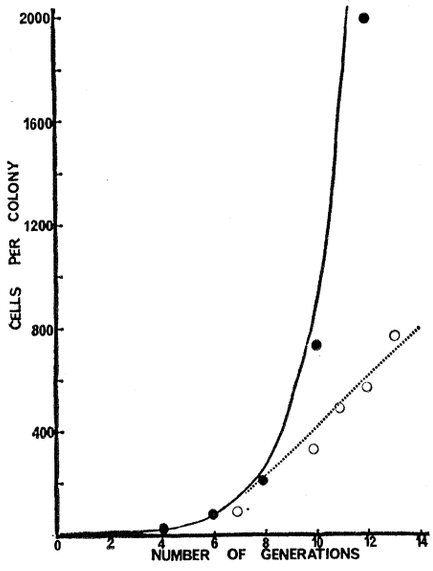

Fig. 2. Growth rates of CHL-1 (solid circles) and 3T3 (open circles). The solid line represents the theoretical exponential curve; the broken line represents the theoretical linear growth-rate starting at about 60 cells per colony.

onies of 3T3 was about 24 hours, whereas that of CHL-1 was about 12 hours.

To determine if the difference in the numbers of cells in the colonies of CHL-1 and 3T3 reflected the effects of contact inhibition, we established

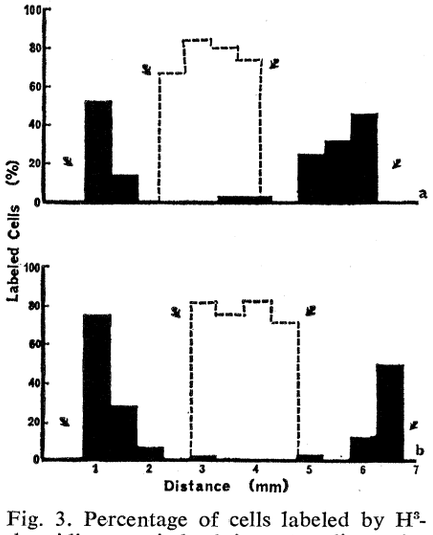

Fig. 3. Percentage of cells labeled by H^3-thymidine, as judged in autoradiographs, as a function of the distance (a) horizontally through a colony and (b) vertically through the same colony. The total cells and labeled cells were counted in adjacent squares (0.5 mm by 0.5 mm). The solid areas represent values for 3T3; under the broken line are the values for CHL-1. Arrows indicate the edges of the colony.

growth curves of the two cell types. With contact inhibition of replication, one would expect that, after the colonies reached a size in which the central cells could not escape contact with their neighbors, the growth rate would be a linear expression of the number of peripheral cells; without contact inhibition in the colonial population, the growth rate would continue exponentially. The results of the cell counts of colonies as a function of the number of generations are presented in Fig. 2. The growth rate of 3T3 colonies became approximately linear; that of the CHL-1 colonies, became approximately exponential. The expression of contact inhibition within the colony was detected from the 60-cell stage. This agrees with the fact that, from the sixth generation on, growth is linear rather than exponential.

To test whether only the cells on the circumference of the colonies were multiplying, we measured DNA synthesis by radioactive labeling and autoradiography. The colonies were grown on glass slides in petri dishes until they reached the desired size. The medium was then changed to Saline F (*4*) with fetal bovine serum and H^3-thymidine; the medium was 2 percent fetal bovine serum and had a final specific activity of 1 μc/ml. The incubation was continued for one generation time, and the radioactive solution was removed. The cells were washed twice with Saline F and fixed with 10 percent formaldehyde. The slides were then rinsed in distilled water, immersed for 1 hour in cold, 5 percent trichloroacetic acid, rinsed in distilled water, and air dried. For autoradiography, the slides were dipped in Kodak NTB-2 liquid photographic emulsion and exposed for 5 to 8 days at 4°C before being developed (*6*).

The autoradiographs of the center of a 3T3 colony had no grains, whereas the CHL-1 cells were labeled throughout (Fig. 1, c and d). The edges of the 3T3 colonies, however, did have labeled nuclei. We determined the distribution of the percentage of labeled cells by scanning the colony in two directions (Fig. 3). Whereas 60 to 80 percent of the peripheral 3T3 cells had labeled nuclei, less than 5 percent of those in the center of the colony were labeled.

Results obtained in analysis of clonal isolates by this method were the same as those obtained with the original 3T3 strain. Analysis of the percentage of cells having the property of contact inhibition in a primary culture is possible with this technique.

