such as storage, reproduction, stimulation, and response (Poste and Allison, 1973). It has been studied in a number of systems, most of which involve whole cells. Several agents have been found to stimulate fusion in these systems. Certain viruses, such as Sendai virus, have been shown to induce cell fusion (Okada, 1969; Poste, 1972). Lysolecithin causes erythrocytes to fuse (Poole *et al.,* 1970), though at the cost of cell viability. Fusion of mammalian cells has resulted from exposure to mixed phospholipid vesicles (Papahadjopoulos *et al.,* 1973), and a host of lipids have been found to induce fusion of hen erythrocytes (Ahkong *et al.,* 1973), among them unsaturated fatty acids, retinol, and α-tocopherol.

No uniform theory of membrane fusion has emerged from these studies, partly due to the inability to characterize at a molecular level the cell components primarily responsible for fusion. In an attempt to overcome this problem, recent investigations have turned to model systems involving phospholipid vesicles of well-defined composition. These structures have been thoroughly characterized (Huang, 1969; Chapman *et al.,* 1967; Johnson, 1973) and are known to undergo fusion processes. Fusion with cells which afterwards retain viability (Grant and McConnell, 1973), with multilamellar or vesicle structures (Papahadjopoulos *et al.,* 1974), and among vesicles (Taupin and McConnell, 1972; Prestegard and Fellmeth, 1974) has been

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demonstrated.

Homogeneous lecithin vesicle fusion has been characterized to the extent that the final structure is a closed bilayer of size greater than 2000 Å and that the vesicles remain closed at least until the instant of fusion (J. H. Prestegard and B. Fellmeth, unpublished results). In addition, spin-label experiments show that the fusion of two or more vesicles entails the mixing of internal contents, as well as significant leakage to the external solution (Taupin and McConnell, 1972).

In a recent paper we indicated that environmental conditions play an important role in accelerating the fusion process (Prestegard and Fellmeth, 1974). Vesicles prepared from impure dimyristoyllecithin (DML)[1] were found to fuse rapidly at 20 ± 2° with a profound fall-off in rate above and below this range. Preliminary indications have shown that pure DML did not exhibit fusion, and that small quantities of other lipids were necessary for fusion. The nature of the lipids involved was not determined, but myristic acid (M. Sheetz, personal communication) or myristoyllysolecithin are probable candidates. Verification of the role of myristic acid and other fatty acids in fusion is of primary concern in this paper.

As in our previous study, we monitor fusion by proton magnetic resonance techniques. In vesicle form, at temperatures above their gel to liquid-crystal phase transition, lecithins yield high-resolution spectra in which choline methyl, methylene, and chain terminal methyl protons are easily resolved (Chapman *et al.*, 1968; Penkett *et al.*, 1968; Horwitz, 1972). These resonances broaden as vesicle size increases (Sheetz and Chan, 1972; Gent and Prestegard, 1975) and become virtually indistinguishable for vesicles far above 2000 Å in diameter. Fusion which produces structures in excess of 2000 Å diameter can therefore be followed as an apparent decrease with time in the intensity of the lecithin high-resolution spectrum. The variation in rate of this decrease for vesicles incubated at various temperatures, and in the presence of various fatty acids, can yield clues to the prerequisites for vesicle fusion.

Materials and Methods

Dimyristoyllecithin (1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine), dipalmitoyllecithin (1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine), and dilauroyllecithin (1,2-dilauroyl-*sn*-glycero-3-phosphorylcholine) were purchased from Calbiochem, San Diego, Calif., and were purified on a silicic acid column by the method of Calacicco (1972). Spectroquality solvents were used whenever available in order to decrease impurities, even though no apparent change in behavior was observed for lecithins purified with reagent grade solvents. Fractions were analyzed by thin-layer chromatography (tlc) with a chloroform–methanol–water (65:25:4) developer. The first and last fractions showing the presence of lecithin were discarded to further guard against contamination that would not appear in this acknowledged low sensitivity analysis. The lecithin preparations are therefore unlikely to contain any free fatty acid or lysolecithin, but the presence of minor contaminants introduced by the sample preparation procedure itself cannot be excluded.

