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Changes in Lipid Ordering and State of Aggregation in Lymphocyte Plasma Membranes after Exposure to Mitogens

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Summary. An electron spin probe study was made of the effect of a number of mitogenic agents on the ordering and state of aggregation of the plasma membrane lipids of lymphocytes. These agents, which included phytohemagglutinin, Concanavalin A, the calcium ionophore A23187 and periodate, caused a 20% decrease in lipid ordering in the region of the bilayer probed by 5-nitroxide stearic acid. The corresponding methyl ester probe showed marked probe-probe interaction under the same conditions indicating an aggregation of lipids in the area probed by this label. Studies with mixed lipid vesicles and ganglioside-free cells indicate that these areas are rich in glycolipids capable of hydrogen bonding to the ester probe. The decrease in ordering and the increase in aggregation of the membrane lipids were correlated with the patching and capping of the ligand-receptor complexes. Furthermore, the disappearance of fluorescent ligand from the surface of treated cells corresponded with the return of the spectral parameters of the probes to control cell values.

It was concluded that glycolipids might play an important role in ligand-induced cell surface changes either as bearers of receptor groups, as in the case of some gangliosides, or in association by hydrogen-bonding with receptor proteins.

The stimulation of peripheral blood small lymphocytes to mitosis by a variety of agents may be regarded as a model of extensive gene activation in higher organisms. Most of these agents appear to act by combining with receptors on the plasma membrane of the cell (Wedner & Parker, 1976). Following cell activation, a number of early changes in the membrane and membrane mediated events have been reported. These

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include an influx of K^+ (Quastel & Kaplan, 1970), Ca^{++} (Allwood et al., 1970, Whitney & Sutherland, 1973), amino acids (van den Berg & Betel, 1973, 1974), glucose and uridine (Peters & Hausen, 1971) as well as increases in membrane phospholipid metabolism (Fisher & Mueller, 1971, Masuzawa *et al.*, 1973). None of these studies, however, throw any light on how combination of a mitogen with the cell surface initiates these changes. Weiss (1973*a, b*) has suggested that aggregation of membrane receptors by ligands could lead to compression of adjacent membrane lipids with consequent transfer and conservation of energy which could affect any nearby membrane enzyme present in an expanded form. Such a compression should be detectable as an alteration in the ordering of membrane lipids. Attempts to demonstrate such alterations by a variety of techniques have yielded conflicting results. Toyoshima and Osawa (1975) found that binding of the mitogenic lectins, *Wistaria floribunda* mitogen and *Lens culinaris* hemagglutinin increased the fluidity of lymphocyte membranes as measured by fluorescence polarization. Barnett, Scott, Furcht and Kersey (1974) also observed a decrease in ordering using spin label lipid probes in the membranes of lymphocytes treated with the lectin of *Phaseolus vulgaris* (PHA). On the other hand, Dodd (1975) was unable to confirm this finding and suggested that the apparent decrease in ordering might have been due to a relative change in the proportions of the three different components of the mixture of spin-labeled probes used by Barnett *et al.* (1974).

To test the hypothesis that receptor-ligand interaction leads to an alteration in the ordering of membrane lipids, we measured the order and motion parameters of a variety of spin labels in the membranes of lymphocytes exposed to mitogenic agents. Our results show that these agents cause significant changes in the ordering and state of aggregation of the different membrane lipids.

Materials and Methods

Cells

Human blood lymphocytes were isolated by centrifugation of freshly-drawn defibrinated blood in a Ficoll-Hypaque (Pharmacia "Isopaque") gradient according to the method of Böyum (1968). The cells were finally suspended at 10^7 ml in Eagles Minimum Essential Medium (MEM, Commonwealth Serum Laboratories, Parkville, Victoria, Australia). Lymphocytes were also prepared from human tonsils within 30 min of resection. The tissue was teased in MEM containing 10% fetal calf serum then strained through gauze. The filtrate was centrifuged briefly (3 min at $200 \times g$) to remove smaller debris and the superna-

tant layered over Ficoll-Hypaque as in the preparation of peripheral lymphocytes. The cells collecting in the leukocyte fraction after centrifugation were very heterogeneous with variable numbers of small lymphocytes, phagocytic cells, plasmablasts, and plasma cells as judged by Giemsa or Schiff-periodate staining. The phagocytes were removed by mixing the cells with carbonyl iron (approximately 5 mg iron powder/10^7 cells in 5 ml 20% fetal calf serum-MEM) and incubating at 37 °C for 45 min with gentle shaking. A magnet bar was then immersed in the cell suspension which was gently agitated for a further 10 min at 37 °C. The numbers of plasmablasts and plasma cells were reduced by further Ficoll-Hypaque density gradient centrifugation. The best preparations contained 70% small lymphocytes with a 75% viability as judged by the Trypan Blue exclusion test.

Normal thymus (T) cells were obtained from 4 to 5 week-old CBA/H/WEHI mice. Congenitally athymic mice homozygous for the mutation "nude" (Pantelouris, 1968) were used as a source of nonthymus influenced, bone marrow-derived lymphocytes (B cells). The mice were killed by cervical dislocation and the spleens and thymuses were minced through a stainless steel mesh into a chilled mixture consisting of 0.02 M phosphate and 0.15 M NaCl buffered at pH 7.2 (PBS). The cells were centrifuged at $300 \times g$ for 10 min. Red blood cells were removed by resuspending the pellet in 0.5 ml distilled H_2O and agitating the pellet for 10 sec. The cells were then resuspended in PBS, stood for 10 min to allow clumps to settle, and the supernatant was removed and adjusted to 10^7 cells/ml.

Rabbit peripheral blood lymphocytes were separated from defibrinated whole blood by the "Isopaque" method, except that the pellet of cells was briefly suspended in 0.5 ml distilled H_2O to remove red cells. The cells were finally suspended at 10^7/ml in Eagle's MEM.

Baby hamster kidney (BHK) cells were the gift of Dr E.L. French, CSIRO Division of Animal Health, Parkville.

Except where otherwise mentioned, human blood lymphocytes were used in our studies.

Because fatty acid spin labels are bound to serum albumin all the cells were washed twice and suspended in MEM without serum for ESR experiments. When cells were kept for more than 120 min in serum-free medium, variable changes were seen in lipid ordering and in the proportion of label remaining in the medium. For this reason all cell preparations were used within 90 min.

