Studies on the Molecular Packing of Mixed Dispersions of Triton X-100 and Sphingomyelin and Its Dependence on Temperature and Cloud Point†

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ABSTRACT: Mixtures of sphingomyelin and Triton X-100 were dispersed in aqueous media and molecular details of the mixed micelles were studied by proton magnetic resonance (^{1}H NMR). Most signals narrowed when the molar ratio of Triton X-100 to sphingomyelin increased, most likely as a consequence of looser molecular packing within the mixed micelle. These changes in molecular packing might be a prime cause for the increased rates of enzymatic hydrolysis of sphingomyelin, observed upon addition of Triton X-100. The changes in the line widths of the various constituents support the model suggested by S. Yedgar et al. [(1974a) *Biochim. Biophys. Acta 363*, 98–111] for the TR–SM mixed micelles. When heated to 65 °C, aqueous dispersions of TR become turbid; this temperature is defined as the "cloud point" of the detergent (T_c). Addition of sphingomyelin decreased the cloud point temperature of the mixed dispersion. Studies of optical properties and ^{1}H NMR spectroscopy suggested that the clouding is a consequence of formation of clusters of mixed micelles of TR and SM. The motional state of the TR molecules in those micelles which are directed toward the bulk aqueous phase does not change as a consequence of the clustering. Kinetic studies were done on the effect of temperature on the rate of hydrolysis of mixed micelles of TR and SM by rat brain sphingomyelinase. Above the cloud point, the effective concentration of substrate, which is available for interaction with the enzyme, decreased, resulting in lower reaction rates at subsaturating concentrations of substrate.

The enzymatic hydrolysis of sphingomyelin (SM)[1] by sphingomyelinase is frequently done in the presence of the nonionic detergent Triton X-100 (TR) (Gatt, 1973; Gatt & Barenholz, 1973). (The formulas of this detergent and of SM are shown in Figure 1.) A previous study (Yedgar & Gatt, 1976) showed that the rates of the enzymatic hydrolysis of SM by rat brain sphingomyelinase increased with increasing molar ratio of TR to SM (herein denoted R). Thus, increasing R from 0.5 to 3.0 resulted in a twofold increase in the rate of hydrolysis of SM. This elevated activity is a consequence of changes in the physical properties of the mixed dispersion of detergent and lipid, as follows. When R equals 0.5 to 4.0 (M/M), the system contains a homogeneous population of SM–TR mixed micelles. Increasing R within this range results in a decrease in the size of the mixed micelles and their shape changes from ellipsoidal to spherical (Yedgar et al., 1974a). At R <0.5, micelles of about 480 000 (similar to those observed at R = 0.5) are present admixed with greater multilamellar aggregates. At R >4.0, the dispersions contain mixed micelles of a molecular weight of about 160 000 (i.e., the same as that observed at R = 4.0), together with micelles of pure Triton X-100 (Yedgar et al., 1974a).

The effect of R on the rate of the enzymatic hydrolysis was interpreted as a result of "loosening" of the molecular packing in the mixed micelle by increasing detergent (Yedgar & Gatt, 1976). This hypothesis accords with previous NMR investigations which suggested that addition of Triton X-100 resulted in a loosening of packing in mixed micelles of phosphatidylcholine (PC) and this detergent (Dennis & Owens, 1973; Ribeiro & Dennis, 1975a,b). These reports discussed in a detailed manner systems containing TR to PC molar ratios greater than 2, where the mixed micelles were considered to be spherical. Size and shape changes were not suggested as a possible explanation for the loosening effect which accompanied the increase in the above ratio.

Studies on the solubilization of SM by TR (Hertz & Barenholz, 1975, 1977) and on its utilization by enzymes (Yedgar & Gatt, 1976) showed several differences between this system and those containing PC (Dennis, 1973). SM–TR mixtures therefore warranted a study to elucidate details of the packing of the lipid molecules in these mixed micelles. This paper reports a proton magnetic resonance (^{1}H NMR) study aimed at this goal. It also elaborates on the effect of temperature on the physical state of the mixed dispersions and on the enzymatic utilization of the lipid substrate. Aqueous dispersions of nonionic detergents become turbid when heated to certain temperature, defined as the "cloud point" (T_c). It is generally accepted that this "clouding" is a consequence of formation of large aggregates, probably due to dehydration of the polar region of the micelles (Nakagawa & Shinoda, 1963; Nakagawa, 1967). The cloud point temperature is influenced by the presence of other compounds which may raise or lower it (Arai, 1967; Nakagawa, 1967; Satto & Shinoda, 1967; Nakagawa & Shinoda, 1963). These changes are not directly proportional to the concentration of the additives and exhibit considerable variability. A general theory that relates the change in T_c to the structure of various additives has not yet emerged. Nevertheless, there seems to be a direct relationship between the capacity of a solubilizate to form hydrogen bonds with the etheric oxygens of a surfactant and its ability to suppress the T_c of the latter (Donbrow & Azaz, 1976).

Dipalmitoylphosphatidylcholine (DPL), 25 mM, lowered the cloud point of 50 mM TR to about 54 °C (Ribeiro & Dennis, 1974). SM, which has two acidic protons in its molecule, might be expected to affect the cloud point of TR even more than lecithin. Such a depression might bring the cloud point within the range of temperature at which the

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[1] Abbreviations used: PL, phospholipid; SM, sphingomyelin; TR, Triton X-100; PC, phosphatidylcholine; $\nu_{1/2}$, the width (in Hz) at half-height of the signal; T_c, cloud point temperature; R, the molar ratio of Triton X-100 to sphingomyelin.

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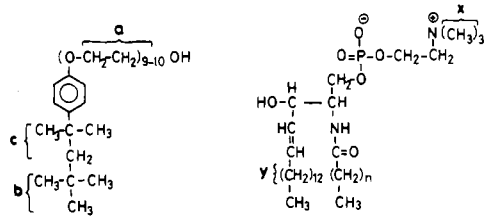

FIGURE 1: Chemical formulas of sphingomyelin and Triton X-100. The assignments a, b, c, x, and y relate each group to its ^{1}H NMR signal as shown in Figure 3.

enzymatic hydrolysis of SM was studied (Gatt, 1973; Yedgar & Gatt, 1976; Gatt et al., 1973; Barenholz et al., 1966).

In this paper we studied the effect of temperature on physical properties of mixed aggregates of TR and SM at various molar ratios of these components. The methods used included turbidity measurements, light scattering, and magnetic resonance spectroscopy. The results of these studies were related to the rate of enzymatic hydrolysis of SM by sphingomyelinase at corresponding temperatures.

