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TEMPERATURE-INDUCED HOMEOVISCOUS ADAPTATION OF CHINESE HAMSTER OVARY CELLS

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Summary

Exponential and plateau phase Chinese hamster ovary cells were maintained for 3 days at 32, 37, 39 or 41°C. The effect of growth temperature on the fluidity and composition of the cellular membranes, and on the ability of the cells to resist a subsequent heat treatment at 43°C, was measured.

Cells grown at temperatures above 37°C displayed increased resistance or tolerance to a 43°C heat treatment, whereas cells grown at 32°C were sensitized to heat. Extensive cell division was not required for expression of heat tolerance.

Membrane fluidity, as determined by the degree of rotational mobility of the fluorescent probe diphenylhexatriene, decreased with increasing growth temperatures, but the relationship did not hold in exponential phase cells grown at 32°C. The cholesterol : phospholipid molar ratio correlated with the fluorescence polarization values, suggesting that the cells are able to adjust membrane fluidity by varying the concentration of cholesterol.

The results are compatible with the concept of homeoviscous adaptation: that organisms strive to maintain an optimal level of membrane fluidity and when grown at a different temperature will alter the lipid composition in order to maintain this level. Up until now, cholesterol has not been implicated in this process.

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Introduction

Membrane fluidity, as assessed by a variety of physical techniques, has been shown to be markedly temperature dependent; a decrease in temperature resulting in decreased membrane fluidity [1,2]. There has been extensive investigation into how cells adapt to temperature-induced alterations in the physical state of their membrane lipids. Mycoplasma [3], bacteria [1,4—6], protozoa [7—9], yeast [10,11], higher plants [12] and animal cells [13—16] have all been shown to respond to changes in environmental temperature by altering the degree of saturation of the fatty acyl chains of their membrane phospholipids. Decreased growth temperature tends to decrease the degree of saturation, and vice versa. Such structural alterations affect the fluidity of the membranes; a greater proportion of unsaturated fatty acids tending to increase membrane fluidity [17]. Thus, when measured at a common temperature, membranes from cold-adapted organisms, which contain a greater degree of fatty acyl unsaturation, are more fluid than membranes from warm-adapted organisms. In situations where these measurements have been made at the temperature of adaptation, the membrane fluidity of organisms adapted at different temperatures tends to be similar [3,4,6,16,18]. The above findings have led to the hypothesis that organisms maintain an optimal level of membrane fluidity and, when grown at a different temperature, will alter their lipid composition in order to maintain this same degree of fluidity. This process has been termed homeoviscous adaptation [4].

It is thought that the utility of this phenomenon lies in providing the optimal lipid environment for critical membrane functions including membrane-bound enzymic activity [17,19], passive permeability [20] and cell-cell adhesion [21]. However, not all cellular membranes adapt completely. In the case of the sarcoplasmic reticulum of goldfish acclimated to different temperatures, homeoviscous adaptation was absent [22]. Also, an *Escherichia coli* mutant defective in the synthesis of *cis*-vaccenate, and thus unable to increase its content of unsaturated fatty acids during growth at lower temperatures, is able to grow at the same rate as the wild-type cells despite the lack of alterations in membrane fatty acid composition [23].

On the other hand, it has been found that other agents that influence membrane fluidity, such as anaesthetics [24], ethanol [25,26], choline analogues [27] and methoxy fatty acids [28] also tend to induce a homeoviscous response.

Membrane fluidity is not influenced only by the degree of fatty acid saturation. Other important lipid factors include fatty acyl chain length, phospholipid headgroup composition and the amount of cholesterol present in the membrane. Cholesterol acts as a regulator of acyl chain packing. Above the phospholipid phase transition temperature it reduces the mobility of the acyl chains [2,29] while below this temperature the presence of cholesterol increases their mobility [30].

Alterations in membrane cholesterol levels have been studied in several biological systems. Changes in membrane fluidity of lymphocytes in leukaemia and lymphoma have been attributed to differences in membrane cholesterol, with a decreased cholesterol to phospholipid ratio resulting in increased fluidity

in the transformed cells [31—33]. However, Johnson and Kramers [34] have attributed this apparent fluidity difference to platelet contamination of the lymphocyte preparations and show no differences in the cholesterol : phospholipid ratio in isolated plasma membranes of normal and transformed lymphocytes [35]. A decrease in membrane fluidity associated with neurite formation in neuroblastoma clone N-18 cells is associated with a small increase in the cholesterol : phospholipid ratio [36]. An increase in cholesterol : phospholipid ratio in synaptosomal plasma membranes and red blood cell membranes was found in ethanol-habituated mice whose membranes had become resistant to the fluidizing effects of ethanol [26]. However, cholesterol has not yet been demonstrated as a modulator of temperature-induced homeoviscous adaptation.