Antisera

Antiserum to murine thymus lymphocytes was prepared by injecting rabbits with 1×10^8 thymus lymphocytes emulsified in Freund's complete adjuvant. Four weeks after the first injection the rabbits received 1×10^8 thymus lymphocytes intravenously for three consecutive days and were bled one week later. The antiserum agglutinated thymus lymphocytes to a dilution of 1:256. The globulin fraction (ALG) was isolated on DEAE cellulose by the method of Levy and Sober (1960).

Anti-rabbit IgM was prepared by injecting guinea pigs with 3 mg rabbit IgM emulsified in 1.5 ml PBS, 0.8 ml complete Freund's adjuvant in three doses at weekly intervals. The guinea pigs were bled 10 days after the last injection. Specific anti-IgM was prepared by absorbing 3 ml of the serum twice with 10 mg of rabbit IgG, prepared by the method of Levy and Sober (1960). The globulin fraction of the absorbed antiserum was isolated on DEAE cellulose by the method of Levy and Sober (1960). The rabbit IgM used for injecting the guinea pigs was prepared by zonal ultracentrifugation and DEAE cellulose chromatography (Curtain & Anderson, 1972).

Lectins and Other Reagents

Concanavalin A (Con-A) and Wheat Germ Agglutinin (WGA) were purchased from Pharmacia (Uppsala, Sweden), colchicine from Calbiochem (Sydney), PHA from Difco,

and the calcium ionophore A23187 was the gift of Dr. R. Hamill of Eli Lilly (Indianapolis). Succinyl Con-A was prepared by the methods of Beppu, Terao and Osawa (1976) and of Gunther *et al.* (1973). Cholera toxin (Batch No. BZ 248) was obtained from Schwarz-Mann (Orangeburg, N.J.). Neuraminidase was obtained from Behringwerke (Marburg), dipalmitoyl lecithin and mixed beef and pig brain gangliosides and cerebrosides were obtained from Serdary Research Laboratories, London, Ontario.

Liposomes

Phospholipid liposomes, with and without added gangliosides, cerebrosides and ceramides, were prepared by the method of Redwood and Polefka (1976). As found by these authors on electron microscope examination, the liposomes were vesicles with a mean diameter of 250 Å.

Spin-labeling

The following spin-labeled fatty acids and their corresponding methyl esters were purchased from the Syva Corporation (Palo Alto, Calif.): 2-(3-carboxypropyl)-2-tridecyl-4, 4-dimethyl-3-oxazolidinyloxyl (5 nitroxide stearate, 5NS), 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl methyl ester (5 nitroxide stearate methyl ester, 5NSMe), 2(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl (12 nitroxide stearate, 12NS), 2(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyl-oxyl methyl ester (12 nitroxide stearate methyl ester 12NSMe), 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyl-oxyl (16 nitroxide stearate, 16NS), and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl methyl ester (16 nitroxide stearate methyl ester, 16NSMe). 2(4-carboxybutyl)-2-dodecyl-4,4-dimethyl-3-oxazolidinyloxyl methyl ester (6 nitroxide stearate methyl ester, 6NSMe) was synthesized by the general method of Keana, Keana and Beetham (1967) where oxazolidines are placed on ketone sites. All the preparations had impurities of 1% or less when examined by thin layer chromatography. Electron-spin resonance (ESR) spectra were recorded with either a Varian V4502 or E4 spectrometer. Both instruments were fitted with a Deltron (Sydney) Model DCM 20 temperature control accessory that maintained the temperature of the sample at ± 0.05 °C of the temperature selected. Spectra were recorded at either 37 or -180 °C. Capillary sample tubes were used each containing 10^7 to 10^8 cells in 100 µl.

Spin label was added to cells in two ways. In the first a thin film of label was prepared by placing 0.25 ml of chloroform containing the desired concentration of spin label in a small vial and evaporating the chloroform with a stream of dry N_2. One hundred µl of lymphocyte suspension was added and the vial shaken at 20 °C for 10 min. Alternatively, the desired concentration of label in 0.1 µl of ethanol was added to 100 µl of cells and the mixture vortexed for 30 sec. Both methods gave identical spectra in all the types of cells used and, therefore, the second was employed in most of the experiments described in this paper. All spectra were recorded immediately after the addition of the label, followed by the mitogenic or other agents, using a 2.5-min scan. The order in which these agents and the label were added did not affect the spectra. In labeled but otherwise untreated cells the spectra were stable, line widths and relative peak heights being unchanged after 1 hr.

Lipid to label ratios were calculated using a lipid value of 4.0×10^{-15} moles/cell (Gottfried, 1972). A 100-kHz modulation amplitude was used at 2 Gauss at a power of 10 mW.

Spectral Measurements

Where the high field hyperfine extremum could be resolved, the order parameter S was calculated as described by Gaffney (1975) from the relation

$$S=\frac{T'_{\parallel}-[T'_{\perp}+C]}{T'_{\parallel}+2[T'_{\perp}+C]}\times 1.723 \tag{1}$$

where $T'_{\parallel}$ and $T'_{\perp}$ are the separation of the outer and inner extrema in gauss of the first derivative spectra and

$$C=(1.463-0.053\{T'_{\parallel}-T'_{\perp}\}) \text{ gauss.}$$

The order parameter of spin-labels is frequently used as a measure of membrane lipid ordering (Kury, Ramwell & McConnell, 1974; Kury & McConnell, 1975). Recent studies have emphasized the need to distinguish spectral changes due to alterations in ordering from those produced by probe-probe interaction which may be facilitated by the concentration of the probes into restricted lipid domains (Verma & Wallach, 1975; Sauerheber *et al.* 1977). For this reason spectra were obtained over a wide range of membrane lipid/probe ratios. Where the spin-label motion was nearly isotropic and the high field hyperfine extremum could not be resolved, an empirical motion parameter τ_o was calculated. This gives an approximation of the rotational correlation time of a spin-label in a given solvent (Keith, Horvat & Snipes, 1974) and is obtained from the relationship

$$\tau_0=6.5\times 10^{-10} W_0[(h_0/h_{-1})^{\frac{1}{2}}-1] \text{ sec} \tag{2}$$

where h_0 and h_{-1} are the height of the mid and high field lines, respectively, and W_0 the width of the mid-field line in gauss.

Probe-probe interaction was estimated by measuring ΔH (equivalent to W_0)

$$\Delta H=\Delta H_0+\Delta H_{dip}+\Delta H_{ex} \tag{3}$$

where ΔH_0 is the line width in the absence of interaction, ΔH_{dip} is the line broadening caused by magnetic dipolar interactions and ΔH_{ex} is contributed by spin exchange (Sackman *et al.*, 1973).