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Plasma Membrane: Substructural Changes Correlated with Electrical Resistance and Pinocytosis

Abstract. *Inducers of pinocytosis in amoeba cause as much as a 50-fold decrease in the electrical resistance of the plasma membrane prior to the formation of the typical tunnels and vacuoles. In this state the thickness of the electron-transparent core or lamella of the unit membrane is at least twice as thick as that of the control. The changes in structure and resistance as well as the induction of pinocytosis are dependent on the initial external concentration of calcium. These changes are rapidly reversed when the concentration of calcium in the external medium is increased.*

Cultures of the amoeba *Chaos chaos* L. (*Pelomyxa carolinensis*) fed washed paramecia were grown in a fluid containing 1.0 mmole of $CaCl_2$, 2.0 mmole of NaCl, and 0.4 mmole of KH_2PO_4-Na_2HPO_4 per liter of ion-free water with a *p*H of 6.9. The test solutions, described by notation of the Ca^{2+} concentration only, were identical except that the concentration of calcium chloride was varied. Solutions were made with ion-free water (resin supplied by Continental Water Conditioning Corp.) and kept in disposable

plastic beakers, and measurements were made in disposable plastic dishes so that low concentrations of calcium were maintained. We measured resistance by inserting two micropipettes filled with 3*M* KCL into a cell and connecting them, through silver–silver-chloride half cells, to appropriate electronic systems. The reference electrode in the bathing solution was a similarly connected pipette filled with 3*M* KCL–agar. Current in the form of a depolarizing or hyperpolarizing ramp (5×10^{-8} amp/sec) of about 5-second duration was supplied to one micropipette through a 100-megohm resistor; the ramp voltage was also used to drive the horizontal sweep of an oscilloscope. The voltage from the other microelectrode was fed through an electrometer preamplifier to the vertical amplifier of the oscilloscope. The resulting current-voltage tracings (I-E plots) were recorded on film. The slopes of these plots were used for the determination of the membrane resistance.

Figure 1a graphically presents three experimental conditions imposed on cells initially bathed in a test solution containing 0.1 mmole of Ca^{2+} per liter. The resistance decreases rapidly when the NaCl concentration is increased from 2 to 7 mmole per liter (+ 5 m*M* NaCl); however, it spontaneously returns towards the control value with time. When the membrane resistance is low, the amoebae cease to stream about the test chamber and begin to exhibit pinocytosis. After the resistance returns almost to normal, they cease to pinocytose and begin moving rapidly in the chamber, at which time most impaled amoebae free themselves from the microelectrodes. When the NaCl concentration is increased by 10 mmole/liter, the decrease in resistance is larger, and the duration of the transient longer. At higher concentrations of calcium, larger increases in the NaCl concentration are required to induce changes in resistance similar in magnitude to those recorded in Fig. 1a, or for NaCl or KCl to induce pinocytosis (*1*). The optimum ratio of NaCl to $CaCl_2$ for evoking the transient resistance changes and pinocytosis is between 100 and 400 to 1. Addition of an equiosmolar concentration of sucrose, which does not induce pinocytosis (*2*), increases the membrane resistance, and the cells continue to move rapidly about the chamber. It is difficult, therefore, to keep the electrodes implanted in order to determine if this increase in resistance is stable. Addition of ribonuclease or lysozyme (0.2 mg/ml), which induce pinocytosis (*3*), evokes a change in conductance similar to that induced by an increased NaCl concentration. Whether the inducing substance is an electrolyte or a protein, the decrease in resistance is dependent in magnitude and duration on the initial Ca^{2+} concentration. When the cells have a low resistance, a tenfold increase in the Ca^{2+} concentration rapidly returns the resistance to the control value or higher and halts pinocytosis (Fig. 1b).