Samples were prepared by dissolving known weights of fatty acids in chloroform, and mixing measured volumes of these solutions with lecithins. The resultant solutions were dried under vacuum for at least 8 hr. When a mixture of two lecithins was prepared, dry weights were mixed, then dissolved in chloroform, and dried for a comparable time. After drying, samples were suspended in a quantity of D_2O–phosphate buffer (pH 7.2 with 0.02% NaN_3 added to retard bacterial growth) sufficient to achieve a total lipid concentration of 1–5%. They were then sonicated in a 40-kHz Branson Model E bath sonicator at 35–40° for at least 2.5 hr until the sample became clear (DPL samples were sonicated at 40–50°). Samples were usually stored at 40–55° for 1 or more hr before proceeding, to allow for equilibration.

Samples for which we anticipated fusion were incubated in constant temperature baths which were controlled to ±0.5° when above 10° and to ±1° when below 10°. For samples which were expected to show no fusion, the effect of incubation at entire ranges of temperature was obtained by incubating in a temperature bath with a motor driven temperature control. Rates of temperature variation were so chosen that the sample spent approximately 1 hr in every one-degree range. It is possible, though, that very slow fusion occurring in a narrow temperature range could be missed. In all experiments the appropriate purified lecithin or lecithins were used as controls by incubating them simultaneously with the fatty acid containing sample under study.

Samples were periodically removed from their incubating baths, and nuclear magnetic resonance (nmr) spectra were obtained on a JEOLCO 100-MHz spectrometer. Areas for choline methyl, chain terminal methyl, and methylenes were measured by cutting and weighing peaks, then examining them relative to the spectrum of a Mn^{2+} doped D_2O–H_2O external standard. The areas were reproducible to better than ±5%. The spectrometer temperature was 37 ± 1° for DML[1] and DLL[1] experiments, and 53 ± 2° for DPL[1] and DPL–DML experiments. Spectra of samples maintained at these temperatures did not change significantly over time.

Half-times for fusion were measured directly from spectra where possible, but in order to improve accuracy and use all data points, a half-time ($t_{1/2}$)[1] for the decay process was also calculated by fitting points to an assumed rate equation. In our previous work (Prestegard and Fellmeth, 1974) a third-order equation was found to give reasonable fit when allowance was made for the fact that choline intensity does not extrapolate to zero. In this study the residual intensity was found to be approximately 20% of the initial. In cases where both procedures could be followed, $t_{1/2}$ values agreed reasonably well. It must be stressed that an assumption of reaction order was adopted merely as a means of data reduction and should not be taken to reflect on the fusion mechanism.

Results

In confirmation of preliminary results reported by Prestegard and Fellmeth (1974), silicic acid purified DML vesicles did not decay significantly over a period of 48 hr when incubated at 20° (the optimum fusion temperature observed for commercially available DML) nor could we detect fusion by slowly increasing the temperature at 1°/hr from 17 to 43°.

Upon addition of myristic acid (C_{14}), making up 2% of the lipid, fusion at 20° was restored to the purified DML vesicle preparation. Typical spectra from such an experi-

[1] Abbreviations used are: DML, dimyristoyllecithin; DPL, dipalmitoyllecithin; DLL, dilauroyllecithin; T_c, gel (solid)–liquid crystalline phase transition temperature; $t_{1/2}$, half-time.

