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Membrane Fatty Acid Replacements and Their Effect on Growth and Lectin-Induced Agglutinability

(agglutination/essential fatty acids)

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ABSTRACT **The growth of 3T3 and SV101 3T3 cells in a lipid-depleted medium is enhanced by the addition of biotin or some fatty acids. The extent of enhancement depends on the fatty acid(s) supplied. The presence of linoleate is unique, since it induces a morphological alteration in 3T3 cells resulting in a cell similar to an SV101-transformed 3T3 cell. Analyses of the fatty acids from the membrane phosphatides show that the exogenously supplied fatty acids are incorporated and alter the fatty acid composition. This is most clearly evident with heptadecanoate-grown cells, in which this fatty acid and its derivatives comprise over 45% of the fatty acids in the phospholipids.**

The fatty acid replacements have a striking effect on the temperature dependence of agglutination by wheat germ agglutinin and concanavalin A, implying that fluidity is involved in agglutination. These temperature dependencies and the effect of fatty acid replacements on them were different for the two lectins, but similar for both transformed and untransformed cells. These observations are interpreted as suggesting that the lipid phase is heterogeneous, and that transformed and untransformed cell membranes have regions of similar fluidity.

Plant lectins are useful probes for detecting alterations in membrane structure involving carbohydrate-containing molecules on the cell surface (1). In spite of the utility of these molecules, relatively little is known about the mechanism of lectin-induced changes in cell behavior. A recurrent theme in these studies is a requirement for a fluid membrane (2–5).

The work of Fox and McConnell (6), Overath and Träuble (7), and their collaborators provides criteria for implicating fluidity in a membrane-related process. These workers correlated the temperature dependence of transport with the physical state of the lipid phase for cells possessing altered fatty acyl compositions. The result of these experiments was the demonstration that a fluid lipid phase is required for transport. We consider such experiments a prototype for implicating membrane fluidity in a physiological process.

The availability of fatty acid auxotrophs of *Escherichia coli*, first isolated and characterized by Vagelos and coworkers (8), was essential for these studies. Techniques for altering the lipid composition of animal cells, therefore, would enable one to study the role of the lipid phase in agglutinin-mediated, as well as in other, cellular phenomena. Previous studies by Bailey (9) and others (10–12) have shown that mammalian cells in culture can take up and incorporate either those lipids present in the serum or those supplied exogenously. These observations have been extended recently by Wisniesky *et al.* (13), who have described a method for altering the fatty acyl composition of cells grown in serum-free medium. Most cells grown in culture, however, require serum from which the cells derive most of their lipid. Therefore, the use of a lipid-depleted serum that can still support growth along with an exogenous supply of selected lipids, particularly those that are not favorable substrates for metabolic conversion once incorporated, provides a suitable method for altering membrane lipid composition and for assessing the role of fluidity in membrane processes.

We present our initial observations of the growth of cells under such conditions, and the effect on agglutination by concanavalin A (Con A) and wheat germ agglutinin (WGA): we discuss these results in the context of a heterogeneous lipid phase.

Abbreviations: Con A, concanavalin A; WGA, wheat germ agglutinin.

MATERIALS AND METHODS

Preparation of Lipid-Depleted Serum was based on the method of Scanu and Edelstein (14) for delipidating serum lipoproteins. In brief, 55 ml of serum was added to a chilled (−25° to −50°) mixture of ethanol (900 ml), diethyl ether (300 ml), and distilled water (7 ml) and allowed to extract with stirring for 2 hr at −25°. The suspension was centrifuged and reextracted in ethanol–ether–water (900:300:62). The mixture was then centrifuged and the pellets washed by suspension in chilled ether before overnight extraction at −25° in about 1.0 liter of ether. The suspension was then centrifuged, and the pellets were washed twice by suspension in chilled ether. The pellets were dried under vacuum, suspended at a concentration of 80 mg/ml in saline (0.9% NaCl), and centrifuged to remove any undissolved material. The yield was virtually quantitative.

The extracted serum contained less than 0.4% of the original (chloroform–methanol-extractable) lipid phosphorus. The total fatty acid contaminants (as determined by gas chromatography, with an odd-chain fatty acid standard) were about 10% of the exogenous fatty acid added to the growth medium.