Materials and Methods

Sphingomyelin was prepared from bovine brain as described previously (Barenholz et al., 1966). Tritium-labeled sphingomyelin of spinal cord was prepared by catalytic hydrogenation with tritium gas in the presence of palladium on charcoal (Gatt et al., 1973) and was diluted with nonradioactive SM of bovine brain. Triton X-100 was purchased from BDH and one lot (no. 30532) was used throughout. Tritium-labeled Triton X-100 was a generous gift of Rohm and Haas; it was further purified and the purity of the two preparations was checked chromatographically in two solvent systems as described (Yedgar et al., 1974b).

Sphingomyelinase of rat brain was an extract of lysosomes of brains of 14 day old rats (Gatt & Gottesdiner, 1976). Its specific activity was about 1000 nmol of sphingomyelin hydrolyzed by 1 mg in 1 h at R = 3 and 37° C.

The rate of enzymatic hydrolysis of sphingomyelin was determined by the method of Barenholz et al. (1966) as modified for assaying the hydrolysis of lecithin (Gatt, 1968). The assay mixtures were prepared by method B of Yedgar et al. (1974b). Incubation time was 1 h at constant temperature. The results reported here are the average of two or more determinations.

D_2O (99.7%) and CD_3COOD were purchased from Merck.

Aqueous dispersions of Triton X-100 and sphingomyelin were prepared by mixing solutions of the two components in chloroform and evaporating the solvent to dryness in vacuo; 0.1 M CH_3COO^- buffer at pH 5.4 or 0.1 M CD_3COO^- buffer at pD = 5.4 (determined by adding 0.4 unit to the pH reading) was added; the tubes were stirred on a cyclomixer and incubated at 37 °C for 15 min. A separation of phases did not occur in these dispersions at any temperature.

^{1}H NMR spectra were recorded on a Jeol MH-100 instrument, at a probe temperature controlled by Jeol JES-VT-3B temperature controller and adjusted by the chemical shift difference between the two signals of external ethylene glycol. Line widths (expressed as $\nu_{1/2}$, the width at half height of the peak) were measured using a sweep width of 3 Hz/cm and a sweep rate of 1 Hz/s. Experimental errors (which are mainly due to base-line determination) depended on the widths and were estimated as ±10% for samples in which narrow lines ($\nu_{1/2}$ < 25 Hz) of the paraffinic protons were observed and up to ±50% of the measured $\nu_{1/2}$ for much broader lines. The line widths were corrected for field inhomogeneity by subtracting the HOD line width from the experimental values $\Delta\nu_{1/2} = \nu_{1/2}(\text{signal}) - \nu_{1/2}(\text{HOD})$. Intensities were measured by weighing paper cutouts of the signals. The errors in these measurements were at least as large as in $\nu_{1/2}$ determinations.

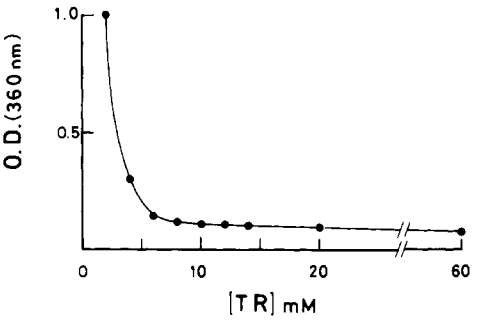

FIGURE 2: Optical density, at 360 nm, of mixed dispersions of SM and TR as a function of the molar ratio of Triton X-100 to SM (R). The concentration of SM was 20 mM.

The turbidities of mixed dispersions of sphingomyelin and Triton X-100 were measured by recording the optical densities at 360, 450, and 540 nm. Diffusion coefficients were measured by quasielastic light scattering as described in detail in an earlier work (Cooper et al., 1974). The focusssed beam of an argon ion laser operating at 4880 A was introduced into the solutions in a scattering cell of 1-cm square cross-section. The light scattered through the adjacent side of the cell, at an angle of 90° from the incident beam (defined by restricting apertures), was detected by an ITT FW-130 photomultiplier tube, and the train of photon-produced pulses was analyzed by a Malvern autocorrelator, operating in "single clipped" mode (Jakeman, 1970).

Results

(1) Spectral Properties of TR–SM Mixed Dispersions at Room Temperature. Figure 2 shows the turbidity of aqueous dispersions containing a fixed concentration (20 mM) of sphingomyelin and varying concentrations of Triton X-100, at 23 °C. In this figure the turbidity was recorded as the optical density at 360 nm, though very similar results were obtained when optical densities were recorded at 450 or 540 nm. As seen in the figure, up to a TR concentration of about 6 mM, the turbidity decreased sharply with increasing TR concentration. At greater concentrations, only minor changes in the turbidity of the dispersions were observed. This may reflect a transition from a liposomal to a micellar state, brought about by the interaction of the detergent with a complex lipid (for comparison, see Yedgar et al., 1974b). The system was further analyzed using the analytical ultracentrifuge, as previously described (Yedgar et al., 1974a,b) and the areas of the schlieren peaks were measured. Using a mixture containing 4 mM TR and 20 mM SM, the dispersion, though not fully homogeneous, consisted of at least 70% of mixed micelles of TR and SM. The s value of these mixed micelles was very similar to that obtained at the minimal detergent concentration, where the system was homogeneous (i.e., 10 mM).

Figure 3 shows ^{1}H NMR spectra of 6 mM TR without and with 20 mM sphingomyelin. Figure 3A assigns the peaks in the spectrum of pure Triton X-100 to the polyethoxy group (a), the terminal *tert*-butyl group (b), and the methyl protons (c) (after Dennis & Owens, 1973). The inset shows peak a, on a tenfold expanded scale. The line shape of this peak indicated that it is broadened by dispersion of chemical shifts of the protons along the polyethoxy chains, as well as spin–spin

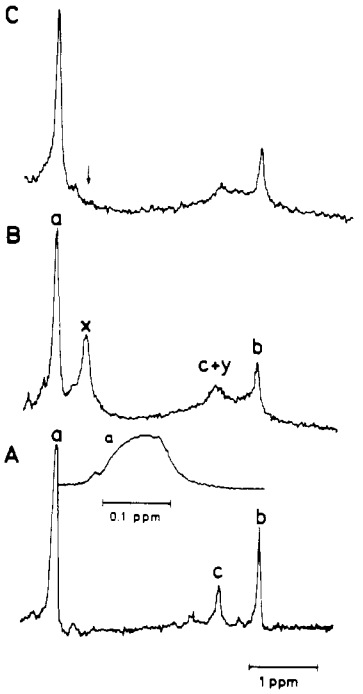

FIGURE 3: (A) ^{1}H NMR spectrum of an aqueous dispersion of 6 mM Triton X-100. Experimental details are described in Materials and Methods. The peak assignment (shown in Figure 1) is based on published data (Dennis & Owens, 1973). The inset represents a tenfold expansion of peak a. (B) ^{1}H NMR spectrum of a mixed dispersion of 6 mM TR and 20 mM SM. (Experimental details in Materials and Methods.) (C) ^{1}H NMR spectrum similar to that of B, except that it was determined following the addition of praseodymium chloride (7.5 mM). The arrow assigns the field at which the choline head group signal (peak x) appeared in B.