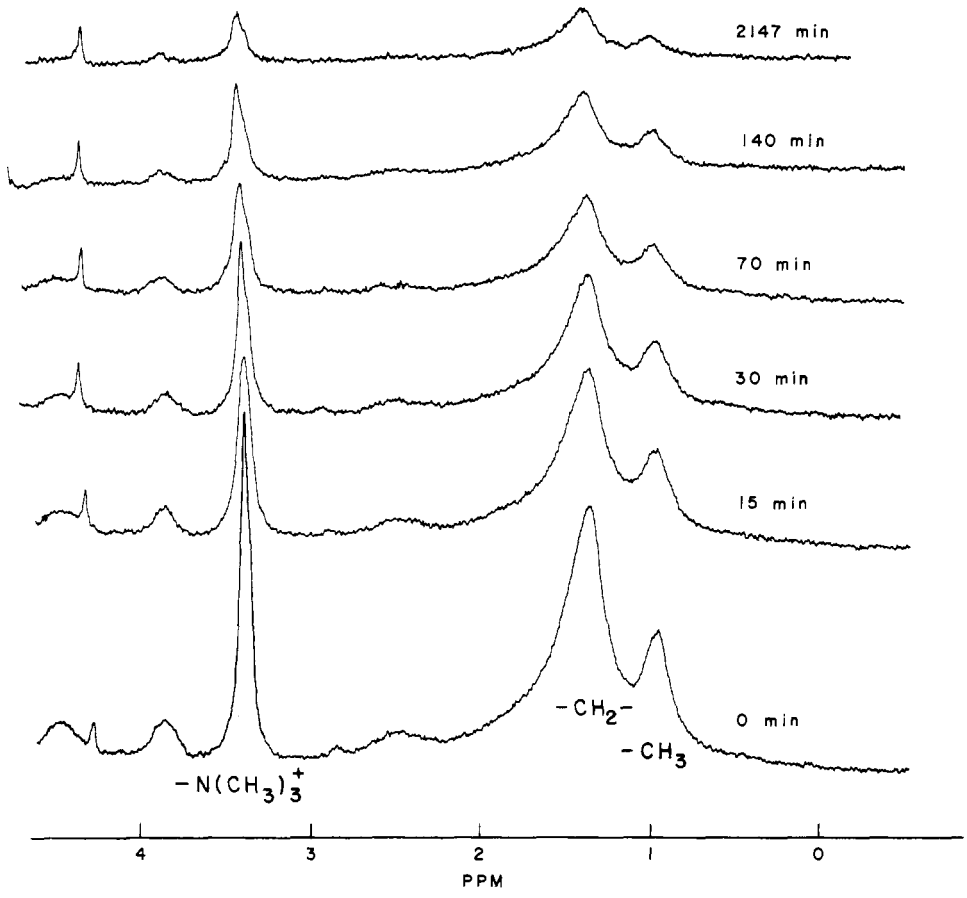

FIGURE 1: Proton nmr spectra of a 5% solution of DML with 2% of the lipid as myristic acid. Samples were incubated at 20° for stated time periods, and then examined at 37°.

ment are displayed in Figure 1. Note that there appears to be a time-dependent decrease in amplitude of the narrow resonances. Analysis of this time dependence at various temperatures shows that the rate of fusion in this system is maximum in the same temperature range as the unpurified commercial DML. Observe, though, that contrary to previous experience the width at half-maximum of the choline peak does not increase with time, suggesting that intermediate size structures, which are known to have broadened nmr peaks (Sheetz and Chan, 1972; Gent and Prestegard, 1975), are much less predominant than indicated in the earlier paper. This difference may be due to the presence of other impurities besides fatty acid, or to the different concentration of lecithin in the previous work.

The quantity of myristic acid necessary to restore fusion did not seem to be sharply defined, but the rate of fusion increased gradually as fatty acid content was increased from 1 to 6%. Beyond 6%, results became quite erratic. A 5% lecithin sample, in which fatty acid comprises 2% of the total lipid, reproduces nearly quantitatively the behavior of the previously unpurified samples.

Total concentration of lipid, at least in the range 1–10%, did not seem to affect initial rates of fusion greatly. This seems to belie the apparent third-order rate observed when a single sample is followed to near complete fusion.

To further elucidate the prerequisites for a fusion inducing agent, a number of other mixed homogeneous lecithin–fatty acid systems, including DML containing lauric (C_{12}) and palmitic (C_{16}) acids, were examined. Samples were 5% lipid in D_2O–phosphate buffer (w/w) with 4% of the lipid being fatty acid. The higher concentration of fatty acid in the lauric acid and palmitic acid samples was adopted to ensure detection of possible slow fusion processes.

