anaerobic bacteria in heartwood accounts for the high gas pressures observed in trees. The factors responsible for proliferation of methanogenic bacteria in trees are questions of considerable biological interest. We have detected methane only in trees that contain wetwood with a distinct volatile fatty acid odor, neutral to alkaline *p*H, and under strictly anaerobic conditions.

The high number of methanogenic bacteria and other anaerobes found in wetwood indicates vigorous microbial fermentation. The presence of these anaerobic bacteria in heartwood may constitute an infection of trees. It is difficult to ascertain whether the wood tissue itself is being decomposed or whether other nutrients serve as the substrates for this methane fermentation. The formation of heartwood in a tree is thought to involve, in part, translocation of excretory products from living xylem tissue toward the center of the stem (*9*). However, wetwood or bacterially infected heartwood from some trees is noticeably weakened (*10*), and this may be an indicator of microbial decomposition of the wood tissue. Methane-producing bacteria are unable to degrade wood, but convert end products from the anaerobic decomposition of organic matter, namely, H_2 and CO_2, into methane. Various kinds of bacteria reside in infested trees in addition to the methanogenic species isolated. Previous investigators (*11*) have described facultative and obligate anaerobic bacteria isolated from wetwood which decompose organic matter. The establishment of an anaerobic bacterial population in trees capable of producing methane probably results from root injury, especially in water-saturated soils, which provides a path of entry for indigenous soil microorganisms.

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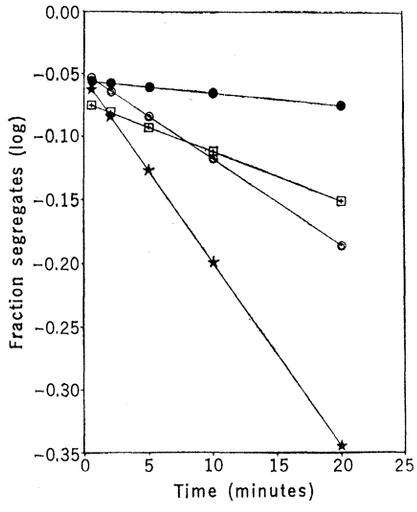
Lateral Phase Separation of Lipids in Plasma Membranes: Effect of Temperature on the Mobility of Membrane Antigens

Abstract. *Cooling populations of newly formed mouse human heterokaryons has effects on the intermixing of mouse and human surface antigens which indicate the occurrence of phase separations in membrane lipids. Antigen mixing, previously shown to be due to diffusion in the plane of the membrane, is retarded when cells are cooled from 37° to 21°C, but is then speeded by further cooling to 15°C. This result is in accord with observations on phase separations of lipids in artificial and bacterial membranes.*

Lipids of artificial and natural membranes have been shown by several physical methods to undergo changes from fluid to solid phase as the membranes are cooled (*1*). In artificial membranes these changes have generally been described as phase transitions, implying that an entire membrane changes from fluid (liquid-crystal) to solid (gel phase) over a narrow temperature range. However, phase changes especially in membranes of mixed lipid species have been discussed in terms of phase separations, rather than as phase transitions (*2, 3*). It has been suggested that several transition temperatures can be defined in mixed lipid membranes, in which classes of lipid molecules coaggregate and solidify when a membrane is cooled, leaving other lipids, with lower melting temperatures still fluid. Phase separations then result in a membrane containing areas of both solid and fluid phase lipids. One consequence of phase transition or phase separation may be an increase in the rate of movement of proteins or ions within or through a membrane as it is cooled. Such increased mobility due to phase separations of lipids has been shown for ion permeation and sugar transport in artificial and bacterial membranes (*4*). We now describe evidence for phase separations of membranes in mammalian cell plasma membranes. A detailed study of the effect of temperature on the intermixing of histocompatibility antigens in the surface membranes of virus-fused mouse and human cells (heterokaryons) shows that within a temperature range of 5° to 6°C cooling the cells speeds the rate of their antigens' diffusion within the plane of the membrane.

