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LATERAL DIFFUSION OF CHOLESTEROL IN MONOLAYERS

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SUMMARY

The surface diffusion coefficient of cholesterol in cholesterol monolayers has been measured as a function of cholesterol surface concentration. Two different radiochemical methods, one integral and the other differential, were developed which gave comparable results. In the integral method two cholesterol monolayers, one of which is radioactive, are isolated on inert hydrophilic supports and then brought into contact. After some time the supports are separated and the radioactivity of the supports is measured. The differential method is an autoradiographic experiment. Two cholesterol monolayers, one of which is radioactive, are separated by means of a thin barrier. Upon removal of the barrier and at later times, an autoradiographic plate is brought to within a fraction of a mm from the aqueous surface and exposed. The plates are developed and analysed.

The data show that the cholesterol surface diffusion coefficient in the dilute monolayers is approximately 10^{-6} cm^2/s and is nearly independent of surface concentration up to a concentration corresponding to an area of 40 Å^2/molecule. As the monolayer becomes compressed beyond this surface concentration, the diffusion coefficient decreases abruptly with the deeply decreasing surface tension to about 10^{-7} cm^2/s, when a fully condensed surface layer of 38 Å^2/molecule is reached. This diffusion coefficient is of the same order of magnitude as the diffusion coefficients measured in lipid bilayers and in membranes.

INTRODUCTION

In the past few years there has been a large body of evidence to indicate that artificial and biological membranes are fluid-like with a large degree of mobility in the plane of the membrane. The mobility of membrane components such as fatty acid chains, proteins and intramembranous particles has been measured by a variety of techniques such as electron spin resonance, nuclear magnetic resonance, fluorescence polarization, high-speed flash photometry, radiochemical diffusion, and others. A substantial amount of the literature has been reviewed by Edidin [1].

The mobility of different molecular residues in the plane of the bilipid layer is

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usually expressed in terms of a viscosity or a lateral diffusion coefficient depending on the technique used. In principle these values can be interconverted. It has been shown that the hydrocarbon core of the lipid bilayer of artificial and biological membranes is more fluid in the center of the bilayer than near the polar heads of the lipid molecules [2–4]. Molecules that diffuse in the plane of the membrane are therefore influenced by the local viscosity in the immediate vicinity of the molecule. These apparent viscosity or diffusion values must be used with caution as they are related to some extent to the system studied. However, as Edidin [1] points out, the calculated viscosities of membranes can give some indication on the general environment of all membrane molecules, while diffusion constants indicate only the motion of particular species in the membrane.

Edidin [1] has tabulated the lateral diffusion data from a variety of sources. These and some recently reported data [5–7] indicate that nearly all the diffusion data are roughly within one order of magnitude from each other. Diffusion data for lipids and steroids are reported in the range from 10^{-7}–10^{-8} cm^2/sec. Diffusion coefficients for smaller organic molecules are approximately 10^{-6} cm^2/s. Although differences exist between sets of data, there appears to be a qualitative agreement between the diffusion coefficients and the size of the molecules and their position in the membrane [1, 6]. Diffusion data obtained by Poo and Cone [8] for the lateral diffusion of the protein rhodopsin in the disk membranes of isolated frog and mudpuppy rods is of the order of 10^{-9} cm^2/s, which is consistent with the previously mentioned results. These diffusion constants may be lower than those measured in aqueous solutions, but on the other hand the physical dimension of most biological membranes is on the microscopic scale e.g. the time of movement across a cell 10^{-3} cm in circumference is about 10 s for $D = 10^{-7}$ cm^2/s and 1000 s for $D = 10^{-9}$ cm^2/s.