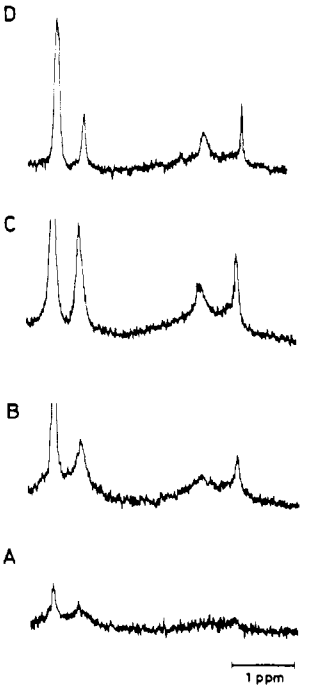

FIGURE 4: ^{1}H NMR spectra of mixed dispersions of TR and SM. SM concentration was maintained at 20 mM. The TR concentrations were as follows: (A) 2 mM; (B) 5 mM; (C) 13 mM; (D) 25 mM. The spectrum in D was recorded at one-fourth of the amplification used for Figures 3A–C.

couplings between some of these protons.

Figure 3B shows a spectrum of an aqueous dispersion of the mixture of TR (6 mM) and SM (20 mM). Two peaks that are absent from Figure 3A can now be seen, one due to the choline head group (x) and the second due to the methylene protons of SM (y) (in parallel to the results on mixed dispersions of TR and phosphatidylcholine, by Dennis & Owens, 1973). It is evident that the TR peaks b and c are broader than the corresponding peaks in Figure 3A, where only Triton X-100 was present. Other signals expected from SM (e.g., the terminal methyl) could not be detected because of overlapping with the above mentioned peaks. They could be detected at higher TR concentrations, where all signals were sharper (cf. Figure 4D).

Addition of praseodymium chloride to the mixed dispersion of Figure 3B resulted in a downfield shift of the complete signal of the choline head group (x). This is expressed as the actual disappearance of this signal (Figure 3C) from the field marked by the arrow. This suggests that at 6 mM TR and 20 mM SM all the choline head groups are available for interactions with the praseodymium ion, thus supporting the conclusion that at this ratio of TR to SM the system is predominantly micellar.

The lack of shifting of other peaks (especially that of peak a) clearly demonstrates that the interaction of the Pr^{3+} ion is associated mostly with the head groups of the phospholipid (presumably the phosphate groups).

Figure 4 shows ^{1}H NMR spectra of aqueous dispersions of sphingomyelin (maintained at a fixed concentration of 20 mM) and Triton X-100, at concentrations varying from 2 (Figure 4A) to 25 mM (Figure 4D). It is evident that increasing the ratio of TR to SM (which results in corresponding changes

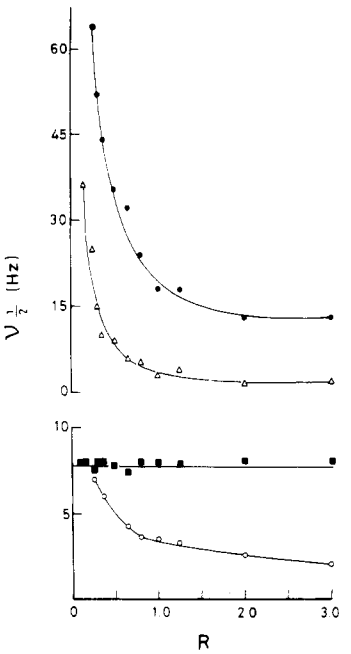

FIGURE 5: Dependence of the linewidths ($\nu_{1/2}$) in the spectra of mixed dispersions of TR and SM on their molar ratio (R). (●—●) Peak c + y; (△—△) peak x; (□—□) peak a; (○—○) peak b. The values were derived from spectra similar to those of Figure 4.

in the micellar composition and structure) markedly affected the line widths of all peaks, except that due to the protons of the polyethoxy groups (peak a, cf. Figure 3).

Figure 5 shows the values of the line widths of the individual peaks as a function of the molar ratio of TR to SM (R), at SM = 20 mM and over the range of TR concentrations from 2.5 to 60 mM. Mixed dispersions with R = 0.5 and R = 3.0 were also prepared with absolute SM concentrations of 10 and 40 mM. The line widths of the various signals in the spectra of these dispersions were essentially the same as in those of

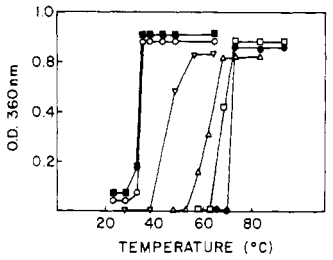

FIGURE 6: The temperature dependence of optical density (OD at 360 nm) for TR (2 mM; ●—●) and mixtures of TR (2 mM) and SM (0.5 mM, □—□; 1.0 mM, △—△; 2.0 mM, ▽—▽; 4.0 mM, ○—○; 6.0 mM, ■—■).

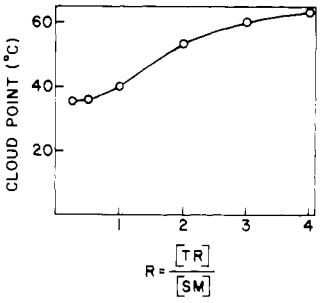

FIGURE 7: The cloud point temperature (T_c) as a function of the molar ratio (R) of TR to SM.

dispersions with the same R value and SM = 20 mM. This indeed suggests that, over the studied range of concentrations, $\nu_{1/2}$ does not depend on the absolute concentration of TR and SM but on the molar ratio of these two components.

It is clear from Figure 5 that the widths of the various signals were not affected uniformly by variation of R. Thus, while most peaks became narrower as R increased, the line width of peak a remained constant througout the entire range. The latter suggests that even at low R, where the signal due to the *tert*-butyl group was so broad that it could not be detected, the segmental motion of the polyethoxy group provided a sharp peak with full intensity. Peak a could therefore be used as an internal standard for determining the intensity of all other peaks. Using the area of this peak as reference, it could be shown that all other peaks appeared in full intensity, provided that the Triton to sphingomyelin ratio exceeded 0.3. It might therefore be concluded that, when R increases from 0.3 to 3.0, the spectral changes primarily reflect variations in the line widths of the entire peaks rather than in their intensities.