Fluidity is usually assessed by measurement of the degree of rotational mobility of fluorescent or paramagnetic probes which are embedded within biological membranes or lipid bilayers [4,12,32]. The greater the rotational mobility of the probe the more fluid its environment is thought to be. In the present studies, the technique of fluorescent polarization has been employed using the probe 1,6-diphenyl-1,3,5-hexatriene incorporated into membrane preparations. The degree of depolarization of the fluorescent emission from diphenylhexatriene in a particular lipid environment depends on the rotational mobility and the excited state lifetime of the probe [32]. In general, given no change in the fluorescent lifetime, the greater the polarization the less mobile the probe, and therefore the less fluid the membrane (or more viscous).

Results of studies into temperature-induced membrane adaptation in mammalian cells in culture, a relatively unexplored area [13], are presented here. We have observed previously that different incubation temperatures of both plateau and exponential phase Chinese hamster ovary fibroblasts result in marked alterations in cellular resistance to heat shock [37]. Adaptation to relatively higher temperatures (39—41°C) results in a marked increase in survival following hyperthermic exposure. We have used both exponential and plateau phase cells to correlate the altered sensitivity to heat treatment at 43°C with changes in membrane fluidity and lipid composition.

Materials and Methods

Diphenylhexatriene and preservative-free tetrahydrofuran were obtained from Aldrich Chemical Company. Egg yolk lecithin and cholesterol were purchased from Sigma.

Cells

The Chinese hamster ovary (CHO) fibroblast line HA-1, was grown in 5% CO_2 in Eagle's minimal essential medium (Grand Island Biological Co.) supplemented with foetal calf serum (Grand Island Biological Co.) and containing streptomycin sulphate (200 mg/l) and potassium penicillin (200 000 units/l). For plateau phase experiments, $(1—2) \cdot 10^5$ cells were plated in 60-mm plastic petri dishes (Lux) and incubated at 37°C for 7 days, at which time they had achieved plateau phase ($27 \cdot 10^6$ cells/dish, approximately 10^4 cells/mm^2); minimal essential medium plus 15% foetal calf serum was renewed daily,

starting on day 3. After 7 days each group of dishes was transferred to incubators at precalibrated temperatures (32, 37, 39 and 41°C) for 3 additional days, and daily feedings were continued. For experiments involving exponential phase cells, (1—6) · 10^5 cells were plated in 100-mm plastic petri dishes (Lux) and were kept at 37°C for 3 h to allow for attachment before being transferred to incubators set at 32, 37, 39 and 41°C for the subsequent 3 days. As growth rate was a function of growth temperature (see Results), the initial plating density was varied to obtain equal cell densities at the time of harvest (approximately 10^3 cells/mm^2).

Survival assays

Cells were exposed to 43°C for various times in specially constructed heat boxes [38]. The temperature was accurate to within ±0.1°C. The pH of the medium was maintained between 7.3 and 7.5 by a regulated gas flow of CO_2 and air. In all experiments the medium was changed just before exposure of cells to heat. Immediately after exposure dishes were rinsed twice with phosphate-buffered saline, the cells trypsinized, counted on a Coulter counter, serially diluted, and plated. The assay for cellular survival was the cloning technique of Puck and Marcus [39]. Clones were allowed to grow for 8—9 days at 37°C. Plating efficiencies were more than 50%.

Preparation of a total membrane fraction

All steps were performed on ice or at 4°C. Each group of dishes (32, 37, 39 and 41°C) were rinsed three times with phosphate-buffered saline (131 mM NaCl in 7 mM phosphate buffer, pH 7.3). The cells were harvested with a rubber policeman into 15 ml of phosphate-buffered saline, spun down in a clinical centrifuge at 2000 rev./min for 7 min, washed with 10 ml phosphate-buffered saline, spun down again, resuspended in 15 ml phosphate-buffered saline and counted on a Coulter counter. They were then sonicated for 3 min with the large probe of a Biosonik II sonicator (Brownwill Scientific) at a setting of 70, keeping the sample on ice during sonication. Phase contrast microscopy showed that cell lysis was over 95%. The homogenate was centrifuged at 2000 rev./min for 10 min to sediment unbroken cells and nuclei, which were discarded. The supernatant from a given temperature group was then divided into at least four separate aliquots and all subsequent steps performed in at least quadruplicate. An additional aliquot was also processed through the subsequent steps and saved for fluorescence polarization measurements. The low speed supernatant was centrifuged at 60 000 × g for 30 min. (30 000 rev./min, T40 rotor, Beckman ultracentrifuge, 4°C) to pellet a total membrane fraction.

The pellets for membrane lipid analysis were extracted with chloroform methanol (2 : 1) and the extract was backwashed with 0.1 M KCl as described by Folch et al. [40]. The lipid extracts and membrane pellets designated for fluorescence polarization were stored under N_2 at −20°C until analysed.

