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MICROVISCOSITY PARAMETERS AND PROTEIN MOBILITY IN BIOLOGICAL MEMBRANES

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SUMMARY

A fluorescence polarization technique with 1,6-diphenyl 1,3,5-hexatriene as a probe were employed to determine the microviscosity, $\bar{\eta}$, in liposomes and biological membranes of different cholesterol to phospholipid mol ratio. From the temperature profile of $\bar{\eta}$ the flow activation energy, ΔE, and the unit flow volume, V, were derived. The increase of cholesterol/phospholipid ratio in liposomes is followed by a marked increase in $\bar{\eta}$ and a decrease in both ΔE and V. Liposomes of the same phospholipid composition as human erythrocyte membranes display in the extreme cases of cholesterol/phospholipid ratios 0 and 1.4 the values of $\bar{\eta}(25\ ^\circ C) = 1.8$ and 9.1 P, and $\Delta E =$ 15.0 and 6.5 kcal/mol, respectively. For most membranes studied the fluorescence polarization characteristics and the corresponding $\bar{\eta}$ values are similar to those obtained with these liposomes when the cholesterol/phospholipid level of the liposomes and the membranes were the same. However, unlike in liposomes ΔE of all membranes is in the narrow range of 6.5–8.5 kcal/mol, regardless of its cholesterol/phospholipid level. It is plausible that this is a general characteristic of biological membranes which originates from the vertical movement of membrane proteins to an equilibrium position which maintains constant ΔE and V values. This type of movement should affect the interrelation between lipid fluidity and protein mobility. Lipid microviscosity and the degree of rotational mobility of concanavalin A receptor sites in cell membranes were therefore determined. The examined cells were normal and malignant fibroblasts, as an example of cells that form solid tumours in vivo, and normal and malignant lymphocytes, as an example of cells that form ascites tumours in vivo. In both cell systems, opposite correlations between the lipid fluidity and the mobility of concanavalin A receptors were observed. In the fibroblasts the malignant cells possess a lower lipid fluidity but a higher receptor mobility, whereas in the lymphocytes the malignant cells possess a higher lipid fluidity but a lower receptor mobility. Thus, in these cell systems the degree of rotational mobility of concanavalin A receptors increases upon decreasing the lipid fluidity and decreases upon increasing the fluidity of the lipid core. This dynamic feature is in line with the above proposal according to which the concanavalin A receptor sites become more exposed to the aqueous surrounding upon increasing the microviscosity of the lipid layer and vice versa.

INTRODUCTION

The fluid nature of biological membranes is now well documented and widely accepted [1–7]. However, the term "membrane fluidity" which is commonly employed in this respect is actually complex and covers different disciplines of the membrane. The most prominent of these are thermal mobility of membrane proteins [1, 3, 6–13] and microviscosity of the membrane lipid layer [4, 14, 15]. These dynamic features, which to some extent are interrelated, are now believed to play a major role in the cellular control mechanisms and to determine normal and abnormal cell growth and differentiation.

Membrane microviscosity, $\bar{\eta}$, and its related parameters could be envisaged as a mechanical barrier which is imposed by the lipid layer and can control transport processes [16] and signal transmissions across the membrane [17]. It is best presented by assuming a complete analogy between the membrane lipid core and a hydrocarbon fluid (e.g. paraffin oil), which enables the application of the well-known treatments and expressions of viscosity, as performed in studies of viscous flow in polymeric materials [18]. The basic microviscosity parameters could thus be evaluated from the expression:

$$\bar{\eta} = Ae^{\Delta E/RT} \tag{1}$$

which actually formulates a Newtonian flow [19]. In this equation T is the absolute temperature, R, is the gas constant, A is a constant characteristic of the system, and ΔE is the flow (or fusion) activation energy. A temperature region with a constant ΔE value defines a singular phase, whereas a phase transition is indicated by a change in ΔE which is displayed by a break in the plot of log $\bar{\eta}$ vs. $1/T$ [20]. The factor A, which is obtained from the intercept of such a plot, contains additional fluidity parameters of the system in its present phase.

The dynamic parameters of lipid layers were studied extensively during the last decade, mostly with liposomes as membrane model systems [2, 21, 22]. These studies have established that three main characteristics determine the microviscosity of lipid regions, and its related parameters. The most prominent of these is the mol ratio of cholesterol to phospholipids. Under physiological conditions and with natural-occurring phospholipids an increase in cholesterol/phospholipid ratio will cause an increase in $\bar{\eta}$ [2, 23–25], and in the degree of order in the system [25], and will gradually abolish phase transitions [26, 27]. The two other characteristics are the degree of unsaturation of the phospholipid acyl chains [21] and the relative amount of sphingomyelin in the system [28].

In the following study we have determined the effect of cholesterol/phospholipid on $\bar{\eta}$ and ΔE, as well as on other fluidity parameters, in a series of systems. The systems studied were liposomes made of phospholipids and cholesterol, single membrane organelles, and the surface membrane of intact cells. The method employed is comprehensively described in previous articles [14, 28] and is based on fluorescence polarization measurements with the probe 1,6-diphenyl 1,3,5-hexatriene (diphenyl hexatriene). This method can yield the absolute value of $\bar{\eta}$ and was shown to be highly sensitive and reproducible.

Since both the proteins and the lipids are integral parts of the membrane, intimate relationship between the membrane microviscosity and the degree of mobility

of its proteins should, in principle, prevail. In an attempt to achieve a better insight into this fundamental problem we have determined the degree of mobility of the concanavalin A receptor sites [12, 13] in cell membranes which were analyzed for microviscosity properties. The cells used were normal and malignant transformed fibroblasts grown in vitro, and in vivo normal and malignant lymphocytes.