In contrast to the rapid fusion observed between 17 and 22° for DML containing myristic acid, when lauric acid (C_{12}) was added to DML, the maximum fusion rate was noted in the temperature range 11–15°, with negligible decay at 20°. Fusion behavior with palmitic acid in DML vesicles departed even more drastically from DML–myristic acid behavior by exhibiting negligible decay when the temperature was slowly varied between 17 and 37°, or incubated for long periods at 20, 35, or 40°. These results are summarized graphically in Figure 2 as a plot of log $(1/t_{1/2})$ *vs.* temperature. The $t_{1/2}$ values have been determined by averaging $t_{1/2}$ values calculated for the decay of the choline and combined methylene–terminal methyl peaks and using these two values as error limits.

The data indicate that the length of the fatty acid not only determines the presence or absence of fusion, but also influences the temperature dependence and relative rate of decay. In reference to the difference in fatty acid concentration between myristic acid and lauric acid containing samples, it is important to note that even though the fatty acid content of the myristic acid–DML sample was one-half that of the lauric acid–DML sample, the former had a minimum $t_{1/2}$ that was approximately 20 times less than the latter.

The chain length of the lecithin also produced a pronounced variation in fusion behavior. Fusion was noted to occur for a DPL–myristic acid sample, well above 20° but below 50°, but not for purified DPL alone in the same temperature range. The 20–50° range was scanned over a period in excess of 24 hr. Preliminary calorimetry results indi-

Table I: Effects of Various Lecithins and Fatty Acids on Fusion.

Sample	Fusion	Temp, °C
DML commercial	Yes	Max at 20
DML purified	No	17–43
DML + myristic acid	Yes	Max at 17–20
DML + lauric acid	Yes	Max at 11–15
DML + palmitic acid	No	17–37
DPL purified	No	20–50
DPL + myristic acid	Yes	20–50 (possible 37° max)
DLL purified	No	0–30
DLL + myristic acid	No	0–30
DML + DPL (1:1, w/w) purified	No	19–43

cate that 37° is the most likely temperature maximum for this decay (S. Mabrey *et al.*, manuscript in preparation). DLL was examined in the temperature range of 0–30° over a 24-hr period, both in the purified form and with myristic acid, without any appreciable change in the nmr spectrum.

An attempt was also made to detect fusion in a mixed lecithin sample containing no fatty acid. DML and DPL were mixed in chloroform, dried and sonicated, and then observed in the temperature range 19–43° over about 24 hr. No demonstrable change greater than the change in controls consisting of pure DML and pure DPL was observed by nmr. This experiment has important bearing on the requirement for a two-phase region which will be discussed later. Results are summarized in Table I.

Discussion

The results clearly indicate that homogeneous lecithin vesicle fusion is greatly enhanced by the presence of a second lipid, in these experiments, a fatty acid. We also recognize that fusion only occurs in narrowly defined temperature ranges. These ranges are different for each sample, but in general they are near the multilayer gel (solid)–liquid crystalline phase transition (T_c) for the lecithin studied (T_c = 23.7° for DML, 41° for DPL, T_c = ~0° for DLL (Chapman *et al.*, 1967)). It is also noted that the approximate range over which fusion occurs is 8° (as seen in Figure 2), a plausible range over which two phases could coexist in a lecithin system (Phillips *et al.*, 1970). These facts suggest that coexistence of two phases, near the gel–liquid crystalline phase transition, one rich in the minor component and one poor in the minor component, may be essential for fusion.