Protein, cholesterol and phospholipid assays

Protein was assayed by the method of Lowry et al. [41], using the particulate fraction remaining after chloroform/methanol extraction. Bovine serum

albumin was used as a standard. Total cholesterol was determined in the lipid extract using the fluorescence assay of Solow and Freeman [42]. The amount of phosphorus in the lipid extract, as determined by the method of Rouser et al. [43] was used as a measure of total phospholipid. All assays for a single replicate were performed in duplicate and at least four replicates were included for any given sample to determine standard errors.

Phospholipid fatty acid analysis

The lipid extracts were fractionated on silicic acid columns (Bio-Sil HA, minus 325 mesh, Bio-Rad). Neutral lipids and glycolipids were eluted with chloroform and 4% methanol in chloroform, respectively. Phospholipids were subsequently eluted with methanol. The methanol eluant was dried under N_2 and transesterification performed in 2% H_2SO_4 in methanol for 1 h at 70°C. The fatty acid methyl esters were extracted with diethyl ether, dried, and resuspended in hexane. Analysis was by gas-liquid chromatography using a Varian 3740 gas chromatograph on 10% SP-2330 on 100/120 Chromosorb W AW (Supelco, Inc.), temperature programming from 170°C to 230°C at a rate of 2 K/min.

Preparation of vesicles

Egg yolk lecithin and cholesterol in chloroform were mixed in various mole ratios, dried under N_2, resuspended in methanol, and dried again by prolonged evaporation under N_2. They were then suspended in phosphate-buffered saline and sonicated on the Biosonic II probe sonicator for 25 min at a setting of 40 until the solution was clear. The final concentration of phospholipid was 500 μM.

Fluorescent polarization

The fluorescent polarization technique was adapted with modification from procedures used by others [32,44,45].

Membrane fractions. The total membrane fraction was resuspended in phosphate-buffered saline by sonicating for about 2 min with the microtip of a sonifier (Branson) at a setting of 7 and then diluted with phosphate-buffered saline to a final phospholipid concentration of 50 μM. At this point the suspension was divided into at least eight 4-ml aliquots and all determinations carried out in at least quadruplicate (four determinations and four background measurements). To 4 ml of this suspension, which was clear but slightly bluish in colour, was added 2 μl tetrahydrofuran containing 0.8 nmol diphenylhexatriene, which had been freshly prepared. This was performed with a Hamilton syringe while the suspension was being vortexed vigorously. The probe to phospholipid ratio was 1 : 250; the high ratio was required because of naturally occurring fluorescence in the membrane sample. Background with this amount of probe was less than 5% of the signal. The dye in the aqueous buffer had no detectable fluorescence. but as it was incorporated into the membranes there was a rapid rise in fluroescence. The solution was maintained at room temperature for 90 min at which time no further increase in fluorescence was detectable. Polarization measurements were performed under constant stirring on a Perkin-Elmer fluorimeter at room temperature (23°C). Excitation was

with polarized light at 360 nm, slit 10 nm; emission was detected at 430 nm, slit 40 nm. Exposure to exciting light was limited to 10 s to prevent photoisomerization [45]. Emission polarization was determined by the formula:

$$P = \frac{I_{/\!/} - I_{\perp}}{I_{/\!/} + I_{\perp}}$$

$$I_{/\!/} = I_{/\!/\mathrm{T}} - I_{/\!/\mathrm{B}}$$

$$I_{\perp} = I_{\perp\mathrm{T}} - I_{\perp\mathrm{B}}$$

Where $I_{\|\mathrm{B}}$ is the intensity of the parallel background emission, $I_{\perp\mathrm{B}}$ perpendicular background emission, $I_{\|\mathrm{T}}$ parallel total emission with probe and $I_{\perp\mathrm{T}}$ the perpendicular total emission.

Vesicles. To 4 ml of phosphate-buffered saline, with vesicles containing a total of 2 μmol egg yolk lecithin plus varying amounts of cholesterol, was added 1 μl tetrahydrofuran containing 2 nmol diphenylhexatriene via a Hamilton syringe during vigorous vortexing. The probe to phospholipid ratio was 1 : 1000. The solution was maintained for 90 min at room temperature before measurement. Polarization measurements were performed on a Spex fluorimeter at 23°C. Excitation was with polarized light at 360 nm, slit 5 nm; emission was detected at 430 nm, slit 20 nm. Background was 0.5%. Polarization was calculated as above. Total fluorescence emission (calculated as $I_{\|} + 2I_{\perp}$) was essentially constant in both membrane and vesicle experiments.

Results

At temperatures less than 32°C or greater than 41°C, growth could not be achieved. Fig. 1 illustrates the increase in cell number with time within this temperature range. Plating efficiency remained constant and greater than 50%. The growth rate was slowest at 32°C. To determine the effect of different growth temperatures on cellular thermal resistance, heat survival assays were performed. Fig. 2 shows survival (clone-forming ability) after exposure to heat at 43°C of cells grown for 3 days at various temperatures. As growth rate was influenced by growth temperature (Fig. 1), the initial cell plating density for each group was determined such that all groups had a similar density at the time of the survival assay. Prior growth at 39°C and 41°C endowed the cells with considerable resistance to killing at 43°C whereas cells grown at 32°C are sensitized to this treatment.