Earlier it was found that the surface antigens of heterokaryons formed from cultured mouse and human cells initially lay in separate areas of the membrane, but rapidly intermixed; antigens of both species come to lie randomly throughout the surface within 1 hour of heterokaryon formation (*5*). Antigen mixing, observed by reacting samples of the heterokaryon population with fluorescent antibodies, was not affected by metabolic or biosynthetic inhibitors but was slowed when the cells were incubated below 37°C after formation. The rapid rearrangement of surface antigens occurring at 37°C appears to be due to their diffusion in the plane of the plasma membrane, lateral diffusion. The temperature dependence of the process would then be due to increases in viscosity of membrane lipids with decreasing temperatures, in agreement with other data on protein diffusion in animal cell membranes (*6*). The relation between antigen mixing and temperature suggested a sharp phase transition in the membrane lipids around 15°C, since there was little or

Fig. 1. Rate of loss of segregates, heterokaryons with separate areas of mouse and human histocompatibility antigens, plotted against time of incubation of the newly formed heterokaryons. Rates of initial loss (the first 20 minutes of incubation) are shown; experiments were continued for up to 24 hours. The curves are least squares regression lines fitted to data from a minimum of three separate experiments at the temperature indicated: stars, 37°C; squares, 21°C; open circles, 15°C; closed circles, 10°C. The standard error of the data in this and Fig. 2 is $\leq$ 10 percent.



no mixing of membrane antigens in heterokaryons held at or below this temperature for 40 minutes; but there was substantial mixing above this temperature. Another suggestion was that the curve reflected a threefold increase in membrane lipid viscosity for every 10°C decrease in temperature (*6*).

Our data were obtained by incubating populations of newly formed heterokaryons at varying temperatures for 1 to 24 hours. At intervals, samples of the population were reacted with fluorochrome-conjugated antibodies to identify mouse H-2 and human HL-A antigens (*7*); by this method the heterokaryons were doubly stained. All doubly staining cells were classified as having antigens completely intermixed or as having separate areas of mouse H-2 and human HL-A antigens; the latter cells are termed segregates. The fraction of all heterokaryons that were segregates at the times indicated are plotted on a semilogarithmic scale (Fig. 1), for four different temperatures of incubation. The slopes of the least squares lines fitted to the data decrease in general with decreased temperature; but one of the lines, that showing loss of segregates at 15°C, is steeper—that is, it shows a faster rate of mixing of antigens than would be expected. A family of lines was determined for intervals of 2° to 4°C, and antigen intermixing rates (the slopes of lines plotted as in Fig. 1) were plotted against temperature in an Arrhenius plot (Fig. 2). The rate of antigen mixing decreased as the temperature was lowered from 42° to around 21°C; this is expected from data on the bulk properties of lipids. However, below 21°C, the rate of antigen intermixing increases, with a new maximum at 15°C. Below 15°C it once more decreases, but at a rate much greater than the other portions of the curve.

We suggest that the changes in rate of antigen mixing imply changes in the membrane surface organization between 21° and 15°C and reflect passage of some plasma membrane lipids through the critical temperatures which define the beginning (t_{li}) and the end (t_l) of lateral phase separations (*8*). Just above 20°C, a class or classes of lipid with high melting temperatures begins to freeze out, the process being complete near 15°C. Antigens in areas of solidifying lipid would be excluded from these phases and forced into the remaining lipids. The increased rate of mixing between 20° and 15°C could be due to either or both of the following: (i) the remaining fluid phase is less viscous at 15° to 20°C than the total membrane lipid below 25°C, or (ii) there is less membrane area available for antigen movement when portions of membrane lipids have solidified. Formation of islands of solid lipid on the molecular scale could canalize diffusion of surface antigens, and on the macroscale of our observation this would result in more rapid intermixing of the antigens observed with the light microscope. Below 15°C it would seem that remaining fluid lipids would increase their viscosity rapidly with temperatures, leading perhaps to formation of a second phase below 0°C, similar to that observed calorimetrically in rat liver microsomes (*9*).