Growth Curves. Swiss 3T3 or SV101 3T3 cells obtained from H. Green were grown as described (15) in 60-mm Falcon tissue culture dishes containing 6.0 ml of Dulbecco's Modified Eagle's Medium supplemented with either 15% (v/v) lipid-depleted or regular serum. The following additions (per 6.0 ml of medium) were made from sterile stock solutions: fatty acids (4 mg/ml or 4 μl/ml in ethanol), 15 μl; biotin (0.5 mg/

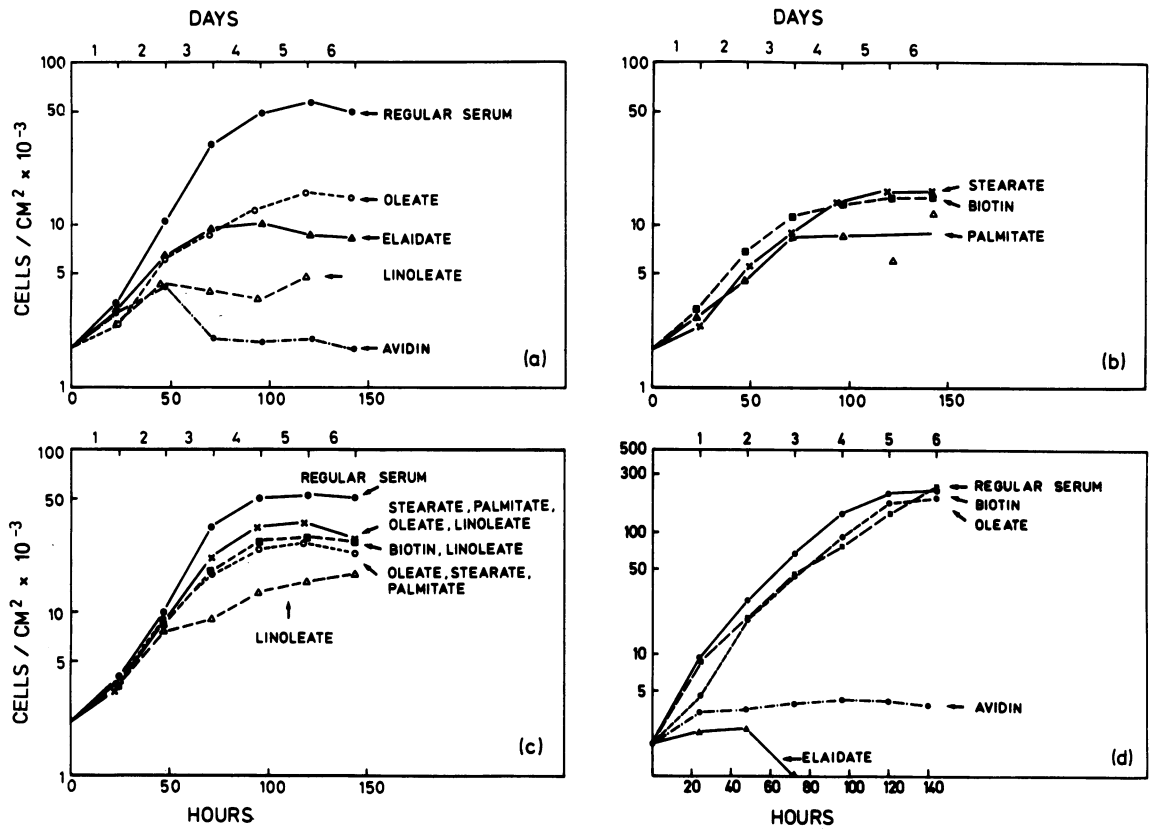

FIG. 1. The growth of 3T3 cells (*a–c*) and SV101 3T3 cells (*d*) in Dulbecco's modified Eagle's medium containing 15% regular calf serum or 15% lipid-depleted calf serum + avidin + the indicated fatty acid. A value of 28.3 cm^2/60-mm plate was used to calculate the cell density.

ml in ethanol–water 1:1), 15 μl; and avidin (12 units/ml in saline), 60 μl. The cells were seeded at either 4.5×10^4 or 6.0×10^4 cells per plate, and in most experiments, the fatty acids were added 1–2 hr after seeding. The cells were checked routinely by [^{3}H]thymidine incorporation for infection with pleuropneumonia-like organisms (PPLO) (16), and were found to be free of contamination.

Agglutinations by the lectins WGA and Con A were performed on cells grown in the presence of the fatty acids under investigation for 3–4 days for 3T3 cells and for 2 days for SV101 3T3 cells. The details of the agglutination assay have been described (17). The temperature was regulated by Tempbloks (Lab Line Instruments, Inc.) immersed in a water bath at the desired temperature.