If the presence of two phases is necessary for fusion, then a rationale can be offered for the shift of fusion temperature ranges observed for the lauric acid–DML *vs.* myristic acid–DML systems. Assuming lauric acid, with its shorter hydrocarbon chain, depresses the melting point of the lecithin–fatty acid phase more than myristic acid, and that these mixed systems behave ideally, a simple phase diagram may be sketched, a small section of which is illustrated in Figure 3. With all other influencing factors held constant, the lower melting point of the lecithin–lauric acid sample would cause the phase lines to have a steeper downward slope than those of the lecithin–myristic acid system, yielding a lower temperature for the two-phase region, and therefore a lower temperature range of fusion.

The coexistence of two phases has been suggested as an important factor in enhanced transport rates in several cellular systems. Transport of β-glucoside in *Escherichia coli* is an example (Linden *et al.*, 1973). The increased compressibility on separation of two phases has been suggested to be the determining factor in these transport phenomena. A similar mechanism may be responsible for fusion.

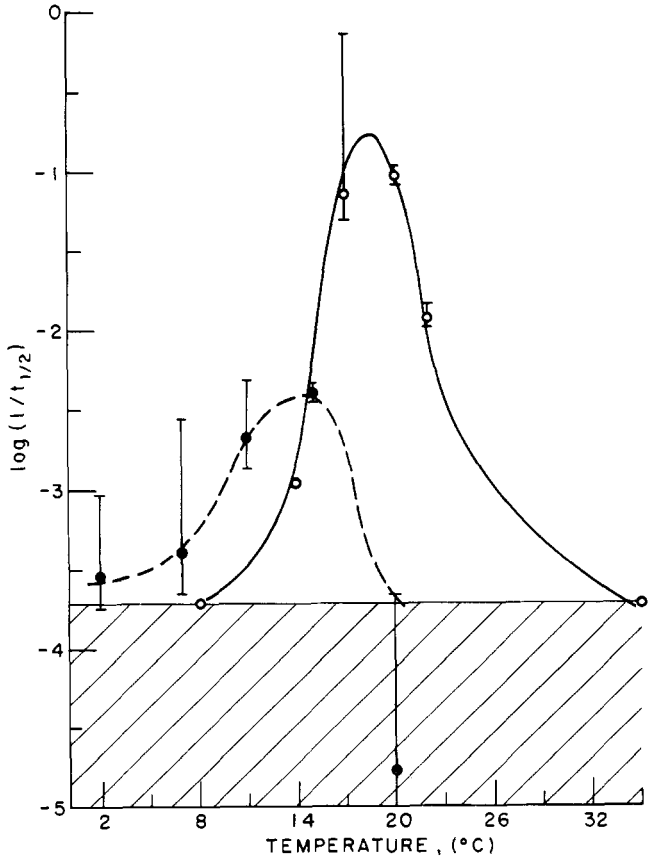

FIGURE 2: Log ($1/t_{1/2}$) ($t_{1/2}$ in multiples of 1 min) determined for fusion of 5% DML with myristic acid being 2% of the lipid (○), and for 5% DML with 4% of the lipid as lauric acid (●). Cross-hatched area indicates values of log ($1/t_{1/2}$) representing rates of sample decay slower than that of controls.

It is clear, however, that for vesicle fusion, the coexistence of two lipid phases is not the only factor essential for enhanced fusion rate. Purified DML vesicles surely exist in two phases at their melting point, and DML–DPL vesicles are likely to have a two-phase region over an extended temperature range (33–38°, extrapolated from data of Shimshick and McConnell, 1973, on unsonicated dispersions), yet results from scanning temperature experiments, within their limitations as stated above, indicate that no fusion occurs for these samples.

Chain length of the fatty acid involved also seems to have a profound influence on the rate of fusion. Myristic acid containing vesicles fuse at about 20 times the rate of lauric acid containing vesicles. Palmitic acid containing vesicles do not appear to fuse at all.

A plausible explanation for the relative rates of the myristic and lauric acid containing samples could involve a difference in effective concentration of fatty acids in the vesicles. Although quantitative consistency could not be found, results with various concentrations of myristic acid show that rates do increase with increasing fatty acid concentration, at least until fatty acid accounts for 6% of the lipid.