MATERIALS AND METHODS

Liposomes. The phospholipids used were all grade I chromatographically pure and were purchased from Lipid Products, England. These were phosphatidylcholine from egg yolk, phosphatidylethanolamine from egg yolk, phosphatidylserine from bovine spinal cord, phosphatidylinositol from wheat germ, sphingomyelin from bovine brain, cardiolipin from ox heart. The phospholipids were kept as stock solutions in chloroform saturated with N_2. The molar concentration of each of the stock solutions was determined by the method of Bartlett [29]. Volumes from the stock solutions were mixed, cholesterol (Sigma CH-S) was added at the proper molar levels, and the solutions were evaporated under N_2 to complete dryness. Phosphate-buffered saline, pH 7.2, was then added to form dispersions of 1 mg/ml phospholipids. The dispersions were sonicated under N_2 for 10 min with Braun-Sonic 300 sonicator and then centrifuged at 30 000 $\times g$ to separate traces of insoluble material.

Membranes and cells. All membranes were isolated by methods which are described in the literature and which yielded the purest preparations. Human erythrocyte "ghost" membranes containing 5 % of the initial haemoglobin were prepared by the method of Dodge et al. [30]. The cholesterol/phospholipid ratio of these membranes is 0.95 ± 0.03 [31]. Chromaffin granules from bovine adrenal medulla were prepared according to Smith and Winkler [32]. These membranes possess cholesterol/phospholipid 0.7 [33]. Mitochondria from bovine heart were prepared according to Fleischer et al. [34]. These membranes are very low in cholesterol with cholesterol/phospholipid 0.03 ± 0.01 [34].

Human normal lymphocytes or leukaemic cells were isolated from freshly drawn peripheral blood from normal subjects and chronic lymphatic leukaemia patients, by Ficoll-Hypaque gradient centrifugation [35]. The cholesterol/phospholipid ratio in the plasma membranes of these cells were taken as 0.75 [36] and 0.38 [37], respectively. The cells were washed twice and resuspended in phosphate-buffered saline to a concentration of $5 \cdot 10^6$ cells/ml. The Burkitt lymphoma cells used in the present experiments were established as an in vitro cell line [38, 39]. The cells were grown in RPMI 1640 with 20 % fetal calf serum and were subcultured every 4–6 days. The cells were washed and resuspended in phosphate-buffered saline at a concentration of $5 \cdot 10^6$ cells/ml. Rat liver cells (hepatocytes) were isolated from a living rat by the collagenase perfusion method of Berry and Friend [40]. The cells were washed several times with phosphate-buffered saline and resuspended to a concentration of $2 \cdot 10^6$ cells/ml. The lipid composition of the plasma membrane of these cells is unusually high in neutral lipids and free fatty acids [41].

Normal fibroblasts were from tertiary cultures of golden hamster embryos. The malignant fibroblasts used were a line derived from a simian virus 40 (SV40)-induced hamster tumour. Normal and malignant fibroblasts were grown in vitro in Eagle's medium with 10 % fetal calf serum; 10^6 cells were seeded in a 100 mm

Petri dish and incubated in a CO_2 incubator at 37 °C. For the experiments, normal and transformed fibroblasts at 3 days after seeding were dissociated with 0.02 % EDTA solution by incubation for 20 min at 37 °C. The dissociated cells were then washed three times with phosphate-buffered saline, pH 7.2. The in vivo cells were normal lymphocytes obtained from lymph nodes of CR/RAR rats of lymphocytes obtained from thymus or spleen of A strain mice. The leukaemic cells were from an ascites form of Moloney virus-induced lymphoma grown in A strain mice [42]. A total of 10^5 lymphoma cells were inoculated intraperitoneally, and the cells were used 10 days after inoculation. The normal lymphocytes and the leukaemic cells were collected from animals and immediately used in the experiments after three washings with phosphate-buffered saline.

Fluorescence labelling and measurements. For monitoring lipid microviscosity we have used as a probe the fluorescence hydrocarbon 1,6-diphenyl 1,3,5-hexatriene. This compound has a series of outstanding spectral properties [28] and is currently the most efficient fluorescence polarization probe available for monitoring fluidity properties of lipid regions. For labelling, a solution of $2 \cdot 10^{-3}$ M diphenyl hexatriene in tetrahydrofuran was diluted 2000-fold by injection into vigorously stirred phosphate-buffered saline. The obtained dispersion of 10^{-6} M diphenyl hexatriene is clear and practically void of fluorescence [14, 28]. The diphenyl hexatriene dispersion was mixed 1 : 1 (v/v) with the liposomes, membranes and cell suspensions and was incubated at 25 °C for 1 h. The incorporation of diphenyl hexatriene into the lipid layer is followed by a steep increase in fluorescence intensity. The liposomes and membranes were measured immediately after incubation whereas the cells were washed and resuspended in the buffered saline to a concentration of 10^6 cells/ml.

In previous articles [14, 17] we presented a series of evidence which suggested that in diphenyl hexatriene-labelled cells the probe is predominantly located in the surface membrane. This is supported by the observed pattern in a fluorescence microscope which is of a glowing periphery. Unfortunately, the labelled cells tend to bleach very rapidly [28] and attempts to photograph them failed. Unless otherwise proven, we will continue to relate the diphenyl hexatriene signal to the cell surface membrane even though conclusive evidence for it are not yet in hand. It should also be noted that the degree of fluorescence polarization is an intrinsic parameter and is therefore independent on fluorescence intensity at a wide range of concentrations. The concentration of probe and lipids used here were around the optimal instrumental response range and more than 100-fold above the ground signal. On the other hand, these concentrations were at least 100-fold below the levels where fluorescence depolarization due to energy transfer [43] could occur.