Fatty Acid Analyses. Cells grown under the conditions described for the agglutinations were washed three times with phosphate-buffered saline (pH 7.4), and were extracted by the method of Bligh and Dyer (18). The neutral lipids were removed by silica gel thin-layer chromatography, with a solvent of petroleum ether–diethyl ether–acetic acid 80:20:1 (18). The resulting mixture was transmethylated with methanolic boron trifluoride, and the fatty acids were analyzed by gas chromatography at 180° with 6-foot columns of 10% EGSS-X and 3% SE-30 on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc.), with a helium flow of 35 ml/min. The relative concentrations of the major fatty acids were estimated by the triangle approximation.

Assays. Protein was determined by the method of Lowry *et al.* (20), and lipid phosphorus by the method of Ames and Dubin (21). Biotin and avidin were assayed by the method of Green (22).

Fatty acids were purchased from Applied Science Laboratories, avidin from Sigma, biotin from Merck, medium and serum from Gibco, and Con A from Miles. WGA was prepared from wheat germ by the method of Bloch and Burger (23).

RESULTS

Effects of Fatty Acid Replacements on Growth. The problems posed by large amounts of lipid in the serum required for the growth of most tissue culture cells are avoided, in large part, by preparation of a lipid-depleted serum. The growth of 3T3 cells in medium containing such a serum is shown in Fig. 1. In the absence of any additions (other than the avidin used to complex traces of biotin in the medium), the cells attach to the substratum, but grow very poorly. This result depends on the density at which the cells are seeded. In contrast, cells grown in a lipid-depleted medium containing biotin, a vitamin required for fatty acid synthesis, grow to an essentially confluent monolayer (Fig. 1). Although growth is stimulated substantially in the presence of biotin compared to that in lipid-depleted medium alone, both the doubling time and the saturation density of the biotin-supplemented cells are lower than those of cells grown in regular serum. This result is not altered by the addition of 1 μg/ml of cholesterol to lipid-depleted medium. The reduced saturation density of cells grown in biotin-supplemented lipid-depleted medium is consistent with the larger and flatter morphology of the cells, as seen in phase contrast microscopy and in cell sizing.

In addition to biotin, certain fatty acids also support appreciable cell growth (Fig. 1). Of the fatty acids studied, those that support growth include palmitate, palmitoleate, stear-

TABLE 1. *Fatty acyl composition of the phosphatides from 3T3 and SV101 3T3 cells grown in regular medium and in supplemented lipid-depleted medium*

Medium supplement	Fatty acid (%) 16:0	16:1	18:0	18:1	18:2	20:4	17:0	17:1	Other
				3T3					
Regular serum	12.0	5.6	24.5	27.2	12.6	11.3			6.7
LDS + biotin	18.8	8.7	17.2	36.5	7.4	2.8			8.6
LDS + palmitate (16:0)	28.2	12.9	14.7	26.7	7.1	5.6			4.8
LDS + oleate (18:1)	7.4	3.6	16.7	55.3	2.0	3.4			11.6
LDS + elaidate (18:1, *trans*)	7.7	1.0	16.8	63.6*	10.8	N.D.			N.D.
LDS + heptadecanoate (17:0)	7.3	2.1	8.7	14.0	5.8	4.1	21.9	24.2	11.8
				SV101 3T3					
Regular serum	13.8	3.9	24.7	29.9	4.6	5.3			17.0
LDS + biotin	27.0	4.0	17.0	51.5	4.5	N.D.			N.D.
LDS + oleate (18:1)	16.6	6.6	10.4	57.0	4.2	5.3			N.D.

LDS, lipid-depleted serum; N.D., not determined.

3T3 and SV101 3T3 cells were grown for 4 days and 2 days, respectively, as described in *Materials and Methods*. Fatty acids longer than 20 carbons were not determined.

* This value represents the total of oleate and elaidate.

ate, oleate, elaidate, linoleate, and heptadecanoate. In most cases, the cells are granulated for the first 2 days and toward the end of the growth period appear to be larger and flatter than cells grown in regular serum. Such cells are viable, but attempts to establish long-term cultures of palmitate- and elaidate-grown cells were unsuccessful. By the second passage, there was little net growth, and many cells appeared to be very flat and large. A change of medium on day four had little effect. These results are in contrast to those with cells grown in medium containing either biotin or both biotin and linoleate, in which the cells could be carried for at least a month. In general, the growth of cells in media containing mixtures of fatty acids exceeds that obtained in media containing any single fatty acid. A mixture of fatty acids similar to those present in serum supports growth at a rate and saturation density closest to that of cells grown in regular serum (Fig. 1). Biotin does not increase the saturation density observed with such fatty acid mixtures.