In order to determine whether replication at elevated temperatures was necessary for development of heat resistance, cells were grown to plateau phase (7 days at 37°C), transferred to incubators set at 32, 37, 39 and 41°C and maintained for the next 3 days, changing the medium daily. As shown in Fig. 3, cell number remained similar at 32, 37 and 39°C but was slightly less after 3 days at 41°C. Plating efficiency remained constant. After the 3 day period of exposure to various temperatures, the plateau phase cultures were heated at 43°C and clone-forming ability assessed (Fig. 4). The results are similar to those found in exponential cells except that cells previously held at 32 and 37°C showed no difference in survival rates. Thus, both exponential and plateau phase cells exposed to mildly elevated temperatures become thermally adapted or tolerant to heat.

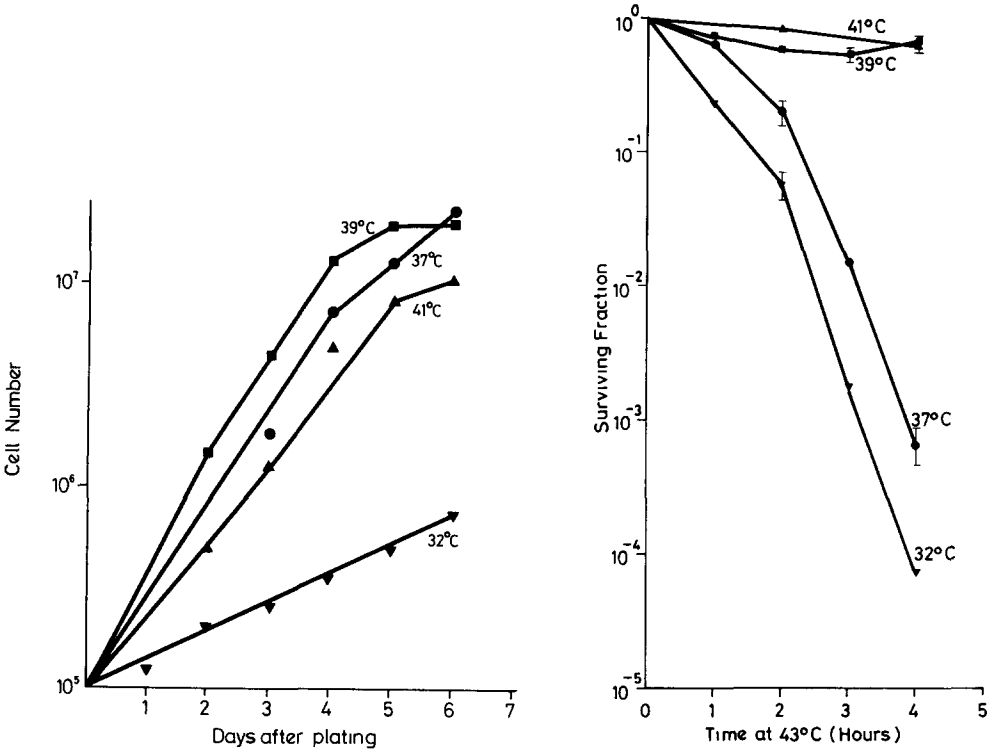

Fig. 1. Growth of CHO-HA 1 cells at various temperatures. The cells were plated at approximately 50 cells/mm^2 and maintained in incubators set at 41, 39, 37 or 32° C. The medium was changed daily starting on day 3.

Fig. 2. Survival of exponential phase CHO-HA 1 fibroblasts exposed to 43° C for various times. Cells had been allowed to attach at 37° C for 3 h following subculture, then grown at 41, 39, 37 or 32° C for the subsequent 3 days.

Membrane fluidity, as assessed by the degree of rotational mobility of the fluorescent probe, diphenylhexatriene, was measured at 23° C in preparations of membranes from cells grown at different temperatures. The polarization values are presented in Tables I and II for exponential and plateau phase cells, respectively. Total fluorescence emission was constant. Generally, polarization values, and hence membrane viscosity, for cells grown at 37° C or above increased with increasing growth temperature. The results at 32° C are more difficult to interpret. For plateau phase cells, the polarization value at 32° C was lower than at 37° C in all experiments except one (Experiment I, Table II). With exponential phase cells, the polarization value at 32° C was similar to that at 37° C and the present data do not establish a clear correlation between growth temperature and membrane fluidity for growth temperatures below 37° C.