For measuring the rotational mobility of membrane proteins 10^7 cells were incubated for 15 min at 37 °C with 1 ml phosphate-buffered saline containing 5 μg fluorescein-conjugated concanavalin A and 5 μg native concanavalin which were obtained from Miles Yeda, Rehovot, Israel. The cells were then washed with phosphate-buffered saline and used in the experiments. The batch of fluorescein-concanavalin A selected for this study showed the greatest difference in fluorescence polarization when free in solution and when bound to the cell surfaces. This batch contained about one fluorescein residue per subunit of concanavalin A.

Fluorescence measurements were performed essentially as outlined before [14, 28] with an instrument which is described elsewhere [44]. For excitation of

diphenyl hexatriene plane polarized 366 nm mercury band was employed. The emitted light was detected in two independent cross-polarized channels after passing through a cut-off filter of 2 M $NaNO_2$. For measurements with fluorescein-concanavalin A-labelled cells the 435 nm Hg band was used for excitation and an aqueous solution of 0.3 % sodium bichromate was used as a cut-off filter. The temperature of the measured sample was controlled with a circulation bath (Haake) and was measured with a thermometer immersed in the sample. When cell suspensions were measured the solutions were gently mixed before each measurement.

Determination of microviscosity ($\bar{\eta}$) and rotational mobility. Fluorescence polarization and intensity measurements of diphenyl hexatriene-labelled systems can yield the absolute value of $\bar{\eta}$ by a method which is described in previous articles [14, 28]. The method is based on the Perrin equation for rotational depolarization

$$\frac{r_0}{r} = 1+C(r)\frac{T\tau}{\bar{\eta}} \tag{2}$$

where r_0 and $r^\star$ are the limiting and the measured fluorescence anisotropies, respectively, T is the absolute temperature and τ is the lifetime of the excited state. $C(r)$ is a structural parameter which varies slightly with r and is obtained from a calibration curve which describes the rotational depolarization of diphenyl hexatriene in liquid paraffin. For the derivation of $\bar{\eta}$ simultaneous determinations of r and τ at a given T were carried out. It should be stressed that like with most spectral techniques the microviscosity determined by this procedure is an average value of all the labelled lipid domains in the system.

The degree of rotational mobility of the concanavalin A receptor was defined by the term ρ_0/ρ where ρ_0 and ρ are the rotational relaxation times of fluorescein-concanavalin A free in phosphate-buffered saline and bound to the cell membrane, respectively [12, 13]. The value of ρ_0/ρ extends from 0, which represents an immobilized state, to 1, which represents the fully mobile state. The ρ_0/ρ was evaluated, as previously described [12, 13].

RESULTS

Mixed liposomes made of phospholipid mixtures and increasing amounts of cholesterol, were employed as model systems for membranes of different cholesterol/phospholipid ratio values. The first type of liposomes contained a mixture of phospholipids (mixture I) identical to that found in human erythrocyte membranes [31], one of the few membranes which were thoroughly analysed for lipid composition. Mixture I was thus comprised of 30 % (mol fraction) phosphatidylcholine, 25 % sphingomyelin, 30 % phosphatidylethanolamine, 13 % phosphatidylserine, 1 % cardiolipin and 1 % phosphatidylinositol. Mixtures II and III differed from mixture I only in their relative amounts of phosphatidylcholine and sphingomyelin; mixture II contained 45 % phosphatidylcholine and 10 % sphingomyelin, whereas mixture III contained 10 % phosphatidylcholine and 45 % sphingomyelin. Mixtures II and III were aimed at testing the

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* $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$ where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities detected through an analyser with an electric field oriented parallel and perpendicular to the electric field of the exciting beam.

effect of the sphingomyelin level [28] in membranes where the ratio of phosphatidylcholine to sphingomyelin can change [31, 45].

The fluorescence polarization of the mixed liposomes labelled with diphenyl hexatriene was found to increase markedly with increase of cholesterol/phospholipid ratio, as might be expected. In all three mixtures this increase obeys a linear relationship when the fluorescence polarization is expressed in terms of $(r_0/r-1)^{-1}$, as shown in Fig. 1. It is apparent from Eqn. 2 that if one assumes that at a given temperature $C(r)$ and τ are constant and independent on r or $\bar{\eta}$, then $(r_0/r-1)^{-1}$ should be directly proportional to $\bar{\eta}$. However, this relation is only approximate since both $C(r)$ and τ increase with r, though much less than $\bar{\eta}$, and as a result $\bar{\eta}$ should be more sensitive to changes in cholesterol/phospholipid ratio than $(r_0/r-1)^{-1}$. The approximate linear relation $\log \bar{\eta}$ (25 °C) = 0.17+0.6 cholesterol/phospholipid ratio which was proposed previously [46] is in line with these considerations. It may be therefore concluded that increasing the value of cholesterol/phospholipid ratio from 0 to 1 in all three mixtures causes an increase of about 4-fold in $\bar{\eta}$. The effect of sphingomyelin on increasing $\bar{\eta}$ [28] at any cholesterol/phospholipid ratio level, is also clearly demonstrated in Fig. 1.

The change of fluorescence polarization with cholesterol/phospholipid in the cells and membranes, which will be dealt with later in this study, are also given in Fig. 1. As shown, except for the rat liver cells, there is good agreement between the results obtained with membranes and with the model systems of mixture I. This is especially pertinent for the erythrocyte membranes which have the same lipid com-

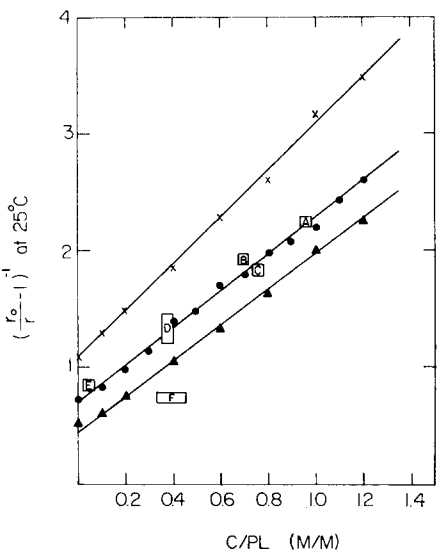

Fig. 1. The dependence of $(r_0/r-1)^{-1}$ at 25 °C on cholesterol/phospholipid ratio (C/PL) in three types of liposomes made from the phospholipid mixtures I (●–●), II (▲–▲) and III (×–×), and in six biological membranes. The membranes presented are human erythrocyte membranes (A), chromaffin granules (B), surface membrane of human normal lymphocytes (C) and of chronic lymphatic leukemia lymphocytes (D), mitochondria (E), and the surface membrane of rat liver cells (F). The latter is presented by cholesterol/total lipid ratio rather than cholesterol/phospholipid ratio. For details see text.