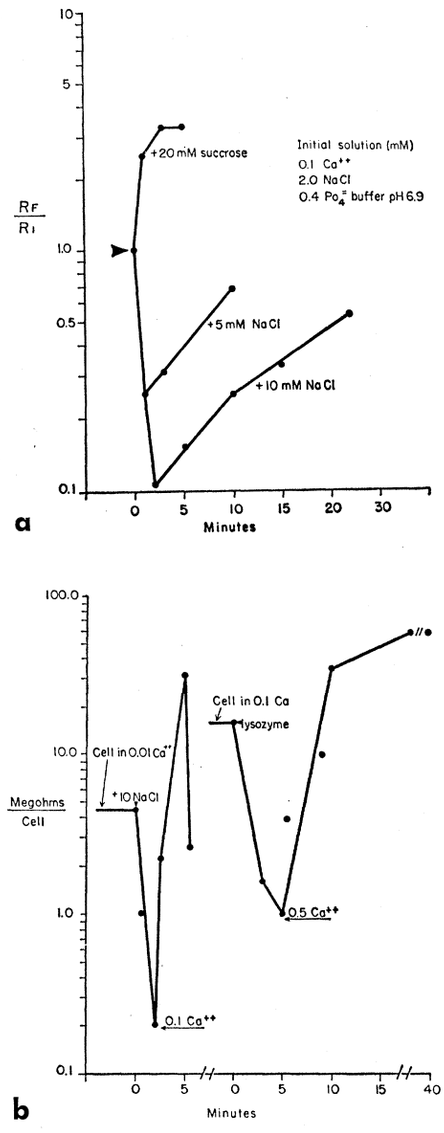

Fig. 1. (a) The arrow indicates when amoebae in the test solution were exposed to the addition of 20 mmole of sucrose per liter, or to 5- and 10-m*M* increases in the NaCl concentration; the relative resistance, R final/R initial, is plotted as a function of time. The sucrose causes an increase in the membrane resistance and does not induce pinocytosis. Increasing the NaCl concentration causes a transient fall in membrane resistance and induces pinocytosis. All the cells had initial resistances between 2.2 and 3.2 megohms. (b) The decrease in resistance induced by an increase in NaCl or by addition of lysozyme (0.2 mg/ml) is reversed when the calcium concentration is increased in the presence of the inducer. The absolute resistance per cell is plotted as a function of time.

In cells with low resistance induced by NaCl, the electron-transparent lamella of the unit (plasma) membrane is twice as thick as that of the control in low concentrations of calcium (Table 1 and Fig. 2). The dimensions of the unit membrane, fixed after the resistance is reversed to normal by an increase in the Ca^{2+} concentration, are identical with those of cells fixed in the culture fluid. Similar structural changes take place when the inducing substances are proteins, and these changes are reversed almost completely by 5-minute washes in the inducing solution with an increased Ca^{2+} concentration. The alterations in the plasma membrane were consistent over the cell surface in all the conditions studied.

The Davson and Danielli (*4*) model of the plasma membrane consists of a bimolecular lipid leaflet with proteins adsorbed to the hydrophylic groups, which face outward. This model has received considerable support from electron-microscopic studies, particularly from those of Robertson (*5*) and Stoeckenius (*6*). Electrophysiological data such as those on resistance and capacitance are also consistent with the gross characteristics of the model. Two main concepts, based on electrophysio-

Table 1. The changes in the unit membrane recorded by electrophysiological and electron-microscopic techniques are compared in this table. The dimensions are averages for ten measurements, and the last column is the difference between the overall membrane thickness and the transparent core (that is, the sum of the thickness of the two dense lines). The presence or absence of induction of pinocytosis is noted by a (+) or a (−), respectively.

Experimental conditions	Pinocytosis	Resistance, d-c (megohm/cell)	Thickness (Å)		
			Transparent lamella	Overall	Dense lamellae
Culture fluid	−	4.5	47	104	57
0.05 m*M* Ca	−	2–8	34	94	58
.05 *M* Ca + 20 m*M* NaCl	+	0.2	78	140	62
.5 m*M* Ca + 20 m*M* NaCl*	−	4.0	51	108	57
.05 m*M* Ca + lysozyme	+	0.4	86	160	74
.5 m*M* Ca + lysozyme*	−	2.	62	124	62
.05 m*M* Ca + ribonuclease	+	0.1	96	183	87
.5 m*M* Ca + ribonuclease*	−		67	124	57

* Cells fixed in this solution had been in the solution having a lower calcium concentration containing the inducing substance.

logical data, of the dynamic functions of the plasma membrane have developed independently of detailed structural evidence, but in the context of the Davson-Danielli model. They propose that changes in permeability result either from alterations in the properties of aqueous holes through the lipid layer (*7*) or from alterations in the configuration and in the dielectric properties of the lipid barrier (*8*). The two mechanisms become indistinguishable as the pores are made smaller and closer together, or they could be coexistent. Since the alteration of the amoeba plasma membrane is uniform, it is simplest to ascribe the change in its resistance to a modification of the dielectric properties of the lipid layer.