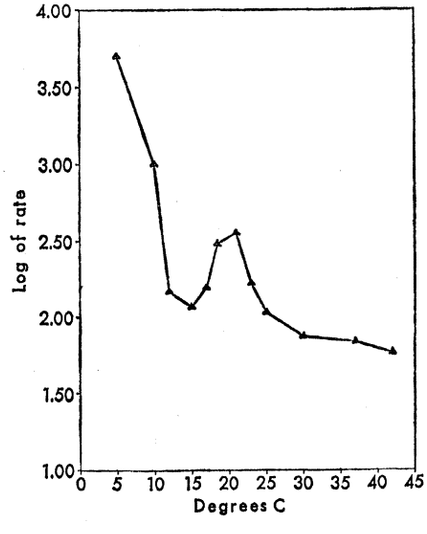


Fig. 2. Change in heterokaryon antigen mixing rate with temperature of incubation. Points indicated are the means of rates derived from the slopes shown in Fig. 1.

Several investigators suggest that cells at physiological temperatures may live "within the phase transition" (*8*, *10*), with their membranes containing mixtures of fluid and solid lipids. Though our evidence for phase separations in mammalian membranes points to their occurrence at temperatures well below physiological, it does show that the cell membrane may retain its integrity while portions solidify. Hence, the possibility remains that mixed lipid phases could occur at physiological temperatures as well. If patches of solid lipids are present in mammalian cells at 37°C, they could play an important role in controlling the mobility and hence the arrangement of a wide range of membrane-intercalated receptors (*3*). Deliberate modification of mammalian cell membrane lipid composition (*11*) ought to permit testing of these suggestions with cells whose membrane lipids are either preponderantly solid or preponderantly fluid at physiological temperatures. The biology of such modified cells might be of special interest with regard to observations on apparent differences in mobility of lectin receptors, surface antigens, and small hydrophobic probes in malignant cells as compared to normal or growth-regulated cells.

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7. Cells of the mouse fibroblast line Cl 1d (derived from the L cell line) and the human epithelioid line VA-2 were grown in Eagle's minimal essential medium supplemented with 5 percent fetal calf serum. Cells from culture plates were trypsinized, washed free of serum in cold Hanks balanced salt solution (BSS) and then mixed with inactivated Sendai virus at a concentration of 200 to 300 hemagglutinating units per milliliter. The suspension was shaken for 10 minutes at 0°C and then was placed at 37°C for 3 minutes to induce formation of heterokaryons. After this brief incubation, virus fusion factor was inactivated by addition of BSS containing 5 percent calf serum, and the cell suspension containing only relatively few heterokaryons was incubated at the desired temperature. At intervals, samples of the suspension were taken and held in ice until the course of incubation was complete. Heterokaryon antigens were visualized by reacting suspensions with mouse alloantibody to H-2 antigen and human alloantibody to HL-A at 0°C, followed by fluorescein-conjugated goat antiserum to mouse IgG and tetramethylrhodamine-conjugated goat antiserum to human IgG. All antibody combinations were checked for specificity of reaction on virus-treated parental cells. Reactions with antibody were carried out in ice. Stained cells were viewed with the microscope and filters described in (*5*).
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Latent Ganglionic Infection with Herpes Simplex Virus Types 1 and 2: Viral Reactivation in vivo after Neurectomy

Abstract. *Inoculation of the cornea, lip, or footpad of mice with herpes simplex virus type 1 resulted in a latent infection of the local sensory ganglia. Inoculation of the vagina and cervix with herpes simplex virus type 2, as well as type 1, also induced a latent ganglionic infection. With the use of sciatic nerve section as a stimulus, a reproducible model of viral reactivation in vivo was established.*

While it has long been suspected that herpes simplex virus (HSV) might reside in latent form in the sensory ganglia of the nervous system (*1*), direct evidence substantiating this theory has only recently been obtained. By means of methods of in vitro organ explantation and cocultivation, HSV has been recovered from sensory ganglia of chronically infected animals (*2, 3*) and from asymptomatic human subjects (*4*).