Lateral diffusion in monolayers on aqueous substrates has not received very much attention. There should be, however, certain similarities between the diffusion process in bilayers and in monolayers. Moreover, measurement of the diffusion coefficients of identical components in monolayers and in bilayers may serve as a powerful tool for the investigation of structural similarities and differences in the two systems. Sakata and Berg [9] measured self-diffusion of myristic acid spread on 0.01M HCl substrates. They reported surface diffusion coefficients of the order of 10^{-4} for dilute films to 10^{-5} cm^2/s for condensed films. These values appeared to be too high, and later work reported by Chung and Berg [10] for myristic acid and also pentadecylic, palmitic and oleic acids were of the order of 10^{-7}–10^{-5} cm^2/s. These values are consistent with those reported by Good and Schecter [11] who gave an upper limit for the selfdiffusion coefficient of stearic acid as 2 or $3 \cdot 10^{-6}$ cm^2/s.

In this work the surface diffusion coefficient of cholesterol in cholesterol monolayers was measured. Cholesterol is an important component of biological membranes and its mobility in membranes can play a role in controlling the physical state of the membrane from point to point. Two new methods, both utilizing radioactive cholesterol, were developed. In principle, in each method two cholesterol monolayers with equal pressures, one of which contained the radioactive cholesterol, were brought into contact for some time. In the integral method the total radioactivity after time t of both monolayers was measured. In the differential method a radioactivity profile was measured at different times.

MATERIALS AND METHODS

Radioactive tritium-labeled cholesterol (spec. act. 30–47 Ci/mmole) and ^{14}C-labeled cholesterol (spec. act. 58 mCi/mmole) were obtained from the Radiochemical Center (Amersham, England). Fresh quantities of the ^{3}H-labed cholesterol were repeatedly obtained from the manufacturer and used within five weeks. The ^{3}H-labeled cholesterol was purified by thin layer chromatography on silica gel, and had a radiochemical purity of 98 % according to the manufacturer. Self-decomposition of this cholesterol does not exceed 10 % per annum (data from manufacturer) so that the self-decomposition for our samples should be less than 1 %. The carbon 14-labeled cholesterol was judged to be pure by thin layer chromatography. Unlabelled cholesterol (purity greater than 99.9 %) was obtained from B.D.H., Laboratory Chemical Division (Poole, England). It was assessed to be pure by thin layer chromatography. Cholesterol solutions for spreading were made by dissolving measured amounts of cholesterol in known volumes of distilled benzene. All solutions were stored at 4 °C. Deionized water was double distilled in all glass apparatus, the first time in the presence of $KMnO_4$. The double-distilled water was the aqueous substrate on which the cholesterol solutions were spread. Prior to spreading, the water surface was cleaned by means of a micropipette connected to an aspirator.

A similar trough was used in both methods and is shown in Fig. 1. In a polypropylene or Perspex plate a square O-shaped trough is milled. The geometry is advantageous for the equalization of the radioactive monolayers. Barriers AB and CD effectively divide the water surface in two halves. After spreading known amounts of solution at equal concentrations on each side of the barriers, and allowing the spreading solution to evaporate, the surface pressure is equalized by slowly removing barrier CD. The experiment is started by removal of barrier AB so that the two monolayers are brought into contact at the same surface pressure.

The barrier AB is a blade barrier with a maximum thickness of 0.03 mm near the edge. The blades were coated with paraffin wax (BDH Laboratory Chemicals Div., Poole, England), rubbed with a cloth, and then repeatedly rinsed with distilled water prior to use. Initially the length of the edge of the blade was situated in the aqueous substrate at a slight angle. The blade was removed by raising one end of the blade while the other end served as a pivot point. As a consequence the blade was removed from the surface in a zipper-like fashion. Observance of the surface showed a minimum of disturbance by this method.

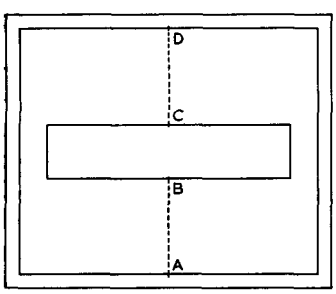

Fig. 1. Top view of the diffusion trough.

The trough was first cleaned by washing with a fine brush under running double distilled water. Then a jet stream of double distilled water was impinged upon the surface of the trough for five minutes. The top of the trough could be closed through the use of snugly fitting covers. Wetted wicks were placed inside the trough to keep the gas phase saturated with water vapor.