Coextensive in time with a decrease in resistance is an increase in the apparent volume of the membrane. If we extend the reasoning and evidence described by Stoeckenius (*6*) to the experimentally thickened electron-transparent zone, then the bulk phase of the lipid in the membrane is the locus of the volume increase. It is difficult for us to explain how the membrane changes volume in a rapid and reversible fashion without concluding that redistribution of electrolytes and water within the membrane are at least partially responsible for these changes. The membrane appears to be thinnest in media with low concentrations of calcium (0.05 m*M*), slightly thicker in those with higher concentrations of calcium (1.0 m*M*), and thickest in inducers such as increased concentrations of NaCl (Table 1). If the resistance proves to be an accurate measure of the dimensions of the membrane, then its volume should be minimal in sucrose since this compound increases membrane resistance (Fig. 1a) and inhibits the effects of inducers. Since the decrease in resistance in the presence of inducers is transient and returns to the resting level, the restabilized membrane must in some way differ from the resting membrane. Whether the accommodation of the membrane to the inducer is due to readjustments in the lipids, proteins, electrolytes, or in their interactions is not known, but problems dealing with this phenomenon may be approached by our experimental method.

The all-pervasive relationship between calcium and the membrane resistance of cells in general (*9*) suggests that the change in resistance in amoebae is basically similar to that of other cells. In our experiments, lowering the Ca^{2+} increased the effects of a given concentration of inducer, and increasing the Ca^{2+} rapidly reversed the effects of the inducer on the membrane resistance and structure.

It has been proposed that the first step in the pinocytosis of proteins is their adsorption to the plasmalemma (*10*). Low resistance of the membrane is accompanied by an active uptake of pinocytosis droplets, and it is suggested that the second step in pinocytosis is a change in membrane resistance due to an interaction with or adsorption of the inducer. The third step is the increased flux of ions across the membrane as a result of the change in permeability. In the fourth step perhaps the cytoplasmic proteins react to the changing ionic environment by gelling and contracting to produce the characteristic ruffling of the surface and to form the tunnels and vacuoles.

There is direct evidence that exchange of sodium and potassium across the membrane is greatly accelerated when the membrane has a low resistance. Bruce and Marshall (*11*) found that addition of 18 mmole of NaCl per liter to the test fluid in the presence of 0.4 m*M* of Ca^{2+} increased the cytoplasmic Na^{+} concentration from 0.3 m*M* to 2.5 m*M*, while a similar con-

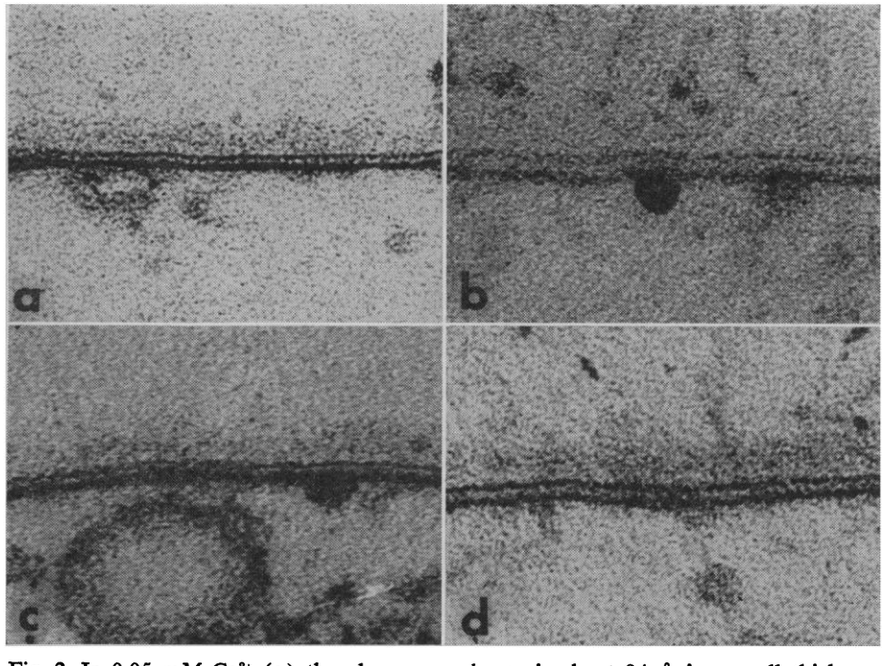

Fig. 2. In 0.05 m*M* Ca^{2+} (a) the plasma membrane is about 94 Å in overall thickness and thickens to about 140 Å when the NaCl concentration is raised to 22 m*M* (+20 NaCl). This is due to an increase (b) in the electron-transparent core, since the thickness of the dense lines is unchanged (Table 1). When the calcium concentration is increased to 0.55 m*M* the unit membrane becomes thinner (c) and has much the same dimensions as that in cells fixed (d) directly in the culture solution (1.0 m*M* Ca^{2+}) (× 216,300).