Lymphocyte Assays

Cyclic-AMP levels in lymphocytes were estimated by a competitive binding assay (The Radiochemical Centre, Amersham). Extracts for assay were prepared by sonicating 10^7 cells in 1 ml of 0.05 M of Tris/EDTA buffer, pH 7.5. Mitogen dose response curves were determined by adding the desired amount of mitogen to 1 ml of 10^6 cells in MEM containing 10% fetal calf serum (Commonwealth Serum Laboratories, Melbourne), incubating for 60 hr, then pulsing with 1 μCi of [6-^{3}H] thymidine (The Radiochemical Centre, Amersham). The cells were incubated for a further 4 hr, collected and washed on glass fiber Millipore filters (Millipore Corp., Bedford, Mass.), and the radioactivity was determined with the filter covered with "Instagel" scintillant (Packard, Inc., Downers Grove, Illinois) in a Packard liquid scintillation counter (Model 2650). Mixed lymphocyte reactions (MLR) were quantitated in the same way, except that 0.5 ml of 10^6 allogenic lymphocytes was added to 0.5 ml of 10^6 lymphocytes and the incubation time before adding the [6-^{3}H] thymidine pulse was 160 hr. Capping of lymphocytes was performed by incubating 10^7 cells with 20 μg of fluorescein-labeled Con-A (Calbiochem, San Diego) for 30 min at 4 °C. The cells were washed twice at 4 °C with MEM and then examined on the warm stage

(37 °C) of a Zeiss Ultraphot microscope using epi-illumination, a HBO400 lamp, 4 mm UG1 and BG38 exciting filters and a 3-mm KV48 barrier filter. Fortyfive percent of the peripheral blood lymphocytes and 33% of the tonsil lymphocytes were capped when examined by this method.

Results

Spin-labeled Probes in Human Peripheral Lymphocytes

The spectral parameters for the 5NS, 12NS and 16NS probes before and after adding 5 μg/ml of Con-A are set out in Table 1. It can be seen that there is a 20% decrease in S, indicative of a decrease in the ordering of the fatty acyl chains of the lipids in the region of the membrane probed by 5NS in the Con-A-treated lymphocyte. There is a similar, but smaller decrease in ordering in the regions probed by 12NS and 16NS. Increases in ΔH indicating electron exchange/magnetic dipole interaction was evident in both Con-A and control cells at lipid/probe ratios of less than 75:1.

Except at high lipid/probe ratios the high field hyperfine extremum was not resolved in spectra obtained with any of the ester probes. Because

Table 1. Spectral parameters for 5NS, 12NS and 16NS in human blood lymphocytes before and after the addition of 5 μg/ml of Con-A

Probe	Lipid: probe	Control		Con-A	
		S	ΔH (gauss)	S	ΔH (gauss)
5NS	300:1	0.568 ± *0.015*	3.99 ± *0.05*	0.461 ± *0.011*	4.06 ± *0.08*
	150:1	0.562 ± *0.021*	3.97 ± *0.04*	0.462 ± *0.016*	4.08 ± *0.07*
	75:1	0.537 ± *0.018*	4.09 ± *0.04*	0.445 ± *0.009*	4.16 ± *0.11*
	35:1	0.512 ± *0.016*	4.75 ± *0.06*	0.421 ± *0.013*	4.85 ± *0.07*
12NS	300:1	0.405 ± *0.009*	2.74 ± *0.05*	0.387 ± *0.006*	2.95 ± *0.05*
	150:1	0.403 ± *0.011*	2.76 ± *0.09*	0.388 ± *0.001*	2.94 ± *0.07*
	75:1	0.395 ± *0.015*	2.81 ± *0.03*	0.376 ± *0.012*	3.01 ± *0.08*
	35:1	0.372 ± *0.007*	2.93 ± *0.09*	0.362 ± *0.005*	3.14 ± *0.06*
16NS	300:1	0.386 ± *0.013*	2.57 ± *0.11*	0.362 ± *0.004*	2.60 ± *0.05*
	150:1	0.388 ± *0.018*	2.60 ± *0.08*	0.375 ± *0.013*	2.63 ± *0.04*
	75:1	0.380 ± *0.006*	2.65 ± *0.03*	0.376 ± *0.005*	2.75 ± *0.03*
	35:1	0.376 ± *0.007*	2.69 ± *0.06*	0.365 ± *0.008*	2.81 ± *0.05*

Spectra were measured immediately after the addition of Con-A. Spectral parameters are averages of three experiments carried out on separate batches of cells from the same individual. Numbers in italics represent range of readings.

Table 2. Spectral parameters for 5NSME, 6NSMe, 12NSMe and 16NSMe in human blood lymphocytes before and after the addition of 5 μg/ml of Con-A

Probe	Lipid/probe	Control		Con-A	
		τ_0 ($\sec \times 10^{-10}$)	ΔH (gauss)	τ_0 ($\sec \times 10^{-10}$)	ΔH (gauss)
5NSMe	600:1	47.61 ± *0.06*	3.99 ± *0.03*	49.36 ± *0.81*	3.95 ± *0.06*
	300:1	48.72 ± *0.43*	4.01 ± *0.05*	156.35 ± *1.65*	4.96 ± *0.03*
	150:1	49.31 ± *0.24*	4.16 ± *0.04*	–	5.63 ± *0.06*
	75:1	–	4.48 ± *0.07*	–	6.51 ± *0.05*
	35:1	–	4.97 ± *0.06*	–	7.61 ± *0.03*
6NSMe	600:1	42.31 ± *0.46*	4.06 ± *0.05*	51.34 ± *0.73*	3.98 ± *0.07*
	300:1	44.16 ± *0.67*	4.16 ± *0.03*	177.19 ± *2.87*	4.85 ± *0.09*
	150:1	47.82 ± *0.65*	4.28 ± *0.09*	–	5.89 ± *0.11*
	75:1	–	5.17 ± *0.11*	–	6.83 ± *0.13*
	35:1	–	5.68 ± *0.06*	–	7.65 ± *0.06*
12NSMe	600:1	19.16 ± *0.47*	2.84 ± *0.09*	19.16 ± *0.75*	2.86 ± *0.05*
	300:1	19.91 ± *0.36*	2.93 ± *0.12*	28.73 ± *1.31*	3.61 ± *0.10*
	150:1	19.53 ± *0.55*	2.86 ± *0.05*	35.84 ± *1.36*	4.05 ± *0.05*
	75:1	21.22 ± *0.61*	3.01 ± *0.07*	–	5.13 ± *0.14*
	35:1	23.26 ± *0.44*	3.15 ± *0.03*	–	6.61 ± *0.06*
16NSMe	600:1	13.51 ± *0.35*	2.53 ± *0.06*	12.85 ± *0.53*	2.61 ± *0.06*
	300:1	14.10 ± *0.61*	2.56 ± *0.11*	16.42 ± *0.66*	2.78 ± *0.05*
	150:1	13.98 ± *0.36*	2.61 ± *0.04*	18.33 ± *0.78*	2.91 ± *0.12*
	75:1	15.10 ± *0.41*	2.67 ± *0.06*	19.13 ± *1.06*	3.16 ± *0.10*
	35:1	15.54 ± *0.27*	2.68 ± *0.05*	19.96 ± *0.83*	3.35 ± *0.09*