The modification of membrane fluidity in response to growth temperature could have been mediated by an alteration in the composition of the cell membranes, and this consideration prompted an investigation of the memebrane lipid composition of these cells. The phospholipid and cholesterol content of a total

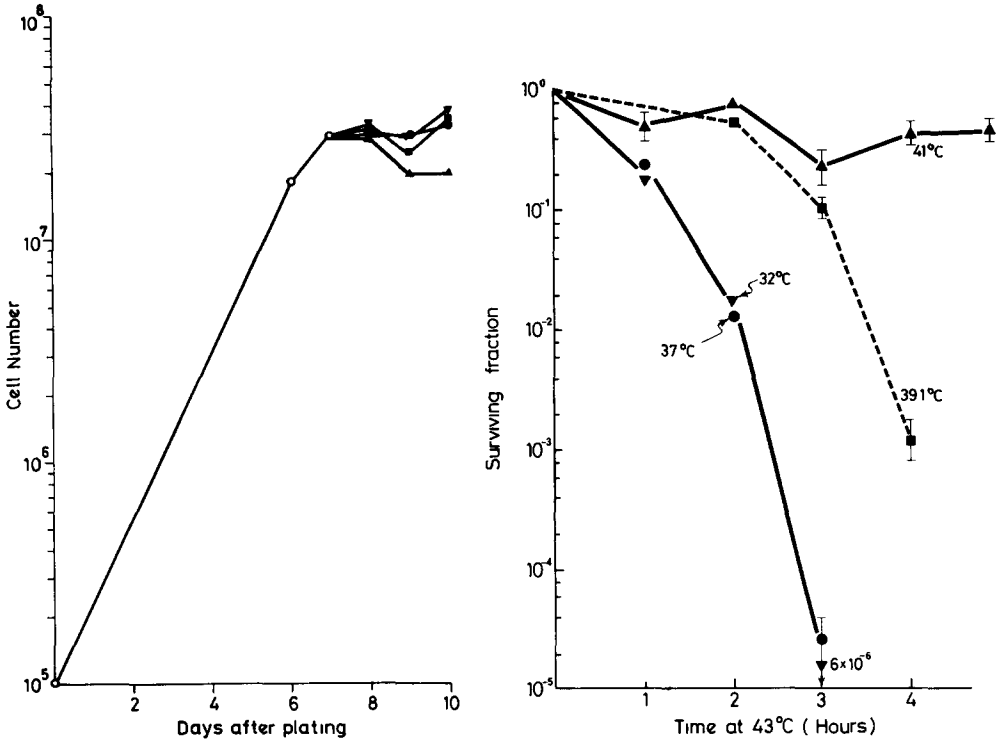

Fig. 3. Growth curve for CHO-HA 1 cells in culture. The cells were plated at approximately 50 cells/mm^2 and maintained at 37°C for 7 days. They were then transferred to incubators set at 41 (▲), 39 (■), 37 (●) or 32°C (▼), and maintained for the next 3 days, changing the medium daily.

Fig. 4. Survival of plateau phase CHO-HA 1 cells exposed to 43°C for various times. Cells had been maintained for 3 days at the following temperatures: 41, 39, 37, 32°C.

TABLE I

CHOLESTEROL AND PHOSPHOLIPID CONTENT, THE CHOLESTEROL : PHOSPHOLIPID MOLAR RATIO AND POLARIZATION VALUES (*P*) AS A FUNCTION OF GROWTH TEMPERATURE FOR EXPONENTIAL PHASE CELLS

The results are expressed as the mean ± S. E. for four or five replicates. n.d., not determined.

Expt. No.	Growth temperature (°C)	Cholesterol/mg protein (μg)	Phospholipid/mg protein (μg)	Cholesterol : phospholipid molar ratio	*P*
1	32	101 ± 2	276 ± 4	0.71 ± 0.01	0.236 ± 0.002
	37	95 ± 3	292 ± 5	0.63 ± 0.02	0.237 ± 0.001
	39	110 ± 5	297 ± 8	0.72 ± 0.01	0.244 ± 0.001
	41	155 ± 2	390 ± 5	0.77 ± 0.01	0.248 ± 0.000
2	32	113 ± 3	261 ± 13	0.82 ± 0.02	n.d.
	37	105 ± 1	295 ± 4	0.67 ± 0.01	n.d.
	39	97 ± 3	223 ± 6	0.82 ± 0.01	n.d.
	41	103 ± 2	295 ± 6	0.66 ± 0.01	n.d.

TABLE II

CHOLESTEROL AND PHOSPHOLIPID CONTENT, THE CHOLESTEROL:PHOSPHOLIPID MOLAR RATIO AND POLARIZATION VALUES (*P*) AS A FUNCTION OF GROWTH TEMPERATURE FOR PLATEAU PHASE CELLS

The results are expressed as the mean ± S.E. with the number of replicates shown in parentheses.