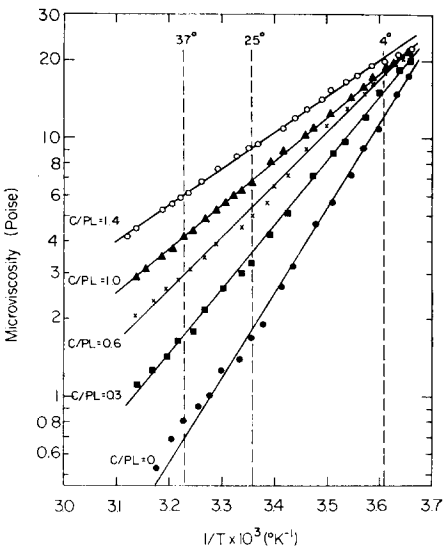

Fig. 2. Temperature dependence of microviscosity presented as $\log \eta$ vs. $1/T$ of liposomes made from mixture I at different cholesterol/phospholipid levels.

TABLE I

MICROVISCOSITY AND FUSION ACTIVATION ENERGY (ΔE) IN THE HYDROCARBON CORE OF LIPOSOMES OF DIFFERENT CHOLESTEROL TO PHOSPHOLIPIDS MOL RATIO

The phospholipid composition is 30 % (mol fraction) phosphatidylcholine, 30 % phosphatidylethanolamine, 25 % sphingomyelin, 13 % phosphatidylserine, 1 % cardiolipin and 1 % phosphatidylinositol. The table includes also the value of the preexponential factor A and the estimated relative volume V_r of the unit flow in the system.

Cholesterol/phospholipid (M/M)	Microviscosity (P)			ΔE (kcal/mol)	A (P)	V_r
	4 °C	25 °C	37 °C			
0	12.3	1.8	0.7	15.0	$2 \cdot 10^{-11}$	1
0.3	15.3	3.6	1.7	11.3	$2 \cdot 10^{-8}$	$1 \cdot 10^{-3}$
0.6	17.6	5.4	2.9	9.4	$9 \cdot 10^{-7}$	$2.7 \cdot 10^{-5}$
1.0	18.4	6.8	4.1	7.8	$1.4 \cdot 10^{-5}$	$1.7 \cdot 10^{-6}$
1.4	20.8	9.1	6.0	6.5	$1.7 \cdot 10^{-4}$	$1.7 \cdot 10^{-7}$

position as the model system, which is in line with the observation that these membranes have a similar microviscosity to that of liposomes made from their lipid extract [47]. The mixture I liposomes can, therefore, represent the approximate dynamic properties of membrane lipid layers with the same cholesterol/phospholipid ratio, and could be used for an approximate estimation of cholesterol/phospholipid ratio in membranes according to their fluorescence polarization values.

Temperature profiles of the microviscosity in liposomes of mixture I with increasing levels of cholesterol/phospholipid are shown in Fig. 2. As demonstrated,

increasing of the cholesterol/phospholipid level is concomitant with an increase in $\bar{\eta}$ and A and a decrease in ΔE (see Eqn. 1). The results obtained from Fig. 1 are summarized in Table I. Similar changes in these dynamic parameters upon changing the cholesterol/phospholipid level were also found in mixtures II and III.

The actual meaning of the preexponential factor A derived from the intercepts in Fig. 1 is not expressed in Eqn. 1. By applying the theory of absolute reaction rates Eyring [48] and later Kauzmann and Eyring [18], derived an analogous expression for a Newtonian flow (Eqn. 2) in which the factor A is explicitly defined:

$$\bar{\eta} \approx \frac{h}{V} e^{\Delta F^{\neq}/RT} = \frac{h}{V} e^{-\Delta S^{\neq}/R} e^{\Delta H^{\neq}/RT} \tag{3}$$

In this equation h is Planck's constant, V is the volume of a unit flow, $\Delta F^{\neq}$ is the free energy of flow activation, $\Delta H^{\neq}$ is the enthalpy of activation and $\Delta S^{\neq}$ is the entropy of activation. $\Delta H^{\neq}$ represents the heat required for flow activation and is practically identical with ΔE and therefore

$$A = \frac{h}{V} e^{-\Delta S^{\neq}/R} \tag{4}$$

According to Eqns 1 and 4 the intercept (log A) in a plot of log $\bar{\eta}$ vs. $1/T$ is equal to log $h/V - \Delta S^{\neq}/2.3R$. The entropy of activation $\Delta S^{\neq}$ is the difference in entropies per mol of flow unit in the activated and the resting states. In a series of analogous systems, like liposomes of similar composition, $\Delta S^{\neq}$ is probably similar in magnitude especially when two adjacent systems in the series are concerned. This assumption leads to the approximate relation $\log A_1/A_2 = V_2/V_1$ which thus permits the evaluation, on a relative scale, of the unit flow volumes, V_r, in the series. The estimated values of V_r which are also given in Table I show that by increasing the cholesterol/phospholipid value of the system its unit flow volume decreases markedly (see below).