(2) Measurements of Optical Density at Various Temperatures. Figure 6 shows the dependence of optical density (OD) of dispersions containing 2 mM TR and increasing concentration of SM (from 0.5 to 6.0 mM) on temperature. For each mixture the cloud point temperature (T_c) is defined as that point on the abcissa where the curve inflects upward. This figure shows an inverse relationship between T_c and increasing SM. In order to examine if the decrease of the cloud point is a function of the absolute concentration of the added lipid or the molar ratio of TR to SM, T_c was measured using varying concentrations of the two constituents, while maintaining a constant molar ratio. Thus, when R equaled 3.0, the TR concentration was varied from 1.5 to 12 mM and, for R = 0.5, the TR concentrations changed from 0.25 to 4 mM. It was found that, over the studied range of concentrations, T_c is a function of the molar ratio, as presented in Figure 7, and not of the absolute concentration of the detergent and lipid. Thus, T_c was 60 °C at R = 3.0 and 36 °C at R = 0.5, independent of the absolute concentrations. It should

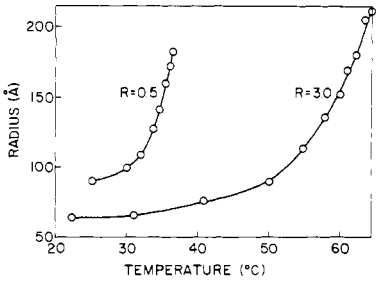

FIGURE 8: The dependence of the Stokes radius (r_s) on temperature for two mixtures of TR and SM (at molar ratios of 0.5 and 3.0).

be emphasized that the clouding occurred only when the concentrations of TR exceeded its critical micellar concentration (about 0.25 mM).

(3) Measurement of Diffusion Coefficients by Light Scattering. The autocorrelation functions of the scattered light were analyzed to determine diffusion coefficients (D) of the mixed dispersions of TR and SM, and the Stokes radii (r_s) were derived, as outlined in an earlier work (Cooper et al., 1974). The values of D at various temperatures are presented in Table I (see paragraph concerning supplementary material at the end of this paper) for dispersions with R = 0.5 and R = 3.0. The Stokes radii of the aggregates, present in the dispersions of these molar ratios, were calculated and are plotted against temperature (Figure 8). It should be emphasized that when the system approached the cloud point, multiple scattering of light was observed. No information is therefore obtainable close to or above T_c, where the size of the aggregates probably increases by more than an order of magnitude. Nonetheless, we may conclude that the increase in the Stokes radius due to increase of temperature from 30 to 45 °C is incomparably greater when R = 0.5 than when R = 3.0; the growth in radius is about 25% when R = 3.0, while when R = 0.5 the radius increases by at least one order of magnitude (as suggested by the steep slope of the respective curve).

The autocorrelation functions indicated polydispersity of the dispersions (Koppel, 1972). This polydispersity did not vary significantly with temperature, as indicated by the variance of the diffusion coefficient with respect to the mean. For R = 0.5, the variance remained close to 30% of the mean for all temperatures and for R = 3.0, the variance was constant around 40%. This variance in the diffusion coefficients is reflected in a similar variance in particle size (Stokes radii) about the mean value.

(4) ^1H NMR Spectra as a Function of Temperature. Figure 9 shows the ^1H NMR spectrum of pure TR (20 mM) and Figures 10 and 11 show spectra of mixed dispersions of TR and SM. In the mixed dispersions, the SM concentration was maintained at 10 mM, while the TR concentration was 30 mM in the dispersion of Figure 10 and 5 mM in Figure 11. The spectra presented here show that, unless T_c was approached, increasing temperatures did not result in broadening of the signals or loss of intensity. On the contrary, some signals became even sharper. This can be exemplified with the signal of the *tert*-butyl group of TR (peak b), the line width of which was 2.8 Hz at 26 °C (Figure 9A) but only 2.0 Hz at 60 °C (Figure 9B, compare also Figure 10A with 10B). Once the temperature reached a value which is close to T_c (Figures 9C, 10C, 11B, and 11C), the intensity of the resonances of the TR as well as of the SM protons decreased. In all dispersions the temperature-induced loss of intensities was more pronounced for the protons of the hydrophobic group (signal b) than for those of the polyetheric chains (signal a)

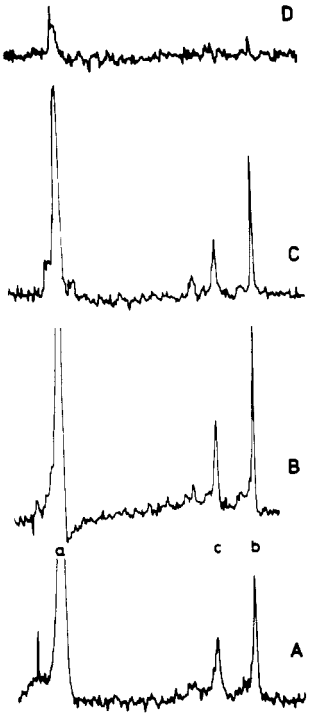

FIGURE 9: ^{1}H NMR spectra of TR (20 mM) at four temperatures (26 °C in A; 60 °C in B; 65 °C in C; and 80 °C in D). Assignment of the various signals is the same as in Figure 3A.

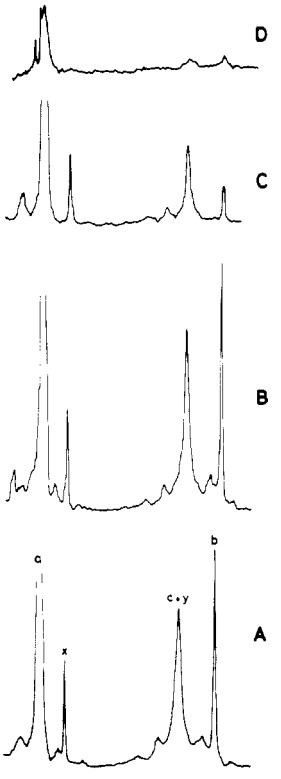

FIGURE 10: ^{1}H NMR spectra of a mixture of TR (30 mM) and SM (10 mM; R = 3.0) at four temperatures (A, 26 °C; B, 60 °C; C, 65 °C; D, 68 °C). Peak assignment is the same as in Figure 3.

or of the choline head group of SM (signal x) (compare Figure 10B with 10C and Figure 6A with 6B). It is of interest that in mixed dispersions of TR and SM, at temperatures near the cloud point, the temperature-induced loss of intensity was accompanied by narrowing of some of the signals (compare the signals x and (c + y) in Figure 11A with the respective signals in Figure 11B). It should be mentioned that in none

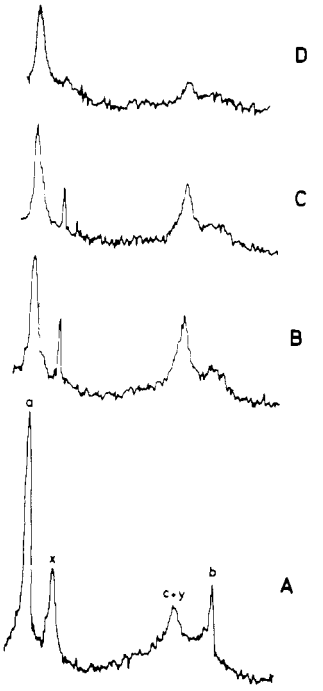

FIGURE 11: ^{1}H NMR spectra of a mixture of TR (5 mM) and SM (10 mM; R = 0.5), at four temperatures (A, 26 °C; B, 33 °C; C, 39 °C; D, 50 °C). Assignment of the various signals is the same as in Figure 3.