The result of any particular experiment can depend on factors such as the particular cell clone used, the density of seeding, the plating efficiency, and the presence of pleuropneumonia-like organisms (PPLO).

Myristate, nonadecanoate, and arachidonate are among the fatty acids that do not support the growth of 3T3 cells under these conditions. Cells grown in lipid-depleted medium containing linoleate alone are unique. About 36 hr after they are plated, the cells undergo a transient morphological alteration that persists for about 48 hr, after which time the original morphology returns. When viewed in phase contrast microscopy, the altered cells appear to be small and contracted and to have few ruffled edges, acquiring an appearance not unlike their transformed counterparts. Cells grown in the presence of linoleate (or arachidonate) together with biotin, however, do not show such a morphological alteration, and reach a saturation density that is higher than that of cells grown either in biotin or in the fatty acid supplement alone.

Evidence for Altered Fatty Acid Composition. The data presented in Table 1 demonstrate that the fatty acids present in the growth medium are incorporated and alter the fatty acyl composition of the membrane phosphatides. A comparison of the gas chromatograms of the fatty acids of the membrane phosphatides from cells grown for about three generations in lipid-depleted medium supplemented with biotin, palmitate, oleate, or heptadecanoate shows that the fatty acid provided in the medium is enriched in the membrane phosphatides. These data also suggest that subsequent metabolism of the incorporated fatty acid depends upon the nature of the fatty acid provided. The clearest indication of the degree of incorporation of exogenous fatty acids is seen with heptadecanoate-grown cells, where this fatty acid and its derivatives comprise over 45% of the membrane fatty acids.

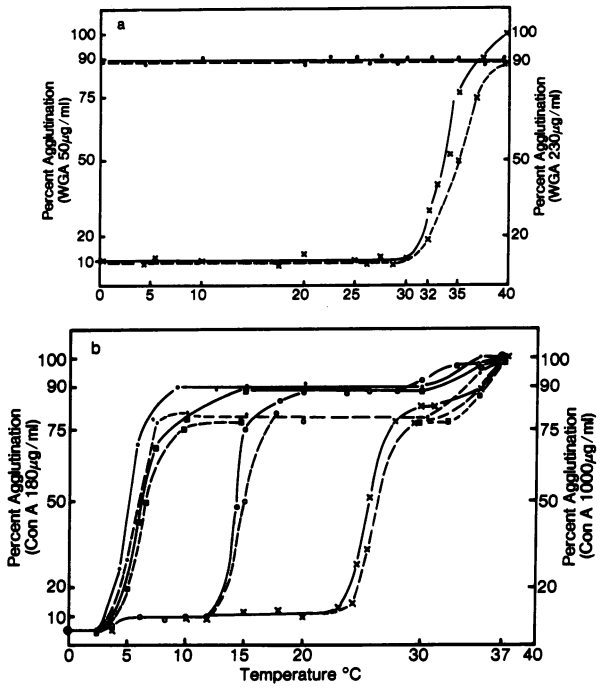

FIG. 2. Effect of temperature on agglutination. (*a*) WGA agglutination: 3T3 cells or SV101 3T3 cells were incubated at the indicated temperature for 4 min with 230 or 50 μg/ml of WGA, respectively. At each point, a duplicate sample was tested at 20°. ●, Agglutination of cells grown in regular serum; ×, agglutination of cells grown in lipid-depleted medium + avidin + elaidate. *Dashed lines*, 3T3 cells; *solid lines*, SV101 3T3 cells. (*b*) Con A agglutination: 3T3 or SV101 3T3 cells were incubated at the indicated temperature for 15 min with 1000 or 180 μg/ml of Con A, respectively. At each point, duplicate samples were tested at 0°, 20°, and 37°. ○, Agglutination of cells grown in regular serum; ●, agglutination of cells grown in lipid-depleted medium + avidin + oleate; ■, agglutination of cells grown in lipid-depleted medium + avidin + biotin; ×, agglutination of cells grown in lipid-depleted medium + avidin + elaidate. *Dashed lines*, 3T3 cells; *solid lines*, SV101 3T3 cells.