Expt. No	Growth temperature (°C)	Cholesterol/mg protein (μg)	Phospholipid/mg protein (μg)	Cholesterol : phospholipid molar ratio	*P*
1	32	125 ± 1 (5)	387 ± 5 (5)	0.63 ± 0.01 (5)	0.239 ± 0.001 (4)
	37	134 ± 3 (5)	389 ± 9 (5)	0.67 ± 0.00 (5)	0.231 ± 0.001 (4)
	39	120 ± 4 (5)	332 ± 10 (5)	0.70 ± 0.01 (5)	0.233 ± 0.001 (4)
	41	123 ± 3 (5)	313 ± 6 (5)	0.76 ± 0.02 (5)	0.244 ± 0.002 (4)
2	32	159 ± 6 (4)	571 ± 18 (4)	0.53 ± 0.01 (4)	0.220 ± 0.001 (6)
	37	151 ± 3 (4)	484 ± 9 (4)	0.59 ± 0.01 (4)	0.223 ± 0.001 (5)
	39	202 ± 11 (4)	551 ± 16 (4)	0.69 ± 0.02 (4)	0.237 ± 0.001 (6)
	41	190 ± 4 (4)	428 ± 8 (4)	0.84 ± 0.03 (4)	0.243 ± 0.001 (6)
3	32	81 ± 2 (4)	317 ± 8 (4)	0.48 ± 0.01 (4)	0.207 ± 0.001 (7)
	37	100 ± 5 (4)	344 ± 8 (4)	0.55 ± 0.03 (4)	0.210 ± 0.001 (7)
	39	107 ± 5 (4)	321 ± 9 (4)	0.63 ± 0.02 (4)	0.212 ± 0.001 (7)
	41	114 ± 5 (4)	325 ± 16 (4)	0.66 ± 0.01 (4)	0.218 ± 0.001 (7)

membrane preparation from the cells is shown in Tables I and II. No definite trends are demonstrated in the phospholipid or cholesterol content, as normalized to membrane protein. These results were the same if the values for cholesterol and phospholipid were normalized to cell number instead of protein, indicating that the results cannot be explained by an alteration in cell protein content as a function of growth temperature.

In all experiments with plateau phase cells, the cholesterol : phospholipid molar ratio increased with increasing growth temperature. In exponential phase cells, the ratio for cells grown at 37°C was lower than that for cells grown at 32°C or 39°C, which had a similar value. At 41°C the results were variable, with some experiments showing an increase in the ratio but an equal number giving a value lower than that at 39°C. Alterations in temperature are known to induce both cell cycle variations and synchrony, and may be responsible for this experimental variability [46,47].

The cholesterol : phospholipid molar ratio shows a positive correlation with membrane viscosity of plateau phase cells where cells rarely undergo division. The correlation is less convincing for exponential phase cells and suggests that factors other than the cholesterol : phospholipid ratio are important in the maintenance of optimal membrane fluidity in rapidly dividing cells.

Tables III and IV show the fatty acid composition of phospholipids from exponential and plateau phase cells, respectively. The relative proportion of unsaturated fatty acids is expressed as the ratio of the double bond index: saturated fatty acids. The double bond index is obtained by summing the products of the percentage of each unsaturated fatty acid and the number of its double bonds [48]. This ratio is used as the fluidity of a membrane will be more dependent on the number of double bonds present than on the the percentage of unsaturated fatty acids. In plateau phase cells and exponential cells grown at 37°C and above, there is a tendency for this ratio to increase

TABLE III

FATTY ACID COMPOSITION OF THE PHOSPHOLIPID FRACTION OF EXPONENTIAL PHASE CELLS AS A FUNCTION OF GROWTH TEMPERATURE

Each result is the average of duplicate analyses and is expressed as a percentage of total fatty acids recovered.

Fatty acid	32°C	37°C	39°C	41°C
14 · 0	2 4	2.8	0.5	2.1
14 : 1	1.0	2.5	0.9	trace
16 : 0	15.2	18 5	18.7	15.7
16 . 1	4.5	6.8	4.4	3.2
18 0	25.5	19.9	26.0	22.0
18 . 1	22.2	20.1	23.6	21.5
18 : 2	2.5	3.6	3.6	3.1
18 · 3	—	—	—	—
20 : 4	21.5	13.2	17.4	20.8
Unidentified	5.3	12.9	5.0	11.7
Total saturated	43.1	41.2	45.2	39.8
Total mono-unsaturated	27.7	29.4	28.9	24.7
Total polyunsaturated	24.0	16.8	21.0	23.9
Ratio double bond index: saturated	2.75	2.17	2.34	2.87

with increasing temperature, due mainly to an increase in the amount of arachidonic acid (20 : 4).

To determine whether the magnitude of the observed changes in fluorescence polarization were compatible with the alterations detected in the cholesterol : phospholipid ratio, the polarization values measured in the total membrane fraction were compared to those of egg yolk lecithin vesicles containing known amounts of cholesterol. The results are shown in Fig. 5. The fluorescence polarization of diphenylhexatriene in egg yolk lecithin vesicles

TABLE IV

FATTY ACID COMPOSITION OF THE PHOSPHOLIPID FRACTION OF PLATEAU PHASE CELLS AS A FUNCTION OF INCUBATION TEMPERATURE

Each result is the average of duplicate analyses and is expressed as a percentage of total fatty acids recovered.