Spectra were measured immediately after the addition of Con-A. Spectral parameters are averages of three experiments carried out on separate batches of cells from the same individual. Numbers in italics represent range of readings.

of this the empirical motion parameter τ_0 and ΔH only are given in Table 2 for normal and Con-A treated cells (5 μg/ml). Again there was a marked broadening effect at the lower lipid/probe ratios, as indicated by an increase in ΔH, and this was apparent at ratios greater than 150:1, much higher than with the corresponding fatty acids. However, the most striking result was the very marked broadening found in the Con-A-treated cells compared with the controls at the same lipid/probe ratios. A pair of spectra for 5NSMe at a lipid/probe ratio of 100:1 are shown in Fig. 1. The broadening occurred with all the methyl ester probes, although to a lesser extent as the label was more remote from the ester groups.

The splitting of the high field line in curve *B* of Fig. 1 indicates partitioning of the ester probe between lipid and polar regions. The

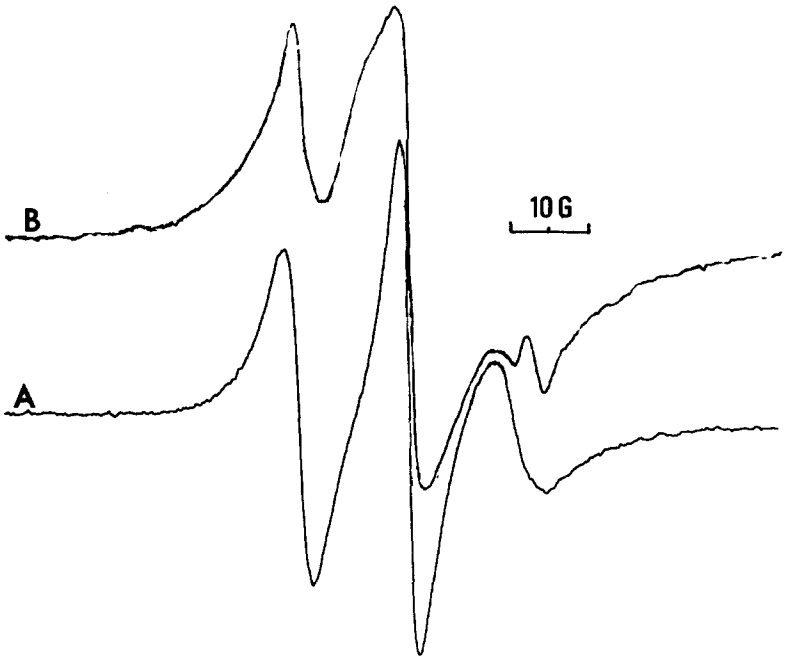

Fig. 1. Spectra of 5NSMe in human blood lymphocytes at 37 °C, before (*A*) and after (*B*) the addition of 10 μg/ml of Con-A. Lipid/probe ratio, 150:1. The splitting of the high-field line in curve *B* indicates partitioning of some of the probe into a polar region

increase in proportion of probe in the polar regions after treatment with Con-A is indicative of an increase in the order of the lipid molecules in the region of the membrane probed by the ester probe in contrast to that probed by the acid probe which showed a decrease in order.

Spin-Labeled Probes in Human Tonsil Lymphocytes

For five different preparations, S for 5NS at a lipid/probe ratio of 300:1 was 0.545 with a range of ± 0.035. The addition of Con-A (5 μg/ml) or PHA (2.5 μg/ml) reduced this value to 0.497 with a range of ± 0.028. For the same preparations, ΔH for 5NSMe was 4.15 ± 0.12 before and 4.48 ± 0.13 after adding the same concentrations of the mitogens at a lipid/probe ratio of 300:1.

Nature of the Con-A-Induced Broadening of Ester Probe Spectra

The spectra for 5NSMe obtained at -180 °C for Con-A-treated and control cells are shown in Fig. 2. It can be seen that the Con-A-induced

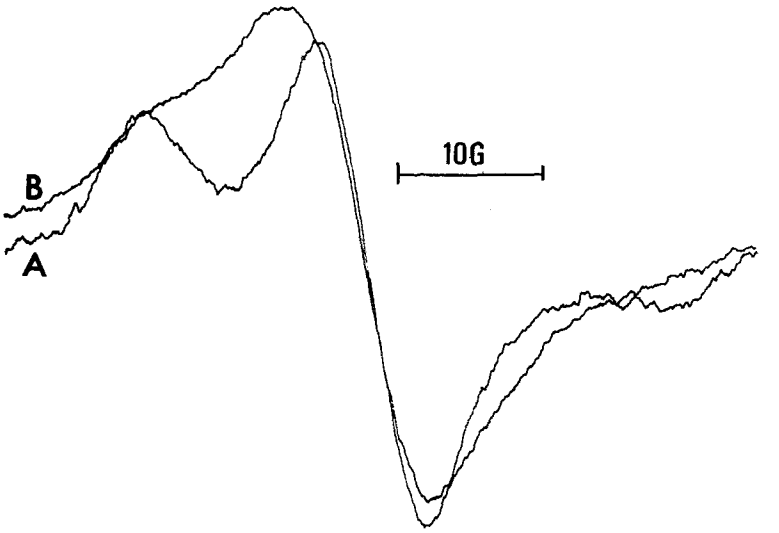

Fig. 2. Spectra of 5NSMe in human blood lymphocytes at −180 °C before (*A*) and after (*B*) the addition of 10 μg/ml of Con-A. Lipid/probe ratio, 150:1