Evidence for Alterations in Agglutinability. The agglutination of SV101-transformed 3T3 cells by WGA and by Con A at several temperatures is given in Fig. 2. The agglutination by WGA is largely temperature-independent, in agreement with previous work from this and other laboratories (1). The agglutination of cells grown in biotin or in oleate is similar to that of cells grown in regular serum. Cells grown in the presence of elaidate, however, show a marked temperature dependence, with the agglutination greatly reduced or absent below 33–35°.

The agglutination by Con A of SV101-transformed cells grown in medium containing regular serum is also shown in Fig. 2*b*. In agreement with previous work, and in contrast to the results with WGA, there is a marked temperature dependence with a reduced agglutination below 14–18°. With oleate- or biotin-grown cells, however, the high level of agglutination extends down to 6–8°, whereas that with elaidate-grown cells already decreases at 26–28°.

Although the untransformed 3T3 cells are considerably less agglutinable than are their transformed counterparts, agglutination can be detected with very high levels of agglutinin (Fig. 2). The effects of both temperature and of fatty acid replacements on the agglutination of these cells are effectively the same as those described for transformed SV101 3T3 cells. We emphasize, however, that the membrane fatty acyl replacements do not change the lectin concentration required for half-maximal agglutination.

DISCUSSION

Cell growth in a medium lacking biotin and containing lipid-depleted serum is greatly impaired when compared to cells grown under normal conditions. The growth stimulation, achieved by addition of exogenous fatty acids, phospholipids, and/or biotin suggests strongly that the absence of lipid synthesis or of a suitable exogenous lipid supply is a major factor limiting growth. This conclusion is supported by the fatty acyl profiles of these cells: the fatty acyl composition of the membrane phosphatides reflects the lipid supplemented in the medium. However, the supplemented fatty acid is never exclusively that found in the membrane phosphatides. Lipid pools, contaminants in the medium, incomplete inhibition of *de novo* synthesis, subsequent metabolism of the incorporated fatty acids, and cellular controls on the extent of fatty acid alteration might contribute to this effect. These results are in agreement with those of other workers (9), and provide an experimental procedure for exerting partial control over the membrane fatty acid composition and for studying the effects of that composition on cellular physiology.

Experiments on the growth of cells in lipid-depleted medium supplemented with fatty acids demonstrate the differential ability of fatty acids and their mixtures to support growth. The saturation density and growth rate of cells grown in medium containing either single fatty acids or mixtures thereof depends on the fatty acid(s) supplemented, and in some cases, on the density of seeding. The fastest growth rate and the highest saturation density (>70 of the control) is observed with a mixture of stearate, palmitate, oleate, and linoleate, a collection of fatty acids found in normal serum and in the membranes of animal cells. The growth of cells in medium supplemented only with biotin is significantly lower, reflecting, perhaps, some dependence on exogenous lipid for optimal growth. No explanation for the different growth rates and saturation densities of cells grown with different supplemented fatty acids can be presented at this time. With palmitate- or elaidate-grown cells, however, the minimal effect either of changing the medium or of replating the cells suggests that the factor limiting cell growth is the fatty acid composition of these membranes.

The effects of linoleate (or arachidonate) are unique among the fatty acids tested. Cells grown in a medium containing only linoleate (and not biotin) undergo a morphological change, with the resulting morphology not unlike that of their transformed counterparts. Arachidonate induces a morphological alteration that is similar to, but more pronounced than, that with linoleate. These molecules are precursors of prostaglandins. Since prostaglandin levels rise on transformation and during mitosis (24, ‡), this suggests a possible mechanism for the morphological alteration.

The use of different fatty acids as growth supplements has a striking effect on the agglutinability by WGA and Con A. In comparison to cells grown in regular serum, growth in elaidate increases the thermal transition temperature§ for Con A agglutination, and possibly creates one for that with WGA, while growth in oleate lowers the thermal transition temperature for Con A agglutination. One can imagine several mechanisms by which an altered membrane lipid composition could change agglutinability. Those mechanisms involving changes in membrane fluidity¶ provide a precedented and more simple explanation for the effects of selective lipid enrichments on agglutination.‖ In this interpretation, the Con A receptors** of cells grown in regular serum are in a more fluid environment at temperatures above 14–18° than below; in elaidate-grown cells, the environment is fluid at 24–27° and above; and in oleate-grown cells it is fluid down to 7° (or below). The agglutination by WGA of cells grown in regular serum, oleate, or biotin is consistent with a fluid environment below zero degrees, whereas in elaidate-grown cells, the environment is fluid at 33–35°.