On the basis of partition coefficient analysis, which is reasonable as the fatty acids are below their critical micelle

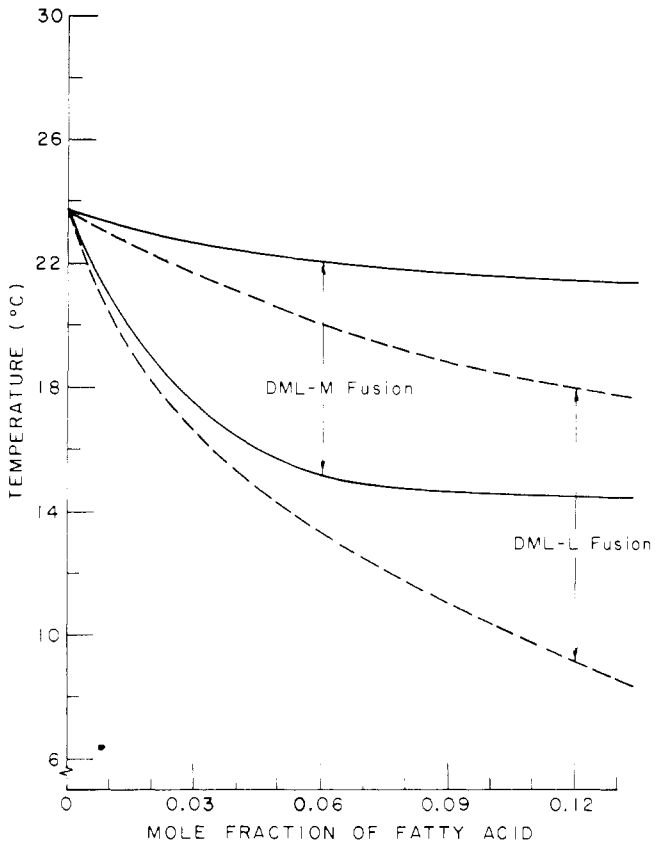

FIGURE 3: Portion of hypothetical phase diagram for aqueous vesicle dispersion of low concentration of DML with myristic acid (—) and DML with lauric acid (- - -).

concentration (Malik *et al.*, 1967), one might expect to find higher effective concentrations for myristic acid in the lipid phase. Data describing the hydrocarbon–water distribution of fatty acids (Smith and Tanford, 1973), extrapolated to vesicle systems, indicate however that essentially all the fatty acid is in the vesicle phase, thereby not accounting for the difference in fusion rates.

One major difference between the palmitic acid system and the myristic acid and lauric acid systems is that the former might be expected to separate a solid fatty acid rich phase, rather than a fluid one upon entering the two-phase region. Suggestions that increased fluidity plays an important part in fusion have been made previously, and would seem to be supported here. It is clear, however, that accurate determinations of phase diagrams and accurate characterization of the fluidity of various phases must precede any firm conclusions.

The nature of the fatty acid may play a more central role in inducing fusion if a phase separates in the form of a molecular complex. Frank and Barton (1973) have observed that alcohols of length C_8–C_{14} in molar ratios less than 1:1 have increased the T_c of DML dispersions. It is thought that these alcohols form stoichiometric complexes with the lecithins by molecular association inducing a more ordered hydrocarbon phase. Similar behavior for fatty acids would be consistent with calorimetric data on DML–fatty acid vesicles (S. Mabrey *et al.*, manuscript in preparation), and could account for increased fusion behavior of vesicles containing only certain fatty acids.

It should be mentioned that our results for DPL are not in strict agreement with the spin-label work of Taupin and McConnell (1972). This could be due to a difference in sample purity or to an effect of the spin label itself. Our results do appear, though, to follow trends observed in the whole cell systems of Ahkong *et al.* (1973), who found that lauric and myristic acids induce fusion of hen erythrocytes at 37° while palmitic acid does not. These results lend support to arguments which extrapolate results to cell systems.