broadening is still evident. Since there is little molecular motion in the membrane at −180 °C the broadening seen at 37 °C must be due to spin-label interaction and not to an increase in ordering. This effect was found with all the methyl ester probes. On the other hand, with the acid probes Con-A-treated and control cells gave identical spectra at −180 °C, indicating that the Con-A-induced decrease in S at 37 °C was due to a decrease in the ordering of the lipids rather than a decrease in spin-label interaction. Another characteristic feature of probe-probe interaction is an increase in line width with temperature in contrast to a decrease in line width due to motional effects. We could not use this criterion, however, because of instability of the cells above 40 °C leading to a rapid decrease in the Con-A-induced spectral broadening. Spectra produced at 37 °C by sweeping between 1400 and 1700 gauss showed no resonances in either control cells or the Con-A-treated samples which had shown line broadening when swept over the normal range. This absence of "half-field transitions" suggests that the broadening is due to electron-exchange rather than dipolar effects.

Distribution of Spin Labels in the Lymphocyte Membranes

The distribution of spin labels in the membranes was investigated by adding 5NS to lymphocytes at a lipid/probe ratio of 30:1. Marked

probe-probe interaction was present ($\Delta H = 5.1$ gauss). After the addition of three aliquots of unlabeled lymphocytes and mixing, the spectral broadening had disappeared ($\Delta H = 3.9$ gauss). Since the broadening remained constant in control lymphocytes with a lipid/probe ratio of 30:1 kept for the duration of the experiment, the elimination of the broadening on the addition of more lymphocytes was due to redistribution of the label between lymphocytes, rather than to redistribution of label within already-labeled cells. This experiment was repeated with 5NSme. At a lipid/probe ratio of 30:1 a ΔH of 6.1 was recorded, and this value was not reduced by the addition of three or more aliquots of unlabeled cells, indicating that the methyl ester probe does not redistribute readily and that it is more tightly bound in the membrane of the original cells than the acid probes.

Mutual interaction was studied by adding acid and ester probes to the same cells. 5NSMe was added to lymphocytes at a lipid/probe ratio of 300:1 and the spectrum gave a ΔH of 4.09 gauss. Sufficient 5NS was then added to bring the total lipid probe ratio to 50:1. The spectrum of this mixture gave a ΔH of 4.5 gauss. For this batch of cells ΔH for 5NS at 50:1 was 4.48 and for 5NSme, 5.93. These results suggest that the two probes are intercalating into separate regions where they do not interact with each other.

Effect of Mitogenic and Other Compounds besides Con-A on the Spectra of 5NSMe and 5NS

Where applicable, each agent was used at the optimum mitogenic dose determined for that batch of cells. Contrasting results were obtained. As shown in Table 3 only the nonmitogenic WGA failed to increase ΔH for 5NSMe. A small but significant increase was observed for the MLR and somewhat larger increases for cholera toxin and the ionophore A 23187. No increase was observed with the latter when 0.01 M EGTA was used with calcium free MEM as the medium. Decreases in S for 5NS were observed for all agents, except WGA, the MLR, and succinyl-Con-A.

Relation between Magnitude of Changes in ΔH and S, Dose of Con-A and Mitogenicity

Values of S, ΔH and [6-^{3}H] thymidine uptake are plotted against added concentrations of Con-A in Fig. 3 which shows that the increase

Table 3. The effect of a range of agents on the spectral parameters of 5NSMe and 5Ns in human blood lymphocytes

Agent	Concn	ΔH for 5NSMe (gauss)	S for 5NS
Control		3.9	0.565
Con-A	5 μg/ml	7.5	0.457
PHA	3 μg/ml	7.1	0.471
A23187[a]	3 μM	5.9	0.526
A23187[b]	3 μM	4.0	0.567
Cholera toxin	8 μg/ml	6.6	0.530
WGA	15 μg/ml	3.9	0.561
Neuramidase	10 μg/ml	6.8	0.493
MLR	(0.5 ml 10^6 allogeneic lymphocytes)	4.3	0.561
KIO_4	0.5 mM	7.3	0.569
Succinyl Con-A[c]	10 μg/ml	4.9	0.523
Succinyl Con-A[d]	100 μg/ml	6.9	0.456

Both probes added to a lipid/probe ratio of 150:1.

[a] In normal MEM.

[b] in Ca^{++}-free MEM with 0.01 M EGTA.

[c] Preparation of Beppu *et al.* (1976).

[d] Preparation of Gunther *et al.* (1973).

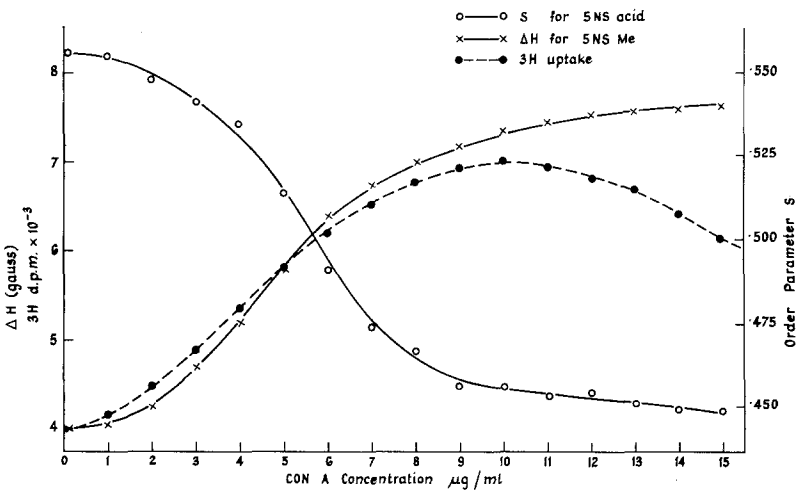

Fig. 3. Relation between the changes in ΔH for 5NSMe, S for 5NS, dose of Con-A, and [6-^{3}H] thymidine uptake in lymphocytes

in ΔH and decrease in S follow the rise in [6-^{3}H] thymidine uptake. However, while the [6-^{3}H] thymidine uptake begins to fall at high Con-A concentrations, the values of ΔH and S remain constant.