Microviscosity parameters of liposomes made from three of the main phospholipids which construct the liposome mixtures studied above, are given in Table II. The phosphatidylcholine and the phosphatidylserine liposomes exhibit similar features to those observed in the mixed liposomes. Thus, the increase in cholesterol/phospholipid ratio in both systems is concomitant with a marked increase in $\bar{\eta}$ and a decrease in ΔE. The liposomes made from bovine brain sphingomyelin display remarkably high microviscosities (see Table II) in addition to a well-distinguished phase transition at 29 °C. These characteristics are similar to those detected in liposomes of sphingomyelin from bovine spinal cord which display a phase transition at 32 °C [28]. At 25 °C, below the phase transition, the presence of cholesterol increases the microviscosity of sphingomyelin liposomes only when cholesterol/phospholipid is smaller than 0.5. At higher cholesterol levels the microviscosity is decreased and the phase transition is gradually abolished (Barenholz, Y. and Shinitzky, M., unpublished results). The value of 29.8 P, determined in the liposomes of cholesterol-sphingomyelin 0.4 M/M (see Table II), is indeed one of the highest $\bar{\eta}$ (25 °C) values ever recorded by us. These results indicate that cholesterol can form a specific complex with sphingomyelin as proposed by Vandenheuval [49].

The temperature profiles of $\bar{\eta}$ in three biological membranes, in the range of

TABLE II

FLUORESCENCE POLARIZATION AND DYNAMIC PARAMETERS OF THE HYDROCARBON LAYER OF LIPOSOMES MADE OF A SINGLE PHOSPHOLIPID (1 mg/ml) AND DIFFERENT MOL LEVELS OF CHOLESTEROL

Phospholipid	Cholesterol/ phospholipid (M/M)	$(r_0/r-1)^{-1}$ (at 25 °C)	$\bar{\eta}$(25 °C) (P)	ΔE (kcal/mol)
Phosphatidyl-choline	0	0.44	0.95	9.0
	0.4	0.88	2.20	8.0
	1.0	1.82	5.50	6.7
Phosphatidyl-serine	0	0.62	1.53	14.0
	0.4	1.09	3.23	11.0
	1.0	2.0	5.90	7.7
Sphingomyelin	0	5.80	18.9	phase transition
	0.4	8.90	29.8	at 29 °C
	1.0	7.70	25.3	

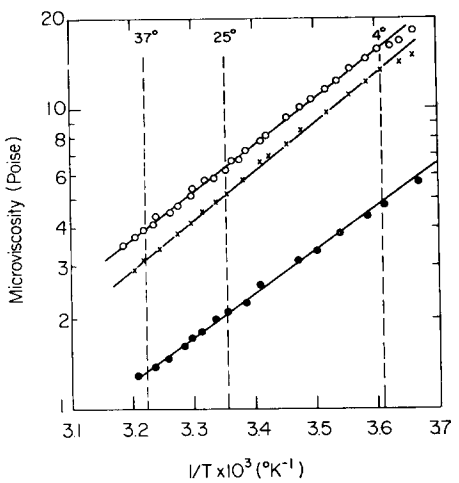

Fig. 3. Temperature dependence of microviscosity in membranes of human erythrocytes (○–○), chromaffin granules (×–×) and mitochondria (●–●).

0–40 °C, are shown in Fig. 3. The linear dependence of log $\bar{\eta}$ on $1/T$ indicates an invariant phase in all three systems at the above range of temperatures. Microviscosities values and related flow parameters of these systems are given in Table III. The analysed membranes differ in their cholesterol/phospholipid ratio (see Materials and Methods) which is reflected in their $\bar{\eta}$ values. However, the ΔE values seem to be unaffected by the cholesterol/phospholipid level unlike in the liposomes. Analogous plots for the surface membranes of four cell types are shown in Fig. 4. The results

TABLE III

MICROVISCOSITY AND RELATED PARAMETERS OF ORGANELLE MEMBRANES AND CELL SURFACE MEMBRANES

For details, see text.

Membrane (organelle or cell surface)	Cholesterol/ phospholipid (M/M)	Microviscosity (P)			ΔE (kcal/mol)	A (P)	V (Å^3)
		4 °C	25 °C	37 °C			
Mitochondria (bovine heart)	0.02–0.05	4.8	2.1	1.3	6.7	$2 \cdot 10^{-5}$	$1.5 \cdot 10^{4}$
Chromaffin granules (bovine)	0.75	13.5	5.2	3.1	7.5	$2 \cdot 10^{-5}$	$3.8 \cdot 10^{4}$
Erythrocyte ghosts (human)	0.9–0.95	16.0	6.3	4.0	7.3	$3 \cdot 10^{-5}$	$2.0 \cdot 10^{4}$
Burkitt lymphoma cells (human)	–	3.0	1.2	0.73	7.2	$5 \cdot 10^{-6}$	$8.3 \cdot 10^{4}$
Liver cells (rat)	0.25–0.45★	4.0	1.7	1.1	6.7	$2 \cdot 10^{-5}$	$1.9 \cdot 10^{4}$
Chronic lymphatic leukemia lymphocytes (human)	0.4	8.8	3.4	2.0	7.7	$8 \cdot 10^{-6}$	$9.3 \cdot 10^{4}$
Normal lymphocytes (human)	0.75	12.3	4.4	2.6	8.2	$5 \cdot 10^{-6}$	$2.3 \cdot 10^{5}$

★ Cholesterol/total membrane lipid ratio.