Fatty acid	32°C	37°C	39°C	41°C
14 : 0	2.8	1.1	1.2	0.8
14 · 1	0.8	—	0.3	1.2
16 . 0	22.5	22.8	21.5	18.9
16 : 1	14.8	10.2	9.7	6.6
18 : 0	13.2	15.1	17.1	17.5
18 : 1	28.7	28.3	28.2	26.3
18 : 2	3.9	5.0	4.9	5.6
18 . 3	1.2	0.8	1.6	1.7
20 : 4	8.5	12.7	11.5	15.2
Unidentified	3.5	3.9	3.8	6.0
Total saturated	38.5	39.0	39.8	37.2
Total mono-unsaturated	44.3	38.5	38.2	34.1
Total polyunsaturated	13.6	18.5	18.0	22.5
Ratio double bond index: saturated	2.33	2.61	2.48	2 99

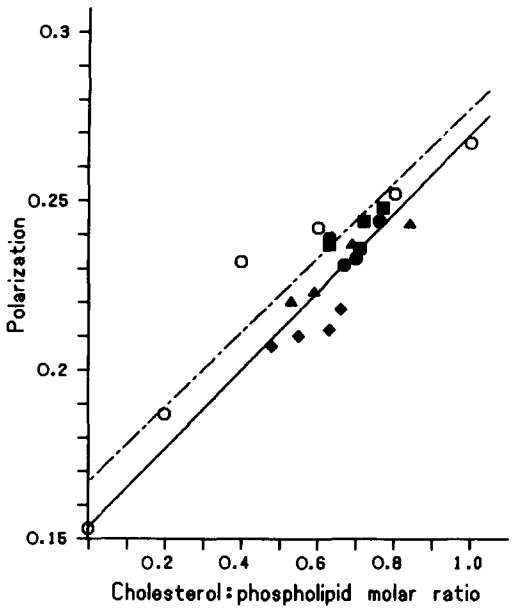

Fig. 5. Relationship between the cholesterol phospholipid molar ratio and membrane fluidity expressed as the fluorescence polarization value at 23° C. Egg yolk lecithin vesicles containing various amounts of cholesterol (○). Total membrane fraction from exponential phase cells (■), (data from Table I) and from plateau phase cells (data from Table II), Expt. 1 (●), Expt. 2 (▲), Expt. 3 (◆). Linear regression of vesicle data points, — · — · —; and of membrane fraction data points, ———.

increases as the cholesterol : phospholipid molar ratio is increased. Also plotted on this graph are the data from Tables I and II. For purposes of comparison, a linear regression analysis through each set of data points is shown. It may be noted that the slopes are approximately parallel. The data suggest that the magnitude of the polarization values as well as the changes in polarization values as a function of the cholesterol : phospholipid ratio are similar in the artificial vesicles and in cell membrane preparations.

Discussion

The results presented here as well as our previous results [37] show a positive correlation between growth temperature and the ability of cells to resist a heat treatment at 43° C. Growth temperatures above 37° C induce heat-resistance both in rapidly dividing cells and in plateau phase cultures where cell cycling is minimal [49]. A similar increase in thermal resistance has been observed by Joshi and Jung [50], who held exponential phase CHO cells for up to 5 h at temperatures of 38—41° C prior to treatment at 43° C, and by Henle et al. [51] who held the same cells at 40° C for 7 h prior to 45° C treatment. Also, V79 cells grown at 33° C were more sensitive to hyperthermic insult than those grown at 37.5° C [52].

At elevated temperatures, the cholesterol : phospholipid molar ratio increases and membrane fluidity, which correlates inversely with the fluorescence polarization value, decreases with increasing growth temperature. Cell

division is not required for expression of these changes in membrane composition as plateau phase cells exhibit alterations of similar magnitude to those in rapidly growing cells.

Cells adapted to growth at 32°C are sensitized to heat and if the correlation is to be extended from cells grown at elevated temperatures, one would predict lower cholesterol : phospholipid ratios and higher membrane fluidity than in cells grown at 37°C. This holds generally for plateau phase cells (Table II). In exponential phase cells (Table I), the cholesterol : phospholipid ratio was higher than at 37°C and the fluidity usually at a similar level. This suggests that factors other than cholesterol are involved in regulation of the physical state of the membrane at subnormal temperatures. The higher proportion of unsaturated fatty acid in cells grown at 32°C compared to those at 37°C (Table III) may explain this observation. However, the situation is more complex than this as the ratios are similar for exponential cells grown at 32°C and 41°C despite the greatly increased heat resistance of cells grown at 41°C.