Relation between Changes in ΔH and S, Cyclic AMP Levels and Patching and Capping

The changes in ΔH for 5NSMe, S for 5NS, and intracellular cyclic-AMP levels in Con-A-treated cells are plotted against time in Fig. 4. Both spectral parameters were immediately changed after the addition of Con-A and then slowly returned to control levels after 60 min. The increase in intracellular cyclic AMP lags behind the changes in the spectral parameters by about 20 min, then begins to fall to control levels after 60 min. With the same batch of cells and fluorescent Con-A, it was observed that capping was complete after 20 min and by 60 min all the labeled lectin had disappeared from the cell surface. Further lectin added at this stage did not bind, nor did it change ΔH or S.

The Effect of Colchicine and Sodium Azide on H and S of Con-A-Treated and Control Cells

A range of concentrations of colchicine and sodium azide were added to lymphocytes immediately after the addition of 5 μg/ml of Con-A and

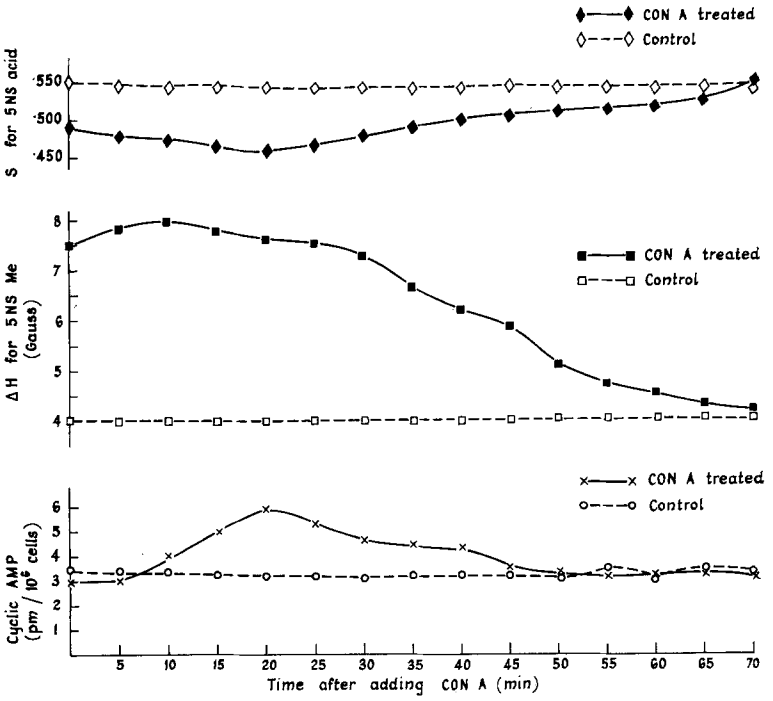

Fig. 4. Time relation between changes in S for 5NS, ΔH for 5NSMe, and cyclic AMP content of Con-A-treated lymphocytes. Lipid/probe ratio, 150:1; Con-A dose, 10 μg/ml

Table 4. Effect of exposure of human blood lymphocytes to colchicine and sodium azide on the spectral parameters of 5NSMe and 5NS in Con-A-treated and control cells

Agent	Conc (μg/ml)	Con-A		Control	
		ΔH for 5NSMe (gauss)	S for 5NS	ΔH for 5NSMe (gauss)	S for 5NS
NAN_3	0	7.12	0.471	4.13	0.565
	100	7.16	0.465	3.92	0.571
	200	7.25	0.466	3.95	0.569
	400	6.86	0.459	3.89	0.563
Colchicine	0	7.12	0.471	4.11	0.568
	2	5.48	0.525	3.87	0.568
	5	5.41	0.537	3.96	0.565
	10	6.82	0.516	5.37	0.541
	20	8.16	0.478	8.26	0.516

Sodium azide and colchicine added after 5 μg/ml of Con-A. Lipid/probe ratio 75:1.

also to control lymphocytes. ΔH and S values for both Con-A-treated and control cells in the presence of azide and colchicine are given in Table 4. At colchicine levels of 2 and 5 μg/ml in the Con-A-treated cells, a significant decrease was observed in ΔH for 5NSMe, compared with Con-A-treated cells without colchicine. At colchicine levels of 10 and 20 μg/ml, marked broadening was observed with the 5NSMe spectra of both Con-A-treated and control cells. With 5NS there was a significant increase in S at 2 and 5 μg/ml compared with cells without colchicine in the Con-A-treated group, while 10 and 20 μg/ml of colchicine decreased S in both the Con-A and control cells. Sodium azide had no significant effect on the values of ΔH or S in either group of cells.

Studies with Mouse and Rabbit Lymphocytes

The effects of Con-A and ALG on the spectral parameters of 5NSMe and 5NS acid in mouse T and B cells were studied. Rabbit peripheral lymphocytes were exposed to anti-rabbit IgM. The values for ΔH and S are given in Table 5. In each case an increase in ΔH for 5NSMe and a decrease in S for 5NS was observed.

Location of the Spin Probes in the Lymphocyte Membranes

It was felt that differences in behavior of the acid and ester probes was probably due to their intercalating into chemically different regions

Table 5. Effect of Con-A and ALG in mouse T and B cells and anti μ-chain in rabbit cells on spectral parameters of 5NSMe and 5NS

Cell	Agent	ΔH for 5NSMe (gauss)	S for 5NS
Mouse T	Control	3.65	0.677
	ALG 20 μg/ml	7.85	0.581
	Con-A 5 μg/ml	8.36	0.581
Mouse B	Control	3.92	0.615
	ALG 20 μg/ml	7.68	0.546
	Con-A 5 μg/ml	8.31	0.516
Rabbit	Control	4.01	0.576
	Anti-IgM 100 μg/ml	7.66	0.493

Lipid/probe, 75:1.