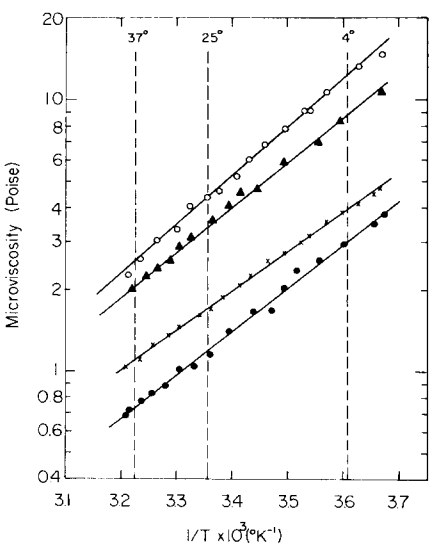

Fig. 4. Temperature dependence of microviscosity in the surface membrane of intact cells. The cells presented are: human normal lymphocytes (○–○), human chronic lymphatic leukemia lymphocytes (▲–▲), rat liver cells (×–×) and Burkitt lymphoma cells (●–●).

obtained are of similar characteristics to those presented in Fig. 3, and are also summarized in Table III. As shown, only $\bar{\eta}$ is affected by the cholesterol/phospholipid level similar to the liposomes.

Based on previous considerations [14, 17] we assumed that with intact cells labelled with diphenyl hexatriene the major contribution to the fluorescence signal originates from the surface membrane (see Materials and Methods). The microviscosities obtained with the human normal lymphocytes, the chronic lymphatic leukaemia lymphocytes and the Burkitt lymphoma cells agree well with a previous observation that the surface membrane of leukaemic cells has a lower $\bar{\eta}$ value than the surface membrane of normal lymphocytes [14]. The increase in fluidity in leukaemic cells is in correlation with the acuteness of the disease [46] and is predominantly caused by decrease of cholesterol/phospholipid ratio in the cell surface membranes. The microviscosity observed in the Burkitt lymphoma cells is unusually low and, according to the results shown in Fig. 4, it may be expected that the cholesterol/phospholipid ratio in the surface membrane of these cells is below 0.2. However, determination of cholesterol/phospholipid ratio in their total lipid extract gave cholesterol/phospholipid 0.4. It might be that the contribution of inner membranes to this value is significant, or that the surface membrane of these cells is particularly rich in neutral lipids and in unsaturated acyl groups or low in sphingomyelin. It is pertinent to note that lymphocytes from other chronic lymphatic leukaemia patients showed similar results to those presented here and in all cases were with microviscosity below that of human normal lymphocytes. In addition, results obtained with other lines of Burkitt lymphoma cells were similar to those obtained with the line presented here.

The cholesterol/phospholipid ratio in the surface membrane of rat liver cells was estimated to be 0.6 ± 0.1 from the data summarized by Ray et al. [41]. However, the fluidity of this membrane is much greater than could be expected from its cholesterol/phospholipid value (see Fig. 4). This could be accounted for by the high content of neutral lipids in this membrane [41]. It is therefore more appropriate to correlate the microviscosity of this membrane with the ratio of cholesterol to total membrane lipids.

The monophasic behaviour of the microviscosity in the membranes, which is characterised by a relatively low and constant ΔE value of 7.5 ± 1.0 kcal/mol, enables us to pursue further the analogy between the viscous flow in these systems and in hydrocarbon liquids. In simple liquids, including hydrocarbons, the following empirical relations were observed [50]:

$$E_{vap}/\Delta E \approx 3.5 \text{ and } E_{vap}/\Delta F^{\neq} (25\ ^{\circ}\text{C}) \approx 2.45 \tag{4}$$

where E_{vap} is the heat of vaporation. A combination of these relations leads to $\Delta F^{\neq}$ $(25\ ^{\circ}\text{C}) \approx 1.4\ \Delta E$, which upon substitution in Eqn. 3, in addition to the other given values, the following approximate relationship is obtained:

$$\log V = \Delta E - 2.2 - \log \bar{\eta}\ (25\ ^{\circ}\text{C}) \tag{5}$$

In this expression V is given in Å^3, ΔE in kcal/mol and $\bar{\eta}$ in P. The unit flow volumes of the studied membranes obtained from Eqn. 5 are given in Table III. If one assumes that the derived volumes are cubic then the range of lengths of the unit flow segments, $V^{\frac{1}{3}}$, is 25–61 Å, which is indeed in the range of chain lengths of membrane phospholipids. A similar approach to derive the absolute V values in the studied mixed lipo-

somes is inadequate because of the large variations in ΔE. The resulting V values fall in some of these cases beyond the limits imposed by the lipid bilayer.

Normal and SV40-transformed fibroblasts, as well as normal lymphocytes and malignant lymphoma cells were examined for the interrelation between the rotational mobility of concanavalin A receptor sites and the membrane microviscosity. The temperature profiles of the microviscosity, $\bar{\eta}$, in the membrane lipid core of these four cell types are shown in Fig. 5. As displayed in the figure, malignant transformation of hamster fibroblasts is characterized by an increase in the membrane microviscosity (decrease in fluidity). Similar results were recently obtained with normal and transformed 3T3 fibroblasts by Fuchs et al. [15] who used the same technique.