The troughs were fastened on top of 10 kilo rectangular aluminum blocks which were standing on four adjustable rubber pods. The rubber pods were designed to absorb vibrations from the surroundings. The whole assembly was located inside a dust-free box. Experiments were conducted at room temperature, $T = 22 \pm 0.5$ °C.

Differential method

In the differential method an autoradiographic plate was placed above the cholesterol monolayer. The amount of liquid in the trough was set so that the surface of the radiographic emulsion on the plate was either 0.1 ± 0.05 mm or 0.2 ± 0.05 mm above the liquid surface. The autoradiographic plates were obtained from Ilford Limited (Ilford, Essex, England). The 1×3 inch glass plates were approximately 1 mm thick and one side was coated with a 10 μm thick emulsion type K2. With appropriate pins and a groove the photographic plate was located in such a manner that the initial boundary line (defined by the position of barrier AB) was midway under the plate and parallel with the sides. Plates were placed for 10 min above the surface and then removed and soaked 30 s in distilled water, developed 30 s in concentrated Ilford Phen-X Developer, placed for 30 s in a 1 % acetic acid bath, fixed in Ilford fixer for 5 min, washed for 2 h in running distilled water, and dried. The plates were viewed with dark field illumination in a microscope (Reichert, Austria) and photographed as a function of position on the plate (normal to the position of AB). The crystal density of each photograph (negative) was measured with the Quantimet Image Analyzer Computer (Model 72) located at the Biological Institute, Ness Ziona.

Integral method

In this method, on both sides of barrier AB two rectangular plates of Perspex 32.0 by 17.0 mm were placed in the trough with their lengths parallel to AB. The top surfaces of the plates have a rim on the widths with dimensions $3.0 \times 3.0 \times 17.0$ mm. On the flat part of each hydrophobic plate (measuring 26.0×17.0 mm) a piece of 26.0×17.0 mm Whatman filter was placed flush with the long edges of the plates. Before spreading the cholesterol solutions the surfaces of the filter papers were 2 mm below the water level. After spreading, the surface pressure was equalized and the water level was slowly lowered through a small drain in the bottom of the trough until the liquid surface was one mm below the upper surfaces of the filter paper. The cholesterol monolayers were now on top of the hydrophillic filter papers which were completely soaked with distilled water. Barrier AB was removed and the hydrophobic supports moved so that the edges of the filter papers were in complete contact. Since the filter papers were cut from one piece and the original cut was rejoined, the fit of the papers was exact. The radioactive and nonradioactive monolayers were in contact and the diffusion process proceeded. After time t the supports were separated, and to eliminate disturbances the barrier AB was replaced. The hydrophobic supports were lifted from the trough and the filter papers were allowed to dry. The dry papers were

placed in 10 ml each of Bray's scintillation liquid and were counted in a Tricarb Hewlett Packard Scintillation Counter.

Measurement of diffusion coefficients

The mass conservation equation for the radioactive tracer is

$$\frac{\partial \Gamma}{\partial t} = D \frac{\partial^2 \Gamma}{\partial x^2} \tag{1}$$

where D is the lateral diffusion coefficient and Γ is the surface concentration. The initial distribution is described by the boundary conditions

$$\Gamma = \Gamma_0,\ x > 0;\ \Gamma = 0,\ x < 0;\ t = 0 \tag{2}$$

The solution for Eqns (1) and (2) is given by Crank [12]

$$\frac{\Gamma(x, t)}{\Gamma_0} = \tfrac{1}{2} \operatorname{erfc}\left[\frac{-x}{2(\mathrm{D}t)^{\frac{1}{2}}}\right] \tag{3}$$

Cholesterol is virtually insoluble in the water subphase; its maximum solubility in aqueous solutions is 1.8 μg/ml [13] at 25 °C. Although sufficient subphase was present to solubilize a significant fraction of the cholesterol monolayer, Miller et al. [14] have shown that cholesterol does not desorb from a monolayer into an aqueous substrate. In this work, Miller's results were confirmed by measuring the subphase radioactivity with time for 24 h. The maximum increase of radioactivity was a few percent of the background which was the same as the order of accuracy of the measurement. Moreover, the considerably lower diffusion coefficients measured in condensed monolayers indicate that the diffusion does not proceed through the subphase.