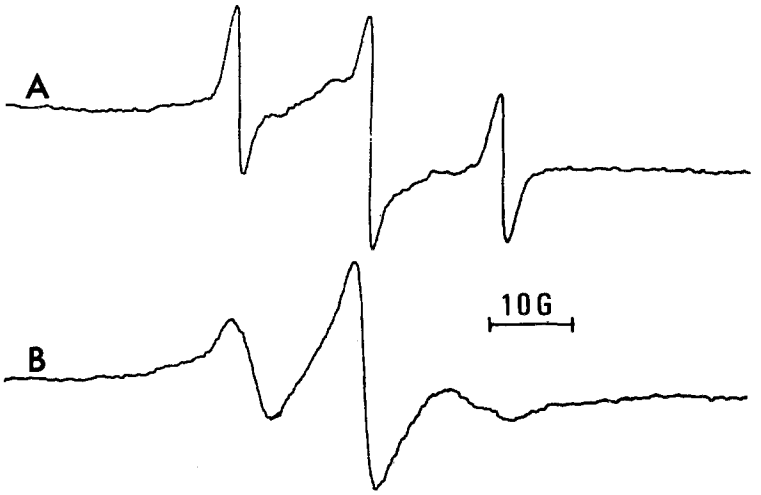

Fig. 5. (*A*): Spectrum of 5NSMe in BHK cells. (*B*): Spectrum of 5NSMe in BHK cells after they had been exposed to gangliosides, then washed. Lipid/probe ratio, 150:1

of the membrane. In an attempt to gain some information about these regions, experiments were carried out with ganglioside-free BHK cells and with mixed ceramide/phospholipid vesicles. It was found that BHK cells gave a large polar component with 5NSMe (Fig. 5*a*) which was eliminated (Fig. 5*b*), by adding gangliosides to the cell and washing (Critchley & McPherson, 1973). This shows that gangliosides increase the solubility of the ester probe in the lipid region of the membrane and suggests some interaction between gangliosides and methyl esters of fatty acids. ΔH for 5NSMe at a lipid/probe ratio of 100:1 in the

Table 6. Spectral parameters for 5NSMe and 5NS in phospholipid vesicles containing varying concentrations of glycosphingolipids

	Conc. % (wt/wt)	5NSMe		5NS	
		ΔH (gauss)	S	ΔH (gauss)	S
Gangliosides	0	2.61	0.367	2.64	0.365
	5	2.83	0.375	2.61	0.374
	10	3.16	0.393	2.65	0.381
	20	3.51	0.411	2.66	0.389
Cerebrosides	0	2.63	0.362	2.65	0.364
0.364	5	2.75	0.378	2.66	0.376
	10	2.87	0.391	2.69	0.382
	20	2.94	0.408	2.71	0.397

Lipid/probe ratio, 150:1.

Table 7. The effect of increasing probe concentration on ΔH of 5NSMe and 5NS in 20% ganglioside and 15% cerebroside vesicles

	Lipid/probe	ΔH for 5NSMe (gauss)	ΔH for 5NS (gauss)
Ganglioside	200:1	3.58	3.56
	150:1	3.63	3.56
	100:1	4.21	3.63
	50:1	4.86	3.66
	20:1	6.31	4.12
	10:1	8.6	4.96
Cerebroside	200:1	2.64	2.81
	150:1	2.59	2.78
	100:1	3.11	2.86
	50:1	3.26	2.93
	20:1	3.65	3.41
	10:1	3.91	3.68
Phospholipid alone	200:1	3.17	3.15
	150:1	3.25	3.20
	100:1	3.24	3.23
	50:1	3.41	3.33
	20:1	3.47	3.41
	10:1	3.66	3.56

ganglioside-treated cells was 4.16 gauss, and this was increased to 6.15 gauss when 5 μg/ml of Con-A was added to the cells. For 5NS at the same ratio S was 0.543 before and 0.485 after Con-A was added to the ganglioside-treated fibroblasts.

Values of ΔH and S for the ester and acid probes were determined in phospholipid vesicles containing varying concentrations of gangliosides or cerebrosides. These are set out in Table 6, and it can be seen that ΔH increases markedly for the ester probes with increasing glycolipid concentration. The ΔH and S values of the acid probes were not affected.

The effects of increasing probe concentrations in 20% ganglioside and 15% cerebroside vesicles are seen in Table 7. ΔH for the ester probes begins to increase markedly at lipid/probe ratios of less than 100:1, whereas broadening is not observed with the acid probes until the ratio approaches 20:1.

Discussion

While spin labels report on the molecular ordering, dielectric properties, and motion of membrane lipids they also form impurity pools within the membrane, and the spectral parameters might not reflect the actual physical state of the membrane lipids. For this reason we have paid special attention to determining spectral parameters at various lipid/probe ratios to overcome the problems associated with spin-spin interaction (Sauerheber *et al.*, 1977).

For example, the decrease in S for 5NS acid on addition of Con-A to lymphocytes (Table 1) is indicative of a change in ordering of membrane lipids rather than an alteration in the interaction between spin probe molecules. This follows from the observations that with lymphocytes, probe-probe interactions occur at lipid-probe ratios of less than 75:1 for acid probes (Table 1) and 150:1 for ester probes (Table 2) and the decrease in S due to Con-A is apparent at lipid/probe ratios of more than 150:1. In addition, the change in ordering induced by Con-A is less marked with probes which reflect the inner region of the bilayer (Table 1), consistent with the fluidity gradient of membrane lipids (Jost, Waggoner & Griffith, 1971). Barnett *et al.* (1974), using a nitroxide label on the 6-position of palmitic acid, found an almost identical decrease in ordering when PHA was added to human lymphocytes, as we observed with 5NS and Con-A. However, Dodd (1975) was unable to detect any change in ordering with 5NS acid in human tonsil lymphocytes treated with PHA. We observed a small but significant decrease in ordering in our tonsil lymphocyte preparations after the addition of mitogens. In our hands tonsils were unsatisfactory as a source of a uniform, reproducible population of cells, only 5 out of 10 resections yielding cells which met our criteria of 70% small lymphocytes with

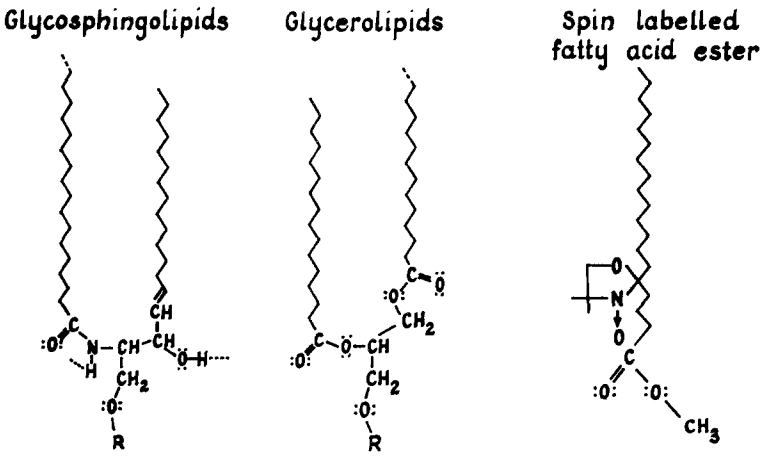

Fig. 6. Structures of glycosphingolipid, glycerolipid, and a spin-labeled fatty acid ester showing hydrogen bond donor and acceptor groups

at least 75% viability. Even these criteria meant that only 52.5% of the cells were satisfactory, which would account for the smallness of the changes observed. Dodd (1975) gave no detailed information about his cells, although his description of a "complex spectrum showing both weakly and strongly immobilized components" for 5NS is reminiscent of the spectra we observed for this probe in peripheral lymphocytes that had been kept for more than 120 min in serum-free media.