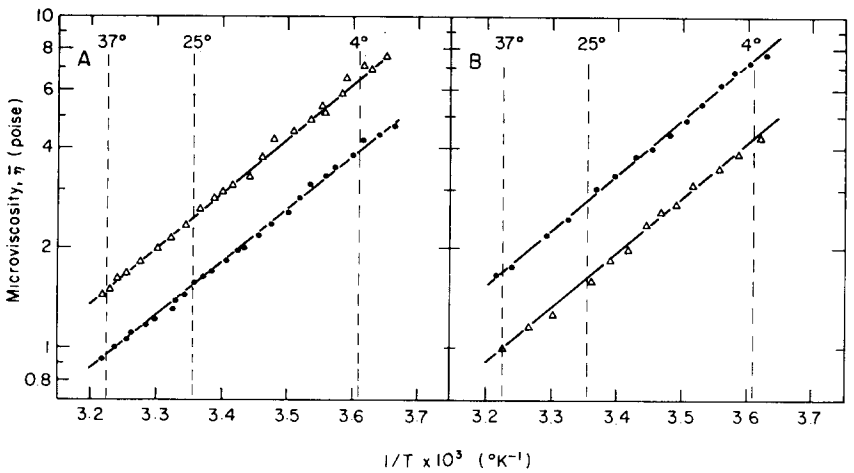

Fig. 5. Temperature dependence of microviscosity, η, presented as log η vs. $1/T$. A, normal hamster fibroblasts (●–●) and hamster SV40-transformed fibroblasts (△–△); B, mouse lymphoma cells (△–△) and mouse normal lymphocytes from spleen (●–●). The results obtained with normal lymphocytes from mouse thymus or from rat lymph-nodes were practically identical with the latter.

In contrast to fibroblasts, Fig. 5 shows that malignancy in the lymphoid system is associated with a decrease in the membrane microviscosity, as has been previously observed [14]. The linear changes of log $\bar{\eta}$ with $1/T$ indicate that the overall lipid phase in the four cell membranes is invariant at the temperature range of 0–40 °C. The slopes of these straight lines yield for all four cells a flow activation energy, E, around 7.5 kcal/mol as observed for the other biological membranes. The values of $\bar{\eta}$ at 25 °C and ΔE obtained from Fig. 5 are summarized in Table IV.

In parallel to the microviscosity measurements, the degree of rotational mobility of concanavalin A receptor sites, ρ_0/ρ, was measured in the four cell types, as previously described [12, 13]. In agreement with our previous results [13] ρ_0/ρ was also found to change upon malignant transformation of the normal cells (see Table IV). Transformation of the fibroblasts is accompanied by an increase in the degree of rotational mobility of the concanavalin A receptors, whereas transformation of the lymphocytes is associated with a decrease in the rotational mobility of concanav-

TABLE IV

MICROVISCOSITY ($\bar{\eta}$) AND FLOW ACTIVATION ENERGY (ΔE) OF MEMBRANE LIPID LAYERS AND THE DEGREE OF ROTATIONAL MOBILITY (ρ_0/ρ) OF CONCANAVALIN A RECEPTORS IN NORMAL AND MALIGNANT FIBROBLASTS GROWN IN VITRO AND IN VIVO NORMAL AND MALIGNANT LYMPHOCYTES

Cells	$\bar{\eta}$ (25 °C) (P)	ΔE (kcal/mol)	ρ_0/ρ (25 °C)
In vitro fibroblasts			
Hamster	1.55	7.4	0.45 ± 0.05
Hamster SV40	2.45	7.7	0.80 ± 0.05
In vivo lymphocytes			
Mouse normal lymphocytes	2.75	7.9	0.85 ± 0.05
Mouse lymphoma cells	1.60	7.6	0.35 ± 0.05

alin A receptors on the cell membranes. Both of these changes are in an opposite direction to the differences observed in the lipid fluidity between normal and malignant fibroblasts and between normal and malignant lymphocytes.

DISCUSSION

Phospholipids and cholesterol are the two major lipid constituents of biological membranes. Cholesterol is distributed in the phospholipid domain where it is only loosely bound and can therefore exchange with exogeneous pools of cholesterol like liposomes [51–54] or serum [55]. Likewise, liposomes and sera can also change the absolute cholesterol level of cell membranes by a simple partitioning mechanism which is directed towards equilibration of the cholesterol/phospholipid in the membrane and the medium [14, 51–58]. As demonstrated in the literature [2, 23–25]. as well as in this study, the microviscosity both in liposomes and in membranes increases markedly with increase of cholesterol/phospholipid ratio. The translocation of cholesterol can thus be utilized to change or control the microviscosity under various physiological conditions. Changes of this kind may induce or alter various cellular functions which depend on the dynamics of the cell surface membrane, such as transport processes, cell division and cell differentiation.

Other factors besides cholesterol/phospholipid can also affect the microviscosity of membrane lipid regions. The most prominent are the degree of unsaturation and the length of the phospholipid acyl chains, the level of sphingomyelin and the presence of neutral lipids like triglycerides. At relatively high levels of neutral lipids, like in the surface membrane of rat liver cells [41], the microviscosity should correlate better with cholesterol/total lipid ratio rather than with cholesterol/phospholipid ratio (see Fig. 1). In all other membranes studied here the amount of neutral lipids is relatively small and the correlation between cholesterol/phospholipid ratio and $\bar{\eta}$ remains valid. As shown in Fig. 1, the variation of $(r_0/r-1)^{-1}$ with cholesterol/phospholipid ratio of these membranes, with the exception of the surface membrane of rat liver cells, is very similar to that obtained with the mixed liposomes made of

mixture I. The phospholipid composition of mixture I is the same as in human erythrocyte membranes, whereas mixtures II and III are pure experimental model systems. The above fitness, therefore, suggests that variations among the various membranes in their phospholipid composition and in the nature of their acyl groups are such that the resulting effects on the gross fluidity properties of the lipid region remain relatively small. This suggestion is supported by the finding that erythrocyte ghost membranes from rat, man and sheep, which differ markedly in their phospholipid composition [31], show relatively small differences in their microviscosity (Barenholz, Y. and Shinitzky, M., unpublished results). These findings further emphasize the major role of cholesterol/phospholipid ratio as a determinant of membrane microviscosity.