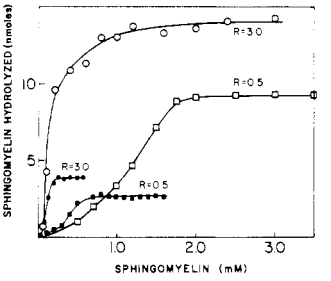

FIGURE 12: The rate of hydrolysis of SM (in nmol per 5 μg of enzyme per 1 h) as a function of SM concentration at 30 °C (full symbols) and 45 °C (empty symbols) at two fixed molar ratios (R = 0.5, squares; R = 3.0, circles).

of the dispersions was a phase separation observed at or above T_c; the turbid dispersions were seemingly homogeneous.

Above T_c only a minute portion of the intensity of the signal due to the polyethoxy group (peak a) could be detected in the ^{1}H NMR spectra of the cloudy dispersions (see Figure 9D, 10D, and 11D). The signal of the *tert*-butyl group of the Triton, as well as the SM signals, were not detectable in these spectra. All signals reappeared immediately upon cooling.

(*5*) *Measurements of Enzymatic Activity.* The rate of hydrolysis of SM by rat-brain sphingomyelinase was measured, using two molar ratios of TR to SM (0.5 and 3.0) and, for each ratio, at two temperatures (30 and 45 °C). At 30 °C, the two respective dispersions were clear, below their T_c. At 45 °C, the mixture of R = 0.5 was cloudy (T_c being equal to 36 °C), while the mixture in which this ratio was 3.0 was still clear, being well below its T_c (which in this case was ca. 60 °C).

Figure 12 shows the dependence of the rate of the enzymatic reaction on SM concentration at the above mentioned conditions. The maximal rates of the enzymatic reactions were three to four times larger at 45 °C than at 30 °C. The relative increase in the reaction rate was about the same at the two respective ratios. A different effect of temperature is observed at low substrate concentrations when R = 0.5. The respective

curves in Figure 12 show that elevation of the temperature from 30 to 45 °C (above T_c at this ratio) lowers the enzymatic utilization of the substrate.

Discussion

(A) Interpretation of Changes in ^1H NMR Line Widths in the Spectra of SM–TR Mixed Micelles. ^1H NMR line widths ($\nu_{1/2}$) have been previously used as an indicator for the motional state of various phospholipids and detergents in lipid bilayers and micelles (Lee et al., 1974; Ribeiro & Dennis, 1975b). Unfortunately, considerable ambiguity might be encountered in the interpretation of line widths of phospholipid dispersions (Bloom et al., 1975; Lichtenberg et al., 1975; Stockton et al., 1976). In large aggregates of phospholipids (e.g., multilayered liposomes), the magnetic dipolar interactions are not fully averaged spatially (Seiter & Chan, 1974). As a consequence, the ^1H NMR line shape, associated with the hydrocarbon chains, is inhomogeneously broadened and, only when the entire aggregate tumbles at a rate sufficiently fast to equalize magnetically all the molecules within the bilayered aggregate, is this heterogeneity canceled. The observed signals are considered homogeneous, in spite of the slight inhomogeneity caused by the chemical shift dispersion of the various protons within the same molecule. Such signals are expected from spherical aggregates having diameters less than 500 Å (Lichtenberg et al., 1975). The SM–TR mixed micelles, whose diameters (at room temperature) do not exceed 200 Å (Yedgar et al., 1974a), most likely give rise to such "homogeneity broadened" ^1H NMR signals.

Above T_c, much larger aggregates are formed (Figure 8) and the ^1H NMR signals might be inhomogeneously broadened. This indeed might explain the broadening (beyond detection) of most ^1H NMR signals observed when the dispersions were heated above their cloud point. The possibility that this "disappearance of intensity" was a result of separation of phases is ruled out since none of these dispersions separated into two phases, neither in H_2O nor in D_2O dispersions (Ribeiro & Dennis, 1974).

On the other hand, the "homogeneous line widths", below T_c, might be determined by both the rate of overall tumbling of the micelles as well as by the local segmental motions of the SM and TR molecules within the mixed micelles. The relative contribution of these two factors to the ^1H NMR line widths must therefore be ascertained before any definite quantitative conclusions can be drawn regarding the changes in molecular packing.

The data of Figure 5 show that changing the TR to SM ratio from 3.0 to 0.5 resulted in threefold broadening of the signal of the SM methylene protons (from 15 to 45 Hz). This value must be corrected since, for quantitative evaluation of this change, one must take into account the broadening of the above lines which results from the dispersion of chemical shifts and from spin–spin couplings of the various protons along the paraffinic chains. Thus, even if the contribution of these two factors to the broadening of the above signals were only 5 Hz, the actual broadening of the signals is from 10 to 40 Hz, namely, a fourfold change. Such a broadening cannot be due to changes in micellar tumbling. This conclusion is based on the following observations and considerations. One important factor in determining the micellar tumbling is the viscosity of the medium. It has been shown that when $R = 0.5$ the reduced specific viscosity had a value of about 6 when the total concentration equaled 1–15 mg/mL (Yedgar et al., 1974a). On the basis of this finding, it can be calculated that, for a dispersion of 20 mM TR and 40 mM SM, the viscosity should be at least double that of a dispersion of 5 mM TR and 10 mM SM (Tanford, 1961). The difference in viscosity might be larger since at a high concentration of TR and SM the variation in the viscosity can be larger (Tanford, 1961). The close similarity of the ^1H NMR spectra of the dispersions therefore suggests that micellar tumbling does not play an important role in determining $\nu_{1/2}$ in the spectra of TR–SM mixed micelles. Thus, it might be concluded that the dominating contributing factor to the widths of the "homogeneously broadened signals" in the spectra of SM–TR mixed micelles below T_c is the local segmental motion of the molecules in the micelles rather than the overall tumbling of the micelles themselves.

This conclusion is considerably strengthened when one considers the broadening of the choline head-group signals. The choline head groups are most probably packed less tightly than the paraffinic chains and their signals should therefore be less sensitive to changes in the rate of overall tumbling (Seiter & Chan, 1974; Lichtenberg et al., 1975). The observed broadening of the choline head-group signal, when the TR to SM ratio was varied from 3.0 to 0.5, was sixfold, again suggesting that the broadening of all the recorded signals must be attributed mainly to changes in packing rather than in the rate of tumbling.