centration of NaCl in a medium with no calcium increased the cytoplasmic concentration of Na^+ to 8 m*M*. An equivalent amount of K^+ was lost in each case, and temperature changes did not affect the results. Chapman-Andresen and Dick (*12*) noted that the amoeba shrinks more rapidly during the first ten minutes of exposure to a hyperosmotic inducer solution than it does when exposed to a similar concentration of noninducer. Thus water permeability is probably increased in the low resistance state. Chapman-Andresen and Holter (*13*) showed that an amoeba is normally impermeable to glucose. However, external glucose is rapidly metabolized by the cells in the presence of an inducer of pinocytosis. Since the pinocytosis vacuoles are surrounded by a segment of plasma membrane internalized with the formation of the vacuole, these workers felt that the only explanation for their results was that there was a rapid change in the permeability of the membrane of the pinocytosis vacuole after it was incorporated into the cytoplasm. It is more probable that the membrane has a low resistance prior to its inclusion with the vacuole. In the experiment cited above, glucose could enter the amoeba cytoplasm by two paths: through the surface membrane and through the vacuole membrane.

The induction of a permeability change by stimulators of pinocytosis increases the flux of water, electrolytes, glucose, and molecules perhaps as large as ribonuclease (*14*) across the plasma membrane of the amoeba. The possible relationship of the induction of a permeability change by proteins in amoebae to the action of hormones such as insulin and antidiuretic hormones is discussed elsewhere (*15*). The similarity of pinocytosis to phagocytosis has been noted (*16, 17*). The consequences of the surface attachment in phagocytosis leads to the same sequence of steps in uptake as that proposed for pinocytosis. In addition general changes in membrane permeability exist (*16*).

It is very provocative to treat the permeability changes induced in different cells by a variety of methods as having a general effect on solute penetration (*15*) rather than an effect on a single molecular species. A locus for change in membrane permeability seems indicated in amoebae by the similar effects on the membrane of two different classes of inducers of pinocytosis, the electrolytes and the proteins, and the all-important role of calcium. Even though these conclusions suggest that the membrane changes structure with a general increase or decrease in permeability, there is reason to believe that the relative permeability in lipids or water, the charge, and the size still regulate the relative rates of solute penetration. The change in permeability in the amoeba plasma membrane upon the induction of pinocytosis primarily involves a visible thickening of the lipid lamella evidently by reversible hydration of this element. Protein stabilization of the lipid lamella (*4*) must be contrasted with the capacity of some proteins to induce pinocytosis, apparently by the same mechanism as monovalent cations do. Stabilization of the amoeba membrane by calcium is a function of the relative concentration of monovalent cations, or appropriately charged proteins, and time. The methods and the results of this study may offer a new approach to the analysis of plasma membranes in general.

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Neoplastic Transformation of Rat Thymic Cells Induced in vitro by Gross Leukemia Virus

Abstract. *Cultures of embryonal rat thymus infected initially with Gross leukemia virus have, at the present time, abundantly replicated infectious virus particles for 20 months. Cells from these cultures, after 3 months in vitro, displayed morphological changes and induced formation of tumors upon isotransplantation. The tumors were serially transplantable, and subsequent transplants continue to carry the initial Gross leukemia virus.*

Gross leukemia virus (GLV) is the inducive agent of spontaneous leukemia in AkR mice (*1*) and has been adapted to produce a similar disease in the rat (*2*). It is, therefore, of interest to develop an in vitro system for studying both replication of this virus and virus-cell interrelationship. Moreover, tissue cultures in which GLV continuously replicates within supporting cells would provide a constant and reproducible source for virus.

Long-term replication of this virus in vitro has been achieved in cultures of both normal and leukemic rat thymus. In work reported previously (*3, 4*), cultures of rat virus–induced thymomas were growing for 4 years, continuously replicating the initial GLV. In another system, normal rat thymic cultures, infected once at the beginning with GLV, have also been replicating the virus for long periods of time (20 months at present) (*4*). In both cases production of infectious virus particles has been demonstrated by means of electron microscopy and bioassays in susceptible animals.

Preliminary evidence indicates that cultures of thymic origin support and replicate GLV better than cultures of kidney and spleen (*4*). This report will deal with a particular event observed in these cultures, namely, that GLV is capable of inducing malignant transformation in long-term cultures of normal rat thymus.

Thymuses of normal W/Fu rat embryos were collected, minced, and suspended in Puck's medium. Gross leukemia virus from stock cell-free filtrates was added to the suspended cells;