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Added in Proof

A later lot of DML (no. 400488) was initially observed not to fuse as readily as the DML (lot no. 300625 and 201408) used in experiments presented here. Upon careful examination, we found a contaminant in this lot which migrated on silicic acid very much like lecithin. Addition of a fraction containing this contaminant to an earlier lot number inhibited fusion. This observation emphasizes the difficulty in obtaining phospholipids of reproducible purity.

References

Ahkong, Q. F., Fisher, D., Tampion, W., and Lucy, J. A. (1973), *Biochem. J. 136,* 147.

Calacicco, C. (1972), *Biochim. Biophys. Acta 266,* 313.

Chapman, D., Fluck, D. J., Penkett, S. A., and Shipley, G. G. (1968), *Biochim. Biophys. Acta 163,* 255.

Chapman, D., Williams, R. M., and Ladbrooke, B. D. (1967), *Chem. Phys. Lipids 1,* 445.

Frank, H. K., and Barton, P. G. (1973), *Biochim. Biophys. Acta 296,* 510.

Gent, M. P. N., and Prestegard, J. H. (1975), *Biochemistry* (in press).

Grant, C. W. M., and McConnell, H. M. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 1238.

Horwitz, A. F. (1972), *in* Membrane Molecular Biology, Fox, C. F., and Keith, A. D., Ed., Stamford, Conn., Sinauer Associates.

Huang, C. (1969), *Biochemistry 8,* 344.

Johnson, S. M. (1973), *Biocim. Biophys. Acta 307,* 27.

Linden, C. D., Wright, K. L., McConnell, H. M., and Fox, C. F. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 2271.

Malik, W. V., Ahmad, S. I., and Jain, A. K. (1967), *Kolloid-Z. Z. Polym. 218,* 155.

Okada, Y. (1969), *Current Top. Microbiol. Immunol. 28,* 102.

Papahadjopoulos, D., Poste, G., and Schaeffer, B. E. (1973), *Biochim. Biophys. Acta 323,* 23.

Papahadjopoulos, D., Poste, G., Schaeffer, B. E., and Vail, W. J. (1974), *Biochim. Biophys. Acta 352,* 10.

Penkett, S. A., Flook, A. G., and Chapman, D. (1968), *Chem. Phys. Lipids 2,* 273.

Phillips, M. C., Ladbrooke, B. D., and Chapman, D. (1970), *Biochim. Biophys. Acta 196,* 35.

Poole, A. R., Howell, J. I., and Lucy, J. A. (1970), *Nature (London) 227,* 810.

Poste, G. (1972), *Int. Rev. Cytol. 33,* 157.

Poste G., and Allison, A. C. (1973), *Biochim. Biophys. Acta 300,* 421.

Prestegard, J. H., and Fellmeth, B. (1974), *Biochemistry 13,* 1122.

Sheetz, M. P., and Chan, S. I. (1972), *Biochemistry 11,* 4573.

Shimshick, E. J., and McConnell, H. M. (1973), *Biochem-*

istry 12, 2351.
Smith, R., and Tanford, C. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 289.
Taupin, C., and McConnell, H. M. (1972), *Mitochondria/Biomembranes, Fed. Eur. Biochem. Soc. Symp. 9th, 28,* 219.

The Specificity of Different Classes of Ethylating Agents toward Various Sites of HeLa Cell DNA in Vitro and in Vivo†