The flow activation energy, ΔE, can be interpreted as the energy required to fuse the flowing segments to furnish a "hole" which is large enough to maintain the flow. This parameter actually relates to the energy of dissociation of a flowing segment from the bulk of the flowing unit, and thus to the complexity of its molecular structure. In linear hydrocarbon liquids of up to 20 carbon atoms ΔE does not exceed 4 kcal/mol [18] but is increased markedly in branched hydrocarbon liquids. In liquid paraffins, for example, it is in the range of 12–16 kcal/mol. It is therefore apparent that ΔE of lipid layers can serve as a criterion for the degree of order in the hydrocarbon core. In all of the three types of mixed liposomes, as well as in liposomes of phosphatidylcholine and phosphatidylserine, ΔE decreases with increase of cholesterol level in the system (see Fig. 6 and Tables I and II) which indicates an increase in order. The decrease in ΔE is concomitant with a marked decrease in both the unit flow volume (see Table I) and the average length of the flowing segments, which is also in accord with the physical meaning of ΔE given above. The interaction of the rigid backbone of cholesterol with the phospholipid acyl chains thus increases the degree of order in the hydrocarbon region and simultaneously reduces the average length of the hydrocarbon segments which participate in the flow process.

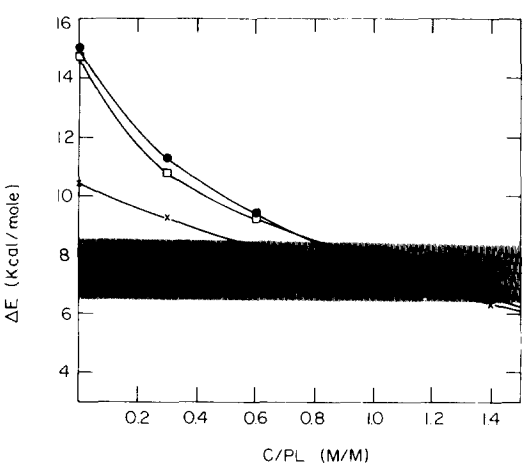

Fig. 6. The change of ΔE with cholesterol/phospholipid ratio (C/PL) in liposomes made of mixtures I (●–●), II (×–×) and III (□–□). The shaded area covers the range of ΔE values determined in biological membranes.

Unlike in liposomes the ΔE values of the membranes studied here are all in the range of 6.5–8.5 kcal/mol (see Figs 3 and 4 and Table III). Thus for membranes of cholesterol/phospholipid < 0.6, ΔE is smaller than could be estimated from an analogous mixed liposomes (see Fig. 6), a deviation which becomes significant at cholesterol/phospholipid < 0.4. This difference between membranes and liposomes could be only partially accounted for by assuming that membranes of a low cholesterol level are also low in sphingomyelin [49] which results in a reduction in ΔE (see Fig. 6) and thus restrains the overall increase in ΔE. However, most of this effect is probably due to membrane proteins, which seem to control a constant ΔE value and the unit flow volume similarly to cholesterol. If this interpretation is correct it leads to the conclusion that the minimum free energy of a membrane like system comprised of phospholipids, cholesterol and proteins is such that ΔE and V remain approximately constant. This equilibrium condition can be reached by the vertical movement of the proteins to the level required for maintaining constant ΔE and V values. This conclusion further implies that membrane proteins can become more exposed to the aqueous surrounding upon increasing the cholesterol level and vice versa. The opposite correlation between lipid fluidity and the rotational mobility of the concanavalin A receptor sites obtained in the two independent cell systems of normal and transformed fibroblasts and lymphocytes (see Table IV) is in fact in line with this hypothesis. This of course will be fully correct if the evaluated ρ_0/ρ and $\bar{\eta}$ refer to the same membrane regions and are therefore interrelated. Means for assessing this question are, unfortunately, not yet available. However, other findings which were obtained with platelets [59] support this viewpoint. Human platelets which acquired about 40 % excess cholesterol become much more sensitive to epinephrine or ADP-induced aggregation. Similar hypersensitivity to these aggregation factors appears in platelets from individuals with familial hypercholesterolaemia [59]. These findings suggest that the receptors for epinephrine or ADP in human platelets become more accessible when the cholesterol level of the membrane is increased.

The opposite change of microviscosity upon malignant transformation of lymphocytes (Figs. 4 and 5) on one hand, and fibroblasts (Fig. 5) or 3T3 cells [15] on the other hand poses a fundamental question as to the role of cholesterol in malignancy. The marked reduction of the molar ratio of cholesterol to phospholipids in membranes of leukaemic cells [46] induces the significant reduction in their membrane microviscosity as compared to normal lymphocytes. Concomitant to the cellular deficiency of cholesterol in the leukaemic cell, the average level of cholesterol in the blood serum of leukaemic patients is also below the average normal level [46]. These observations indicate that similar to erythrocytes [55, 60] and platelets [59], cholesterol in lymphocytes partitions efficiently between the cell membranes and the serum. It is therefore plausible that a significant part of the cholesterol in the lymphocyte membranes originates from its serum environment. In cells which form a solid tissue in vivo, represented here by the hamster fibroblasts, the contact with the cholesterol pool of the blood serum is poor and indirect. The main source of cholesterol in such cells is from intracellular biosynthesis which presumably is of a higher turnover than that in lymphocytes. This argument may explain the opposite change in microviscosity which follow malignant transformation in the lymphocytes and the fibroblast systems. Solid tumours like hepatoma [61] and bowel cancer [62, 63] were shown to contain elevated cholesterol contents. Moreover, epidemiologic

evidence suggest that bowel cancer is dependent on dietary cholesterol [62]. It has been recently recommended that the "Prudent Diet" [64], which is believed to be important for the reduction of cardiovascular disease, may be of beneficial effect towards a reduction of colon cancer [63]. These associations between cholesterol and malignancy, which are in an opposite direction in leukaemia and in hepatoma or bowel cancer, are in line with the differences in the membrane microviscosity between the fibroblast system and the lymphoid system reported here. This observation indicates that the formation of solid and ascites tumours are of different mechanisms as far as the dynamic behaviour of cellular membrane components is concerned.

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