As far as changes in packing are concerned, it has been previously shown that the radius of curvature is a major parameter in determining molecular motions (and thereby the ^1H NMR line widths) of PC molecules in spherical vesicles (Sheetz & Chan, 1974a; Lichtenberg et al., 1975). The model of Yedgar et al. (1974) proposed that, when SM is solubilized by TR, the curvature of the aggregate changes considerably. This model suggests that the curvature of the surface, along which SM molecules are arranged, increases with increasing molar fractions of Triton X-100. It is thus possible that the variation of the line widths of the SM signals in the spectra of SM–TR mixed micelles is similarly a consequence of the variation of curvature.

To test this possibility we attempted to calculate (see supplementary material) the radius of curvature of the surface of the oblate SM–TR mixed micelles proposed by Yedgar et al. (1974a). The calculations resulted in the approximation $a = (1/30)(r^2 - 90r + 2250)$ for the radius of curvature (a), where r is the radius of the mixed micelle. On the basis of this approximation, it might be concluded that, for mixed micelles in which the TR to SM ratio is 1:2 ($r = 90$ Å), the average radius of curvature (a) is about 75 Å. Consequently, the ^1H NMR line widths of the SM protons in these micelles should be somewhat narrower than those of SM in its smallest, bilayered vesicles ($r = 120$ Å). In the latter case at 30 °C, the peak of the choline methyl group has a width of about 20 Hz and the line width of the methylene signal is 45 Hz (Schmidt et al., 1977), whereas the corresponding signals in TR–SM mixtures of ratio 1:2 have widths of 9 and 36 Hz, respectively.

The significance of the radius of curvature (a) in determining the line width in the spectra of mixed micelles is further emphasized by evaluating the implication of increasing r on the radius of curvature of the mixed micelle. Increasing r by 10 Å (from 90 to 100 Å) will increase the radius of curvature (a) from 75 to about 110 Å. This considerable increase in the radius of curvature might therefore constitute the major contributing factor to changes in the line widths of the ^1H NMR signals.

(B) Variations in Molecular Packing within Various Zones of the Mixed Micelles. The observation that the line width of the signal due to the polyoxyethylene chain protons of the

Triton did not change at all suggests that the motion of these groups was not affected by the packing variations of the hydrophobic core. This is in line with the model suggested by Yedgar et al. (1974a) for the mixed micelles of TR and SM, according to which the polyethoxy groups of the Triton protrude into the aqueous bulk solution.

The above model also proposed a higher local concentration of the TR molecules in the curved portion of the oblate elipsoidal mixed micelles. Figure 5 shows that the signal of the terminal *tert*-butyl group of Triton X-100 was broadened from 2 to 6 Hz when the TR to SM ratio changed from 3.0 to 0.3. This broadening was significantly smaller than that observed for the signals of sphingomyelin. In the latter case and over the same range, the signal due to the choline head group broadened from 1 to 15 Hz and that due to the chain protons from 13 to 51 Hz.

Previous results suggested that molecular packing in phospholipid bilayers becomes looser with increased curvature, resulting in narrowing of the NMR lines (Sheetz & Chan, 1972; Seitzer & Chan, 1974; Lichtenberg et al., 1975; Petersen & Chan, 1977). The fact that the line widths of the signal due to the terminal *tert*-butyl group of the Triton changed less than those due to the paraffinic protons of SM supports the assumption of an uneven distribution of these two components in the mixed micelles, in agreement with the above model.

Close examination of Figure 5 shows that, when the ratio of TR to SM exceeds the value of 1.5, the sensitivity of the line widths to changes in R is reversed, namely, the line width of the *tert*-butyl signal is more sensitive to changes in the ratio of these two components than that of the SM methylenes. It is therefore probable that there are two types of effects of the detergent on the packing of the phospholipid, one predominating below and one above this ratio. At ratios greater than 1.5, where the mixed micelles are assumed to be spherical, the spectral changes due to addition of detergent might reflect "dilution" of the micelle with detergent. Such changes will affect the environment of the TR more than that of SM, whose molecules are anyway mostly surrounded by TR molecules at a ratio close to TR:SM = 1.5. Only for $R < 1.5$, the major consequence of varying the detergent concentration is a drastic change in line widths (mostly those of the SM protons), probably due to changes in the radius of curvature (see supplementary material).

(*C*) *Comparison of TR–SM with TR–PC Mixed Micelles.* Triton-induced loosening of packing of phospholipid molecules was reported for mixed micelles of this detergent with lecithin (Dennis & Owens, 1973). However, the dependence of the spectrum on the molar ratio of the detergent to phospholipid differed considerably from the case of SM. The intensities of the signals in the spectra of the mixed micelles having TR and PC were very low up to a TR:PC ratio of about 0.8 and the full intensities were obtained only when this ratio exceeded 1.6. A detailed analysis of the data presented by Dennis & Owens (1973) discloses that between ratios of 0.8 and 1.6, the widths of the signals of both the *tert*-butyl protons of TR and the PC methylene protons were not affected by TR concentration. Further increase in the relative TR concentration resulted in narrowing of these two "indicator peaks", the signal of the *tert*-butyl protons being more sensitive to the change in the TR:PC ratio than that of the PC methylenes, similar to what was observed with SM–TR at $R > 1.5$ (Figure 5).

The above differences between SM and PC might be explained if the transformation of phospholipid bilayers by Triton X-100 into mixed micelles starts after the bilayers are saturated with TR (Yedgar et al., 1974b).

SM molecules, being more lipophilic than PC, are packed in their bilayers tighter than PC. It is thus plausible that SM bilayers can accommodate less molecules of Triton than PC bilayers before saturation is reached and solubilization starts. This corresponds with the finding that 1 mol of TR, which was sufficient to solubilize 2 mol of SM (Yedgar et al., 1974a), solubilized only 0.5 mol of PC (Dennis & Owens, 1973). Since PL molecules which are aggregated in multibilayers do not contribute to the high resolution ^{1}H NMR spectra (Seiter & Chan, 1974), the ^{1}H NMR intensities reflect the fraction of PL molecules which are solubilized by TR in mixed micelles. Correspondingly, with PC–TR dispersions at $0.8 < R < 1.6$, the increase of intensities without narrowing of the ^{1}H NMR line width (Dennis & Owens, 1973) may reflect the formation of more mixed micelles of the same composition at the expense of bilayers. At this range, multibilayers of the PL coexist with mixed micelles and the ratio of TR to PC in the system differs from the actual ratio in the mixed micelles. Only at $R = 1.6$ a homogeneous PC–TR mixed micellar system is obtained, and the micelles are assumed to be spherical at this stage (Ribeiro & Dennis, 1975b). On the other hand, with TR–SM dispersions, nearly full intensities were obtained already at $R = 0.3$ where the system practically contains a homogeneous population of elipsoidal mixed micelles (Yedgar et al., 1974a). Addition of detergent above this ratio increases TR:SM ratio in the mixed micelles, causing a reduction of their size, an increase in the curvature of the micellar surface and consequently a narrowing of ^{1}H NMR signals. This system therefore can demonstrate the role of curvature in the TR-induced loosening of packing, within the hydrophobic core of TR–PL mixed micelles.