L. Sun and B. Singer*

ABSTRACT: The sites and extent of ethyl products of neutral ethylation of HeLa cell DNA by [^{14}C]diethyl sulfate, [^{14}C]ethyl methanesulfonate, and [^{14}C]ethylnitrosourea have been determined in vitro and in vivo, and found to differ significantly depending on the ethylating agents. Diethyl sulfate and ethyl methanesulfonate ethylate the bases of HeLa cell DNA in the following order: 7-ethylguanine > 3-ethyladenine > 1-ethyladenine, 7-ethyladenine > 3-ethylguanine, 3-ethylcytosine, O^6-ethylguanine. Ethyl bases accounted for 84–87% of the total ethyl groups associated with HeLa cell DNA. Ethylnitrosourea, in contrast, has particular affinity for the O^6 position of guanine. It ethylates the bases of HeLa cell DNA in the following order: O^6-ethylguanine, 7-ethylguanine > 3-ethyladenine > 3-ethylguanine, 3-ethylthymine > 1-ethyladenine, 7-ethyladenine, 3-ethylcytosine. Ethylation of the bases only accounts for 30% of the total ethylation in the case of ethylnitrosourea. The remaining 70% of the [^{14}C]ethyl groups, introduced in vivo and in vitro, are in the form of phosphotriesters which after perchloric acid hydrolysis are found as [^{14}C]ethanol and [^{14}C]ethyl phosphate. In contrast, phosphotriesters amounted to only 8–20% of total ethylation in in vivo or in vitro diethyl sulfate and ethyl methanesulfonate treated HeLa cell DNA, and 25% of the total methylation in in vitro methylnitrosourea treated HeLa cell DNA. Alkylation at the N-7 and N-3 positions of purines in DNA destabilizes the glycosidic linkages. Part of 7-ethylguanine and 3-ethyladenine are found to be spontaneously released during the ethylation reaction. Incorporation of the ^{14}C of the alkylating agents into normal DNA bases of HeLa cells can be eliminated by performing the alkylations, in the presence of cytosine arabinoside, for 1 hr.

Different classes of alkylating agents differ in their affinity for a site on a nucleic acid. There are three types of alkylation that can occur in a nucleic acid, namely, base, ribose, and phosphate alkylation. However, in the case of DNA, ribose alkylation is not possible. Methylation of the bases in DNA has been studied extensively by using a variety of methylating agents both in vitro (Lawley and Brookes, 1963; Lawley et al., 1969; Lawley and Thatcher, 1970; Lawley et al., 1973; Lawley and Shah, 1973; Margison and O'Connor, 1973) and in vivo, either in animals (Lawley et al., 1968; Craddock, 1973; O'Connor et al., 1973; Kleihues and Magee, 1973) or in tissue culture (Lawley and Thatcher, 1970; Walker and Ewart, 1973). Phosphate alkylation was not studied in detail until Rhaese and Freese (1969) found that methyl methanesulfonate and ethyl methanesulfonate at neutrality rapidly alkylated the phosphate of dTMP and oligo(dT) forming phosphotriesters. Bannon and Verly (1972) later reported that not only were phosphotriesters quite stable in alkylated DNA, but also presented data that treatment of T7 phage or T7 phage DNA with ethyl methanesulfonate caused formation of 15 times more triesters than treatment with methyl methanesulfonate (Bannon and Verly, 1972; Verly et al., 1974).

Singer and Fraenkel-Conrat (1975) have treated TMV-RNA with diethyl sulfate, ethyl methanesulfonate, and ethylnitrosourea and found that these ethylating agents differed in their specificity. Diethyl sulfate and ethyl methanesulfonate predominantly ethylated the bases and produced relatively small amounts of O^6-ethylation on guanine and phosphate ethylation. Ethylnitrosourea, on the other hand, had a strong affinity for oxygen and O^6-ethylguanine was the major product of base ethylation. Moreover phosphotriesters represented up to 60% of the total ethylation. In this work we are dealing with the effects of these three types of ethylating agents acting on DNA. HeLa cell DNA has been treated with these reagents in vitro and in tissue culture. In both cases, the extent and sites of base ethylation and phosphate ethylation were determined and the reagent specificity was studied.

When Goth and Rajewsky (1974a,b) investigated the carcinogenic effect of ethylnitrosourea in animals they found that base ethylation amounted to only 20–30% of the total radioactivity, while the remainder (70–80%) which has not been described by other investigators, was not bound to the cation-exchange column and thus possibly represented phosphotriesters. The present study on ethylation of HeLa cell DNA in vitro and in vivo with ethylnitrosourea identifies such products as phosphotriesters.

We also present evidence for the first time that a significant part of the total 7-ethylguanine and 3-ethyladenine is released from DNA during the period of ethylation.

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