In contrast with the lack of interaction with acid probes, methyl ester probes show a marked increase in probe-probe interaction on addition of mitogenic agents to cells. This increase in interaction leads to a broadening of the spectra which gives extremely large increases in the motion parameter because ΔH is equivalent to W_0 in Eq. (2). Since the observed spectral broadening indicates that the probes have come to lie within $< 15 \times 10^{-8}$ cm of each other (Devaux & McConnell, 1972), it appears that the lipids associated with these probes are aggregated by the action of mitogens. The observation that the Con-A-stimulated probe-probe interaction is reversible with time, paralleling the disappearance of receptor-ligand complexes from the cell surface suggests that the ester probes are located in lipid regions which are closely associated with the receptor. The ester probes, being hydrogen bond acceptors, are more likely to associate with glycosphingolipids (Fig. 6) which are hydrogen bond donors than with glycerolipids which are not (Pascher, 1976). Loss of the aqueous component of the spectra of 5NSMe labeled, ganglioside-free BHK cells (Fig. 5*a*) on addition of gangliosides (Fig. 5*b*) is indicative of a preference for the ester probes to locate in regions rich in glyco-

sphingolipids. This is confirmed by the effect of adding cerebrosides and gangliosides to phospholipid vesicles on methylester probe-probe interaction (Table 7). Further evidence for the involvement of gangliosides in the ligand-induced ester probe-probe interaction is found in the effect of cholera toxin on both lymphocytes and ganglioside-treated BHK cells. We found that the toxin, which has been shown to aggregate its Gm_1 ganglioside receptors (Révész & Greaves, 1975, Craig and Cuatrecasas, 1975) causes probe-probe interaction in both types of cell. That the ester and acid spin probes locate into different regions of the membrane has been shown independently by Zenser, Petrella and Hughes (1976) with the observation that nitroxide fatty acids inhibit rat-thymocyte-associated adenylyl cyclase, whereas the corresponding ester did not. This concept is further supported by our observation that in double-labeled membranes, spin-spin interaction between one type of probe is independent of the concentration of the other. This would be consistent with the acid probe being evenly distributed among the phospholipids. The ligand-binding induced decrease in the order parameter of acid probes may therefore be a consequence of an increase in fluidity of the remaining membrane lipid following condensation of the glycosphingolipids in the receptor sites. This comes about because of the separation of the more rigid acyl chains of the glycosphingolipids from the bulk membrane lipids. When monitored at the same position on the fatty acid chain, glycosphingolipids are more ordered than phospholipids, indicating a closer packing of head groups for the former lipids compared to the latter (Sharom *et al.* and Grant, 1976; Feinstein, Fernandez & Sha'afi, 1975). Furthermore, Sharom *et al.* (1976) noted that the decreased fluidity of glycosphingolipids compared with phospholipids was most pronounced when the spin probe was close to the head group. This increased order of the glycosphingolipids agrees with our interpretation of the presence of the polar component of spectrum *B* in Fig. 1 as indicating an increase in order in the regions in which the ester probes were aggregated.

It is of interest to consider possible mechanisms for the association of the glycosphingolipids with receptors. It is becoming increasingly evident that the glycosphingolipids can present a range of functional groups on the cell surface. The cholera toxin receptor has been well characterized as Gm_1 ganglioside (King & Van Heyningen, 1973, Hollenberg *et al.*, 1974), tetanus toxin and serotonin receptors as neuraminidase-labile, disialosyl gangliosides. Van Heyningen (1974), and Esselman and Miller (1974) have presented evidence that the BAθ antigen of the mouse is

G_{D1b} ganglioside. Complex glycosphingolipids with 30–50 sugar residues have been isolated from the human erythrocyte membrane by Gardas and Koscielak (1973, 1974), and Slomiany and Slomiany (1977) have isolated two blood group-A active glycolipids with 12 and 18 sugar residues from pig gastric mucosa. It is, therefore, conceivable that many ligands, particularly the sugar specific lectins, would be capable of binding to glycosphingolipid saccharide groups. The glycosphingolipids could also be associated with membrane proteins and glycoproteins, bearing receptors either by lateral hydrogen bonding or interaction between sugar residues. Evidence for such an association in the human red cell membrane has been presented by Ji (1974). Under these circumstances, ligand-induced cross-linking of receptor-bearing proteins would aggregate the glycosphingolipids. Colchicine which is thought to affect lymphocyte surface modulation by disrupting microtubules (Edelman, 1976) had varied effects on probe-probe interaction in the presence of Con-A (Table 4), reducing it at concentrations of 2–5 μg/ml but causing a marked increase in interaction in both Con-A-treated and control cells at concentrations of 10–20 μg/ml. Possibly the breaking of microtubular anchorages by colchicine may increase the extent of protein-protein interaction in the membrane, causing aggregation of the protein-associated glycosphingolipids.

There is a substantial literature showing that changes in lipid fluidity affect membrane-associated enzymes and could be involved in the initiation of cell activation (reviewed by Farias *et al.*, 1975). We find that changes in lipid ordering were induced by all the mitogenic agents tested (Table 3). The nonmitogenic lectin WGA, on the other hand, had no effect on lipid ordering or aggregation. No evidence is available on the patching ability of WGA, although it should be noted that it reacts with ganglioside-loaded phospholipid vesicles (Redwood & Polefka, 1976). The finding that ALG caused an increase in lipid aggregation and decrease in ordering in mouse B cells suggests that the lipid changes alone are not sufficient to cause mitogenesis because ALG does not activate B cells.

On the present evidence we cannot say whether an increase in order in the bulk of the membrane lipids or an increase in restriction among the aggregated glycolipids could be responsible for the activation of an effector enzyme. One possibility is that the glycosphingolipids act as a bridge between the receptor molecules and the effector enzyme; another is that the glycosphingolipids are the receptors or the glycosphingolipids could be hydrogen-bonded to receptor glycoprotein.

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