For the differential method it was assumed that the concentration profile of the silver crystals corresponded to the radioactivity profile. The broadening of the radioactivity profile with time was fitted to Eqn (3) and the diffusivity calculated. Γ_0 was obtained from the uniform silver crystal density at large distances (x) from the boundary (AB). In the integral method, the amount of radioactive cholesterol $M(t)$ that has diffused in time t into an initially inactive surface is related to the diffusivity by

$$D = \frac{\pi}{t}\left[\frac{M(t)}{L\Gamma_0}\right]^2 \tag{4}$$

where L is the length of the paper strip, and Γ_0 was ascertained from measurements of the radioactive strip at time zero. As an additional check, the total radioactivity of both strips at time t should be equal to $\Gamma_0 \cdot W \cdot L$ where W is the width of one strip.

RESULTS

Differential method

The detection by a device of the radioactivity of an experimental system is usually expressed in counts per minute and for a radioactive monolayer the count rate can be expressed as [15]

$$R = 2.22 \cdot 10^{12}\, \eta_B \sigma S \Gamma \tag{5}$$

where S is the surface area, σ the spec. act. of the labeled material, R is the count rate, and η_B the detection coefficient. Exposure times for the nuclear plates should be of the order of minutes for a diffusivity of order 10^{-6} cm^2/s, so that the material of choice for this experiment was ^{3}H-labeled cholesterol by virtue of its high activity. Since the maximum penetration depth of tritium β-particles in air is of the order of 6 mm, the nuclear plate must be brought as closely as possible to the surface in order to increase the detection coefficient η_B such that R is statistically significant. The contribution of radiation from the bulk substrate on the counting rate can be ignored because cholesterol is virtually insoluble and the maximum penetration depth of tritium β-particles in water is only 6 μm [15].

Since radioactive tracer methods are usually performed under isotopic dilution, preliminary experiments were performed to determine the suitable concentration of the radioactive material in the monolayer. Glass slides were coated with monolayers by the Blodgett-Langmuir technique as modified by Kuhn et al. [16]. It was found that a glass slide coated with a double layer of a 20 : 1 mixture of arachidic acid to radioactive cholesterol had a specific activity of approximately 2 μCi/cm^2. A 10 min exposure of this slide to a nuclear plate at a distance of 0.1 mm produced a density of approximately 2000 silver crystals per (200 μm)^2 above background (50–100 crystals per (200 μm)^2). In preliminary experiments with the trough, it was found, however, that a monolayer which was 40 to 50 % radioactive was required to give adequate discrimination. This high concentration is necessary for two reasons. First one should be able to measure much lower concentrations as diffusion starts. Secondly, to eliminate water vapor condensation on the nuclear plate, which would absorb radiation, the effective exposure time had to be kept as short as possible.

Results of typical diffusion experiments are shown in Figs 2 and 3. The zero point of the x-axis at time zero is defined at the $\Gamma/\Gamma_0 = 0.5$ value, and this position was found to be consistent with the initial position of the AB barrier to within a fraction of a mm. The drift of the boundary in the surface could not be always eliminated but it did not seem to affect the concentration gradient. For approximately half of the experiments performed, the surface drift was so large that within 3–4 h the radioactive