The direction of these changes in agglutination is consistent with the alterations in transport transitions seen in analogous experiments with bacteria (6, 7), as well as in physical studies on model systems (25–27). That is, higher proportions of shorter and *cis*-unsaturated fatty acids lower the transition temperature for phosphatide melting.

A particularly interesting result from these experiments is that the temperature transitions for the agglutination by Con A and WGA are different and that the effect of fatty acid replacements on these transitions is not the same. One likely possibility is that the two lectin receptors are situated in different lipid environments possessing different transition temperatures.†† A similar observation and conclusion have

‡ S. Hammarström and M. M. Burger, unpublished observation.

§ We use empirically the expression "transition temperature" to refer to the temperature region in which the agglutination is altered.

¶ We use the expression "fluid" to refer to the quantity and physical state of the melted regions of the lipid phase, and reserve the term "mobile" for describing the rotational, lateral, and transverse motions of single membrane components.

‖ In the event that WGA and/or Con A receptors are glycolipids, the fatty acid replacements could affect the receptors as well as the surrounding lipid environment.

** These experiments pertain directly to the lectin–receptor complexes, and only implicitly to the receptors themselves. For convenience, we define the lectin receptor as those molecules involved in the agglutination reaction.

†† More general models of the phase equilibria expected from these multicomponent membranes will have a temperature region (that might even extend through the growth temperature) in which both solid and liquid phases coexist (25, 27). If, by virtue of structural differences, the WGA and Con A receptors associate differently between the solid and liquid phases, one could account for the two transitions. This would be the case even if the receptors were in similar lipid environments at the growth temperature. When one considers, however, the sharpness of the transitions and the differential magnitude of the shift in transition temperature of agglutination resulting from the fatty acid replacements, it is most likely that the strong selectivity of the receptors for two different phases at the lower temperature is reflected in their local lipid environments at the growth temperature. Of course, if the addition of lectin were to differentially alter the respective receptors, the conclusion about the environments of the native receptors might be weakened. A conclusion regarding the nature of the lipid heterogeneity is premature. It could be local or extensive, but in any event, if one or both of the lectin receptors span the membrane, one must consider both bilayer surfaces (30, 31).

been discussed by Mavis and Vagelos (28) for membranous enzymes in *E. coli*. These interpretations are supported furthur by magnetic resonance studies of sarcoplasmic reticular (29) and model membranes (27, 30). These studies also suggest the presence of a heterogeneous lipid phase.

In contrast to the differences in agglutinability between WGA and Con A, the transition temperatures and the effects of fatty acyl replacements on these transition temperatures are the same for both transformed and untransformed cells. This finding implies that the two cell types have membrane regions with similar fluidity. This observation bears on an explanation for the differences in the concentration of lectin needed to agglutinate transformed compared with untransformed cells. Among the many possibilities, the increased agglutinability of transformed cells can be explained by an increased fluidity or mobility of membrane components. However, even in the most fluid membrane of untransformed cells, achieved with oleate enrichment, the level of agglutinability is not altered. For a fluidity involvement, therefore, there would have to exist a region of the membrane containing a majority of the lectin receptors that is not altered by our fatty acyl replacements. Alternatively, mobility differences could arise from one of several possibilities, e.g., protein aggregation, association with a solid phase (if one exists), interaction with the cytoskeleton, etc. However, in considering such possibilities, one should be aware of the effect of particle size on mobility. The rotational diffusion constant of a molecule or a complex of molecules, in simple approximation, is proportional to the molecular weight of the molecular complex, whereas the translational diffusion constant depends on its cube root. Thus, if any significant change in the lateral diffusion of the receptors in transformed compared with untransformed cells were responsible for the difference in agglutinability, the difference in the sizes of the complex of molecules containing the receptor in the two cell types would necessarily have to be quite large, unless it were anchored to a stationary element of the cell, e.g., the cytoskeleton.

Preliminary studies using a radiolabeled Con A binding assay (32, 33) show that at 0° the number of receptors in oleate- or elaidate-grown cells is the same as that of cells grown in regular serum. An increase in binding is observed at the agglutination transition temperature, however, in all three cases. These data suggest that the physical state of the membrane lipids control the availability or conformation of lectin receptors (33, 5).

In light of our experiments, therefore, one should consider, in addition to the effect of fluidity, other factors that can control membrane mobility as well as alternative agglutination mechanisms discussed elsewhere (1).

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