Our study was initiated to determine whether latent infection of local sensory ganglia could be induced after exposure of diverse epithelial surfaces to HSV type 1 (HSV-1). In addition, because HSV type 2 (HSV-2) differs from HSV-1 in a number of biological properties (*5*), the ability of HSV-2 to establish a latent ganglionic infection was also examined. Vaginocervical inoculation was chosen as the route of infection with HSV-2 because it is this serotype which is usually responsible for herpetic infection of the human genitalia (*5*).

Further studies were undertaken to develop a model of in vivo viral reactivation. Previous attempts to simulate the human condition of herpes reactivation with a reproducible animal model have met with only limited success (*6–8*). Since nerve section provokes herpetic lesions in man (*9*), sciatic neurectomy was chosen as the stimulus for effecting viral reactivation in latently infected dorsal root ganglia (DRG) of mice.

Pools of stock virus HSV-1 and HSV-2, which had been grown on primary rabbit kidney cells (*10*), contained approximately 4.0×10^8 and 1.3×10^7 plaque-forming units per milliliter, respectively. Female mice (4 to 6 weeks old) (*11*) were inoculated with undiluted virus at one of four sites, the methods varying according to the site. For corneal inoculation a drop of virus was placed on each eye, and both corneas were scarified with a hypodermic needle. For inoculation of both the right hind footpad and the lips, 10 percent saline was first injected locally, and 4 to 6 hours later the skin was abraded and virus was rubbed into these areas (*12*). Vaginocervical infection was accomplished by abrasion of the mucous surfaces followed by intravaginal insertion of a virus-soaked cotton pledget.

Trigeminal ganglia and lumbosacral DRG were removed under sterile conditions, washed three times, and then homogenized or explanted. The ganglia were disrupted with a Ten Broeck homogenizer; the homogenate was frozen and thawed three times, and samples were plated on duplicate monolayers of primary rabbit kidney cells. Explantation was achieved by placing either intact DRG or fragments of trigeminal ganglia on primary rabbit kidney monolayers. Cultures were observed for the appearance of typical HSV cytopathic effect. Isolated virus was identified by neutralization with specific rabbit antiserum to HSV.

Animals used for in vivo reactivation experiments were inoculated in the right hind footpad with HSV-1 at least 3 weeks before surgery. All surgical procedures were done on the animals' right sides under ether anesthesia. Sciatic neurectomy was performed peripheral to the DRG (that is, outside the spinal column) by cutting the lumbosacral roots of the sciatic nerve close to the exit foramina of the spinal column. Sham operations consisted of skin

Table 1. Latent infection of ganglia with HSV-1 and HSV-2. Virus was inoculated into the cornea, lip, footpad, and vagina. Sensory ganglia were removed bilaterally at intervals after infection and were assayed for virus by homogenization and explantation. Results are given as the ratios of the number of animals positive to the number of animals assayed.

Inoculation site	Virus type	Ganglia assayed	Method of assay	Assay after inoculation of virus on days: 4	7	10–12	14–28
Cornea	HSV-1	Trigeminal*	Homogenate	14/16	11/16	3/16	0/39
			Explant	8/16	8/16	9/16	31/39
Lip	HSV-1	Trigeminal	Homogenate	15/16			0/12
			Explant				14/24
Footpad	HSV-1	Dorsal root	Homogenate	7/10	8/10	0/10	0/34
			Explant				40/41
Vagina	HSV-1	Dorsal root	Homogenate		5/7		0/8
			Explant				7/15
Vagina	HSV-2	Dorsal root	Homogenate		22/32		0/27
			Explant				13/43

* In the corneally infected animals, trigeminal ganglia were removed bilaterally and divided into fragments of approximately 0.5 mm. Pieces were equally apportioned for explantation or homogenization. Figures for this group represent results of these two methods on the same animal. In all other experiments mice were divided into groups to be explanted or homogenized; only one method of assay was employed for each animal.