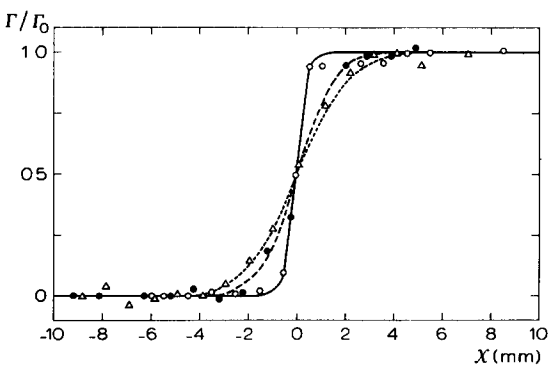

Fig. 2. Radioactive ^{3}H-labeled cholesterol diffusion experiment. –○–, initial distribution; – – – ● – – – distribution at time $t = 15.5$ h, surface diffusion coefficient $D = 6.1 \cdot 10^{-8}$ cm^2/s; - - - △ - - -, distribution at time $t = 41.5$ h, surface diffusion coefficient $D = 5.8 \cdot 10^{-8}$ cm^2/s. The maximum concentration Γ_0 was approximately 900 crystals per (200 μm)^2.

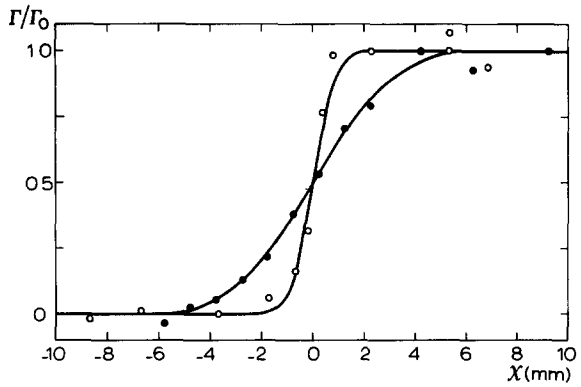

Fig. 3. Radioactive ^{3}H-labeled cholesterol diffusion experiment. -○-, initial distribution; -●-, distribution at time $t = 19.5$ h, surface coefficient is $2.0 \cdot 10^{-7}$ cm^2/s. The maximum concentration Γ_0 was approximately 1100 crystals per (200 μm)^2.

front was near the edge of the fixed photographic plate so that later measurements were not possible. A drifting phenomenon was also observed by Good and Schecter [11] who used an identical trough.

The experiments shown in Figs 2 and 3 have a reasonably sharp front with a very steep concentration gradient at zero time. The zero time gradient which occurs over a distance of approximately 1 mm is probably determined by the resolution of the method which improves when diminishing the distance between the plate and the radioactive layer. In these experiments the plates were located 0.1 $\pm$ 0.05 mm from the monolayer. In order to calculate the surface diffusion coefficient it was assumed that a step function in concentration did exist at time zero in spite of the gradient of the silver grains on the plate. Therefore, the curves were first fitted to a function of the form:

$$\frac{\Gamma(x,t)}{\Gamma_0} = \tfrac{1}{2}\,\mathrm{erfc}\left[\frac{-x}{2(\kappa t)^{\frac{1}{2}}+\tau}\right] \tag{6}$$

where τ and κ are fitting parameters determined from the $t = 0$, and the $t = t$ curve, respectively. The curves shown in Figs 2 and 3 were obtained from Eqn 6. The difference in distance between the $t = 0$ curve and the step function was subtracted from the $t = t$ curve. The final resulting curve was then matched with Eqn (3) in order to calculate diffusivity. This procedure is justified because any broadening of the radioactivity curves is due to diffusion. The values obtained by this method from the data presented in Figs 2 and 3 are of the order of 10^{-7} cm^2/s.

For experiments where the plate was separated by 0.2 mm $\pm$ 0.05 mm from the surface, a much broader initial distribution of the radioactivity as shown in Fig. 4 was observed. This confirms our assumption that the sharpness of the initial distribution is determined by the resolution which decreases with the distance of the nuclear plate from the monolayer. The number of spots per unit area of the photographic plate decreased with the distance. As with the experiments at the shorter distance (0.1 mm) the background spot density was subtracted from the spot counts. In Fig. 4 the drift of the monolayer was quite high, it moved 1.6 mm in 1h. The flow, however, does not appear to disturb the initial distribution. For Fig. 4 the time was too short to ob-