(*D*) *Effects of Temperature and Cloud Point on SM–TR Mixed Dispersions.* As can be seen in Figure 6, the optical density of the mixed dispersions remained low and showed no changes when the temperature was increased up to T_c, when the optical density increased abruptly. The more sensitive laser light scattering showed that the radii of the TR–SM mixed micelles did actually increase with temperature even well below T_c. Thus, when R equaled 3, the radius at 55 °C (i.e., 10 °C below T_c) was about double that measured at room temperature (Figure 8). This indeed suggests that the response of the aggregates to temperature changes is gradual and not an "all-or-none-transition", though at T_c the change in size exceeded by far the size increase which occurred at lower temperature.

The size increase below T_c was not reflected in the linewidths of the various ^{1}H NMR signals, which became sharper at elevated temperatures (cf. Figures 11A and 11B). Since an increase in size causes a decrease in the rate of Brownian tumbling of the aggregates, a broadening of the ^{1}H NMR signals could have been expected. Nonetheless, increasing temperatures also increases the rate of molecular motions within these aggregates and the observed narrowing suggests that the latter factor predominates.

At a temperature just below T_c, the ^{1}H NMR signal of the choline head group of sphingomyelin (peak x) was narrowed, while its intensity was reduced (compare Figure 11A with 11B and the latter with 11C). The line width of this peak is inversely proportional to R (Figure 5), whereas T_c is directly proportional to this ratio (Figure 7). It is therefore probable that when T_c is approached the micelles with R values smaller than the average undergo clouding first and do not contribute to the ^{1}H NMR spectra. Consequently, only mixed micelles with R values larger than the average and which do not yet undergo clouding contribute to the ^{1}H NMR spectra. This

indeed results in narrowing of the signals.

At T_c, much larger aggregates are formed. The broadening (beyond detection) of most signals in the ^1H NMR spectra, which accompanies this aggregation, may be explained by the consequent drastic reduction in the rate of overall tumbling. Nonetheless, it might also be due to a change in molecular packing into a more organized state.

It should be emphasized that this broadening does not provide meaningful information about the mode of aggregation. In the formation of large aggregates, two different processes might be involved. One possibility is that larger mixed micelles are formed, in which the identity of the original micelles is changed. On the other hand, it is possible that, at T_c, the original micelles undergo flocculation (Ottewill, 1967); namely, they are organized into clusters (flocks) of micelles, in which they preserve their identity. One major difference between these two possible modes of aggregation is that, upon formation of larger separate micelles, all the polyethoxy groups of TR protrude into the aqueous solution whereas in a cluster of micelles only the molecules of micelles on the outside of the cluster do. Thus, if the clouding forms larger separate micelles one should expect all the TR molecules to be equivalent in terms of their packing. In this case, it would be difficult to explain the fact that the signals of most of the polyetheric protons were broadened beyond detection by the clouding, while the rest of these groups remained unchanged in their widths even 20 °C above T_c (compare Figure 9C with 9D). The observed differentiation of TR molecules could indeed be a result of polydispersity of the Triton, as was suggested by Ribeiro & Dennis (1974). However, the sharper change in our spectra indicates that in our study the polydispersity was smaller. A straightforward explanation for the appearance of a fraction of peak a at a temperature high above T_c is offered by the flocculation model. According to this model, those polyetheric groups of molecules on the outside of the cluster will remain free to undulate in the aqueous solution and give rise to relatively sharp ^1H NMR signals.

In dispersions of TR–SM mixed micelles, the clouding is accompanied by a decrease in the intensity of all the peaks, both those of the TR as well as of SM. Again, as expected on the basis of the above discussion, the larger loss in intensity is of protons located in hydrophobic regions of the micelles (see Figures 10 and 11). In any event, it is clear that both the detergent and the lipid are involved in the clouding process. This conclusion is also in accord with the observation that T_c depends on the TR to SM ratio and not on the absolute concentrations of the constituents.

The model suggesting micellar flocculation at the cloud point is also in accordance with the data on the enzymatic hydrolysis of SM. In the "multimicellar" aggregate, the SM molecules located in the inner core of the cluster will be less accessible to the enzyme than those on its outer surface.

As a result, when the concentration of sphingomyelin is subsaturating, the clouding results in a decrease of the rate of enzymatic reaction, whereas it shows no effect at saturating substrate concentration. It is worth mentioning that, in a previous communication (Yedgar & Gatt, 1976) where TR–SM mixtures were used above the cloud point, substrate concentrations were saturating throughout. The cloud point therefore had no effect on the results reported in that paper.

Elevation of temperature indeed increases the enzymatic reaction rate as expected from thermodynamic considerations. This, along with the effect of clouding, explains the results presented in Figure 12. In the curves of $R = 0.5$, which compare enzymatic activity below and above the cloud point, the decrease in the effective concentration of the substrate is expressed by the fact that above the cloud point lower reaction rate observed at low SM concentrations is required for saturation of the enzyme and reaching the maximal activity. The maximal reaction rate is, as expected, increased by elevation of temperature. With the curves of $R = 3.0$, both below T_c at this ratio, the main difference observed is the general increase of enzymatic activity at the higher temperature (Figure 12).

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Supplementary Material Available

A calculation of the curvature in mixed micelles of phospholipids and Triton X-100 and its effect on molecular motions within the mixed micelles and Table I which contains diffusion coefficients of mixed dispersion of Triton X-100 and sphingomyelin (4 pages). Ordering information is given on any current masthead page.