The correlation between the cholesterol : phospholipid ratio and resistance to a heat treatment at 43°C suggests a possible relationship between cholesterol content and thermal resistance. Cress and Gerner [53] have recently demonstrated a correlation between cholesterol, standardized on a cell protein basis, and heat resistance in five unrelated cell lines, although the correlation did not extend to the cholesterol : phospholipid molar ratio or the ratio of stearic to oleic acid. Demonstration of a causal relationship between alterations in lipid composition and increased survival after heat shock requires further investigation.

In the present work, cholesterol ester and free cholesterol were not quantified individually. Since cholesterol was being measured in a membrane extract, it is likely that the values represent the free sterol since sterol esters are found only in trace amounts in membranes [29]. Also, the presence of sterols other than cholesterol, such as desmosterol or cholestanol, was not determined. Although cholesterol is always the major sterol present in mammalian cells [29], the possibility exists that the observed changes were due to other sterols or sterol esters.

As a peripheral observation in this study, we noted that there were no striking differences in the cholesterol : phospholipid molar ratio or fluorescence polarization values between exponential (low density) and plateau (high density) phase cells that had the medium renewed daily, although the double bond index to saturated fatty acids was lower in exponential cells. However, if exponential phase cells are maintained without renewal of the medium, the cholesterol : phospholipid ratio increases markedly as the cells approach confluency (unpublished observation). Other studies employing polarization with diphenylhexatriene as the probe molecule [54,55] have indicated that normal cells, i.e. those exhibiting density-dependent inhibition of growth, show a decrease in membrane fluidity with increasing cell density.

Recently there has been an increase in knowledge of the behaviour of diphenylhexatriene. This has included pulse fluorimetric findings of complex decays of both total fluorescence and emission anisotropy in lipid vesicles and cellular membranes [56—60]. It has called into question the interpretation of steady-state data in terms of a 'microviscosity' measured in poise. Therefore,

we express our values only in terms of polarization as an index of the average motion of the probe and a qualitative indicator of the physical state of the membrane [61]. It is unlikely that variations in excited state lifetimes influenced the polarization values as total fluorescence emission ($I_{\parallel} + 2I_{\perp}$) was essentially identical in all membrane measurements. Also there was no significant variation in total fluorescence emission in the lecithin vesicle measurements. It has been found that diphenylhexatriene not only localizes in the plasma membrane but in all cellular membranes as well as in non-membranous sites, including storage lipids [44,62,63]. In order to limit probe distribution to cellular membranes, fluorescence polarization was measured in a membrane preparation.

To ascertain that the small changes in polarization observed were consistent with the differences detected in the cholesterol : phospholipid ratios, the data was compared with polarization values obtained in egg yolk lecithin vesicles containing varying amounts of cholesterol (Fig. 5). The relationship between polarization and cholesterol : phospholipid ratio is not necessarily linear; for purposes of comparison, however, linear regression analyses were performed on each set of data. Although there are obvious differences in composition between egg yolk lecithin vesicles and the CHO cell membrane preparation, it should be noted that the polarization values are comparable and that the slopes of the linear regression are approximately equal, indicating that the degree of change in polarization values as a result of changing cholesterol content is similar in both systems. This similarity suggests that although the changes in polarization seen in the cell membrane preparations are small, they are of the expected order of magnitude. Furthermore, it would suggest that the observed temperature-induced changes in phospholipid composition are not as significant an influence on fluidity as the cholesterol content.

As the fluorescent properties of the probe molecule, diphenyl-hexatriene, vary with absolute temperature, it was not possible to equate directly the polarization values measured at different temperatures. To compare such polarization values, time-resolved fluorescence studies are required, where the average fluorescence life-time of the probe can be measured. For this reason, we were unable to directly test Sinensky's [4] concept of homeoviscous adaptation, but the range of polarization values obtained in this study both for plateau phase cells at all temperatures and exponential cells grown at elevated temperatures suggest that homeostasis of membrane fluidity is the aim of the cell.

It is noteworthy that an alteration in membrane cholesterol content is apparently participating in this process. Homeoviscous adaptation has previously been correlated with changes in the degree of phospholipid fatty acid saturation, in a wide variety of organisms [14]. Cossins [64], in looking at homeoviscous adaptation in goldfish adapted to different growth temperatures, demonstrated that the degree of fatty acid unsaturation was increased in the synaptosomal membranes of cold-adapted fish but was unable to show any change in the cholesterol content. Few tissue culture studies have been performed. An increase in the degree of fatty acyl unsaturation in cells grown at 28°C compared to those at 37°C was noted in LM cells in culture [13] but cholesterol was not measured in that study.

The significance of homeoviscous adaptation in mammalian cells is not clear.

Mammals are homeothermic and thus are not normally required to respond to fluctuations in environmental temperature except in special circumstances such as hibernation. It is possible that the genetic capacity to respond in this way may have persisted during the evolution of homeotherms.

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