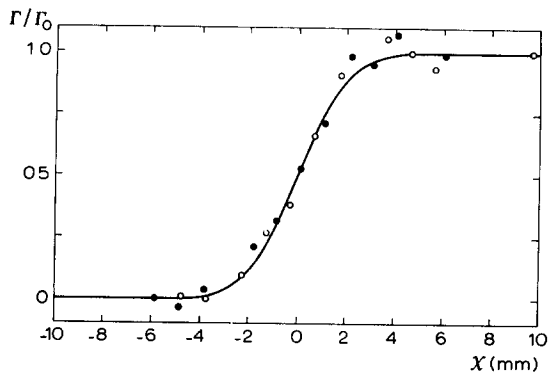

Fig. 4. Initial distribution of ^{3}H-labeled cholesterol (○), and the distribution after one hour (●). The ● points have been moved 1.6 mm to the right in order to coincide with the $\Gamma/\Gamma_0 = 0.5$ point. Γ_0 is approximately 250 crystals per $(200\ \mu m)^2$.

tain an accurate value of the diffusion coefficient and the drift was too rapid to perform a measurement at later times.

Several experiments were performed which gave a diffusivity range from approximately $6 \cdot 10^{-8}$ cm^2/s to $5 \cdot 10^{-7}$ cm^2/s. All experiments performed with the differential method were done on fully condensed monolayers at 38 Å^2/molecule (see later discussion).

Integral method

Experiments were conducted at times of either 5, 18, or 24 h. Measured diffusion coefficients were independent of time, but at longer times the reproducibility of the experiments was better. Most experiments were conducted at $t = 24$ h. ^{14}C-labeled cholesterol was used in these experiments and isotopically diluted to a 10 or 20% concentration. Fig. 5 shows the results of a series of measurements of the surface diffusion coefficient of cholesterol versus the surface concentration. At 38 Å^2/molecule the cholesterol monolayer is fully packed and causes a maximal surface tension reduction of water by 40 dynes/cm [17]. At 40 Å^2/molecule the cholesterol monolayer is still closely packed but the surface tension reduction drops below one dyne/cm. The experiments indicate that beyond 40 Å^2/molecule the lateral diffusion coefficient is nearly independent of surface concentration. Below 40 Å^2/molecule the lateral diffusivity appears to be a strong function of surface concentration, decreasing from (2 ± 1.2) 10^{-6} cm^2/s at 40 Å^2/molecule to $4 \pm 2 \times 10^{-7}$ cm^2/s at 38 Å^2/molecule.

In both methods the specific area per molecule was obtained from the known area of the trough and the concentrations of the spreading solutions. Surface tensions were measured with the Wilhelmy slide as a function of surface concentrations. At 38 Å^2/molecule (as calculated) the surface tension reductions varied from 15 to about 40 dynes/cm. At 40 Å^2/molecule the surface tension reduction was several dynes/cm and at lower concentrations it was nearly zero. To ensure that the monolayer at 38 Å^2/molecule was fully compressed a slight excess (1 %) of spreading solution was added to the liquid surface on the CD side of the trough.

DISCUSSION

The photographs of the inspected nuclear plates showed that the silver particles were randomly dispersed. Very few of the particles were in contact with each other. If each developed silver crystal has been struck by only one β-particle, then the surface radioactivity would be directly proportional to the concentration of the developed silver crystals. Inspection of the plates further showed that in nearly one quarter of all experiments attempted, the initial radioactive boundary was grossly disturbed. Bands of crystal density higher or lower than the average crystal density were observed and occasionally low density patches were noticeable. The first exposed plate therefore can serve as a control to see if the initial boundary condition is spread correctly. In the differential method, the material of interest must have a sufficiently high specific activity in order to shorten the exposure time to a few minutes. It is technically difficult to further diminish the distance between the plate and the surface to allow a lower radioactivity and to improve the resolution.

The filter paper used in the integral method was not a completely flat supporting surface, i.e., there may be some surface roughness. The increase in surface area allowed by the surface tension is, however, negligible. By assuming that the surface variation can be modelled as a wave, it was estimated that the length of the wave is one order of magnitude larger than the height.