References

Arai, H. (1967) *J. Colloid Interface Sci. 53*, 348–351.
Barenholz, Y., Roitman, A., & Gatt, S. (1966) *J. Biol. Chem. 241*, 3731–3737.
Bloom, M., Burnell, E. E., Valie, M. I., & Weeks, G. (1975) *Chem. Phys. Lipids 14*, 107–115.
Cooper, V. G., Yedgar, S., & Barenholz, Y. (1974) *Biochim. Biophys. Acta 363*, 86–97.
Dennis, E. A. (1973) *Arch. Biochem. Biophys. 158*, 485–493.
Dennis, E. A., & Owens, J. M. (1973) *J. Supramol. Struct. 1*, 165–176.
Donbrow, M., & Azaz, E. (1976) *J. Colloid Interface Sci. 57*, 20–27.
Gatt, S. (1968) *Biochim. Biophys. Acta 159*, 304–316.
Gatt, S. (1973) *Metab. Inhibitors 4*, 349–387.
Gatt, S., & Barenholz, Y. (1973) *Annu. Rev. Biochem. 42*, 61–90.
Gatt, S., & Gottesdiner, J. (1976) *J. Neurochem. 26*, 421–422.
Gatt, S., Herzl, A., & Barenholz, Y. (1973) *FEBS Lett. 30*, 281–285.
Hertz, R., & Barenholz, Y. (1975) *Chem. Phys. Lipids 15*, 138–156.
Hertz, R., & Barenholz, Y. (1977) *J. Colloid Interface Sci. 60*, 188–200.
Jakeman, E. (1970) *J. Phys. A: Gen. Phys. 3*, 201–215.
Koppel, D. C. (1972) *J. Chem. Phys. 57*, 4814–4819.
Lee, A. G., Birdsall, N. J. M., & Metcalfe, J. C. (1974) *Methods Membr. Biol. 2*, 1.
Lichtenberg, D., Petersen, N. O., Girardet, J.-L., Kainosho, M., Kroon, P. A., Seiter, C. H. A., Feigenson, G. W., & Chan, S. I. (1975) *Biochim. Biophys. Acta 382*, 10–21.
Nakagawa, T. (1967) in *Non-Ionic Surfactants* (Schick, J. J., Ed.) pp 558–572, Marcel Dekker, New York.
Nakagawa, T., & Shinoda, K. (1963) in *Colloidal Surfactants* (Shinoda, K., Ed.) pp 129–131, Academic Press, New York.
Ottewill, R. H. (1967) in *Non-Ionic Surfactants* (Schick, J. J., Ed.) pp 627–682, Marcel Dekker, New York.
Petersen, N. O., & Chan, S. I. (1977) *Biochemistry 16*, 2657–2667.
Ribeiro, A. A., & Dennis, E. A. (1974) *Chem. Phys. Lipids 12*, 31–38.
Ribeiro, A. A., & Dennis, E. A. (1975a) *Chem. Phys. Lipids 14*, 193–199.

Ribeiro, A. A., & Dennis, E. A. (1975b) *Biochemistry 14*, 3746–3755.
Rosch, M. (1967) in *Non-Ionic Surfactants* (Schick, J. J., Ed.) pp 753–773, Marcel Dekker, New York.
Satto, H., & Shinoda, K. (1967) *J. Colloid Interface Sci. 24*, 10–15.
Schmidt, C. F., Barenholz, Y., & Thompson, T. E. (1977) *Biochemistry 16*, 2649–2656.
Seiter, C. H. A., & Chan, S. I. (1974) *J. Am. Oil Chem. Soc. 95*, 7541–7553.
Shah, D. O., & Schulman, J. H. (1976) *Lipids 2*, 21–27.
Sheetz, M. P., & Chan, S. I. (1972) *Biochemistry 11*, 4573–4581.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954–966.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Chapter 6, Wiley, New York.
Yedgar, S., & Gatt, S. (1976) *Biochemistry 15*, 2570–2573.
Yedgar, S., Barenholz, Y., & Cooper, V. G. (1974a) *Biochim. Biophys. Acta 363*, 98–111.
Yedgar, S., Hertz, R., & Gatt, S. (1974b) *Chem. Phys. Lipids 13*, 404–414.

Cytoplasmically Inherited Respiratory Deficiency of a Mouse Fibroblast Line Which Is Resistant to Rutamycin†

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ABSTRACT: Mouse fibroblasts resistant to the drug rutamycin were isolated and found also to be respiratory deficient. These cells produce large amounts of lactic acid, and oxygen consumption data indicate that the first complex of the electron transport chain, NADH–coenzyme Q reductase, is defective. Levels of rotenone-sensitive NADH–cytochrome *c* reductase and pyruvate decarboxylase of the pyruvate dehydrogenase complex are markedly depressed in the mutant cells. Other components of the electron transport chain appear to be fully functional. The mutant cells were enucleated and fused with another cell line, and the resulting cybrid demonstrated a similar pattern of respiratory deficiency as did the original mutant. These results indicate that this defect in respiration is a cytoplasmically inherited characteristic in this cell line.

Respiratory deficiency has recently been studied in several mammalian cell lines (DeFrancesco et al., 1976; Soderberg, et al., 1977). One has been shown to be defective in NADH–coenzyme Q reductase (DeFrancesco et al., 1976) and another lacks succinate dehydrogenase activity (Soderberg et al., 1977). These cell lines all exhibit a high rate of glycolysis during growth and consume oxygen at significantly lower rates than do the parental cell lines. We have recently isolated a line of mouse fibroblasts resistant to the drug rutamycin (Lichtor & Getz, 1978), an inhibitor of mitochondrial ATPase. Four subclones of this mutant were derived from the initial population, and all of these were also found to be respiratory deficient. The mutagenesis procedure involved selectively introducing BrdUrd (5-bromodeoxyuridine) into the mitochondrial genome of a line of mouse fibroblasts (clone 1 D) lacking cytoplasmic thymidine kinase activity. The ATPase activity of mitochondria isolated from these cells was resistant to rutamycin. The rutamycin resistant mutants were enucleated with cytochalasin B and fused with mouse A 9 cells resistant to 8-azaguanine and sensitive to rutamycin. Cytoplasmic hybrids or cybrids were selected as cells resistant to rutamycin and 8-azaguanine and appeared at a high frequency. ATPase activity of mitochondria isolated from these cybrid cells was also resistant to rutamycin. Other fusions between rutamycin-resistant nucleated cells and A 9 resulted in many fewer resistant colonies, and only a small number of these continued to grow for any length of time. Finally, fusions between enucleated clone 1 D cells and A 9 cells produced no rutamycin-resistant colonies. These results indicate that rutamycin resistance is a cytoplasmically inherited characteristic in this cell line.

In this paper the respiratory characteristics of both the original mutant and the cybrid cells are described. The capacity to grow respiratory-deficient cells in tissue culture in reasonable yield indicates the possibility for the isolation and study of mitochondrial mutants.

Experimental Procedures

Growth Conditions. Cells were grown on Corning flasks or glass roller bottles in RPMI medium 1640 supplemented with 10% fetal calf serum, 2.0×10^{-3} M L-glutamine, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin in a 37 °C incubator. The cultures were periodically checked for mycoplasma, and any contaminated cultures were discarded. It was determined that clone 1 D is capable of growing in the presence of rutamycin, an analogue of oligomycin, at concentrations up to 1.0×10^{-8} g/mL; however these cells were not able to divide even once in the presence of rutamycin at a concentration of 1.0×10^{-7} g/mL.

Growth Curves. A number of 25-cm^2 Falcon flasks were seeded with 1.5×10^5 cells each. On the following day varying concentrations of antibiotics were added. Cells from two flasks containing each concentration of the drug being tested were harvested and counted in a Coulter counter at intervals of several days.

Isolation of Mitochondria. The harvesting of cells, their storage, and isolation of mitochondria have been described (Lichtor & Getz, 1978).

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