The long incubation time ($\sim$ 24 h) required in these experiments raises a question if oxidative degradation of cholesterol may have taken place. In previous experiments in this laboratory [14], it has been shown that cholesterol maintains constant surface pressure and constant surface radioactivity over many ($\sim$ 10) h. No decrease of surface radioactivity was observed in this work over a 24 h period. This suggests that if oxidation took place, it did not produce highly volatile or soluble products. However, Smith et al. [18] exposed cholesterol to air at room temperature for a period of one month and could detect only trace amounts of oxidation products. Consequently for the time span of these experiments no significant oxidation took place.

Investigation of the result of the two methods show that the results are comparable for the fully condensed monolayer at 38 Å^2/molecule. Yun and Frederickson [18] have reported a value of $1.20 \cdot 10^{-5}$ cm^2/s for the diffusivity of cholesterol in toluene at 20 °C. The lower diffusivities of cholesterol in cholesterol monolayers are consistent with a less fluid system. The lateral diffusivities reported here are roughly from 10^{-6} to 10^{-7} cm^2/s. For fully packed monolayers of cholesterol (38 Å^2/molecule) the value of 10^{-7} cm^2/s is of the same order of magnitude as the widely spread values (10^{-7}–10^{-8} cm^2/s) reported for lipids and steroids in membranes, bilayers, multilayers, and monolayers.

Unfortunately none of the systems reported in the literature can be compared directly to the system studied here. Träuble and Sackman [20] reported a lateral diffusion coefficient of $1.0 \cdot 10^{-8}$ cm^2/sec for spinlabeled androstan, a cholesterol analogue, in lecitihin-cholesterol bilayers at 50 °C which is somewhat lower than the values reported here. However, Träuble [21] in another publication has asserted that the value of the lateral diffusion coefficient for spin-labeled androstan reported by Träuble and Sackman [20] must be considered only as a minimum value.

Chung and Berg [10] and Good and Schecter [11] also observed that the lateral diffusion coefficient is a function of surface concentration. The surface concentrations

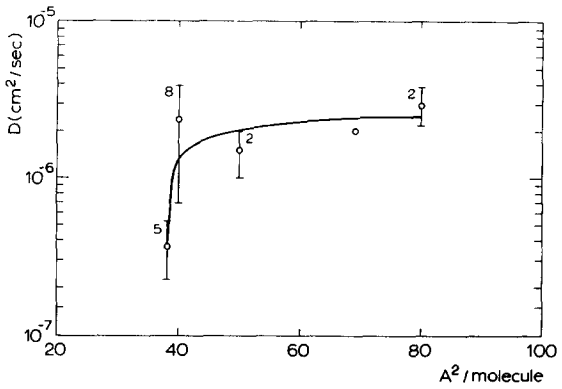

Fig. 5. Results of the ^{14}C-labeled cholesterol diffusion experiments with the integral method. The bar on the data points gives the variation of values observed and the number the total experiments. Data points are averages.

investgated were limited, however, so that no direct comparisons are possible in the low concentration range, where no significant change was observed. Cholesterol is believed to exist in a condensed-expanded state at a surface concentration below a packed monolayer (> 40 Å^2/molecule). In the condensed-expanded state, islands (clusters) of cholesterol molecules are present in a random distribution on the surface. The region between islands contains only a low concentration of cholesterol. The maximum diameter of such islands is considerably smaller than the penetration depth of radioactive cholesterol into the nonradioactive cholesterol monolayer during the course of the experiment. If we consider the monolayer of cholesterol in these surface concentrations to be in a two-phase state, condensed and expanded, then the results of Fig. 5 suggest that the mass transfer rates in both phases are nearly equal.

The surface diffusion coefficient becomes a strong function of the inverse of surface concentration below 40 Å^2/molecule as shown in Fig. 5. Below 40 Å^2/molecule the cholesterol monolayer becomes tightly packed and the lowest area per molecule is roughly 38 Å^2. Apparently, this close packing hinders the movement of the cholesterol molecules. Perhaps close packing plays also an important role in biological membranes to control lateral transport.

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