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Response of endocytosis to altered fatty acyl composition of macrophage phospholipids

(membrane fluidity/pinocytosis/phagocytosis/activation energy)

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ABSTRACT **Mouse peritoneal macrophages incubated in serumless medium containing a 19:0 or *trans*-18:1 fatty acid complexed to bovine serum albumin incorporate the exogenous fatty acid supplement into cellular phospholipids. Within 8 hr, 25% of the total phospholipid fatty acids are derived from the supplement, with cell viability remaining >95%. The incorporation of either of these supplements increases the saturated/unsaturated fatty acid ratio in the phospholipids 2-fold over that of cells cultured in serum and effects striking changes in endocytic activities. The levels of both fluid-phase pinocytosis and receptor-mediated phagocytosis are decreased at all temperatures examined between 15° and 37°. The increased degree of saturation of cell phospholipids correlates with decreased endocytic rates for both processes and with increased activation energies (E_{act}) for phagocytosis. The E_{act} values for phagocytosis, which range from 54 to 90 kcal/mol, depend on the supplementation conditions used. Although the levels of pinocytosis are depressed, the E_{act} values for pinocytosis (17–25 kcal/mol) are not strikingly affected by saturated fatty acid enrichment. These observations suggest that the degree of lipid fluidity of macrophage membranes influences both phagocytosis and pinocytosis in macrophages.**

The mouse peritoneal macrophage serves as a useful tool for examining many aspects of plasma membrane physiology (1). Homogeneous, nonreplicating populations of resident cells can be easily cultured in simple media and express high levels of endocytosis and secretory activity (2). During endocytosis, the plasma membrane is interiorized to form a vesicle that then migrates into the cytoplasm and fuses with preexisting primary and secondary lysosomes. This process initiates the digestion of the vesicle content (3), whereas the membrane is largely recycled back to the cell surface (4). Prior studies have described sensitive, quantitative techniques for examining fluid-phase pinocytosis (the uptake of liquid droplets from the external environment) and receptor-mediated phagocytosis (the recognition and internalization of specific extracellular particles) (5, 6).

Under steady-state conditions in 20% fetal calf serum, fluid-phase pinocytosis effects the interiorization of an amount of plasma membrane equivalent to the entire surface area of the macrophage within 33 min. This occurs by means of vesicles whose average diameter is 0.2 μm and whose average surface area is 0.2 μm^2 (4).

The rate of membrane interiorization during receptor-mediated phagocytosis is dependent upon the size and number of particles ingested per unit time. However, the uptake of one sheep erythrocyte with a diameter of 5.2 μm and a surface area of 67 μm^2 (7) results in the internalization of approximately 7% of the macrophage surface area.

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Although there is considerable information concerning the role of membrane receptors in endocytosis (8), little information exists on the importance of membrane composition. In this article we focus on one component of the membrane—i.e., lipids. Using enrichment techniques, we found that raising the content of saturated fatty acids in cellular phospholipids is associated with a striking reduction in the rates of both fluid-phase pinocytosis and receptor-mediated phagocytosis.

EXPERIMENTAL PROCEDURES

Cell Culture. Primary cultures of peritoneal macrophages were prepared from the resident cells of female NCS mice weighing 20–30 g (9). Cells were plated on culture dishes (3×10^7 per 60-mm dish; 10^7 per 35-mm dish) for lipid analyses and pinocytosis assays or on 12-mm^2 glass cover slips (10^6 cells) for phagocytosis assays. Cultures were incubated for 16 hr in Eagle's minimal essential medium supplemented with 20% fetal calf serum and then divided into three groups. One set was rinsed, reincubated for 8 hr in Neuman–Tytell serumless medium prepared without biotin or methyloleate (GIBCO serumless medium), rinsed again, and cultivated for an additional 8 hr in serumless medium supplemented with fatty acid–bovine serum albumin complex. Final concentrations were: fatty acid, 10 μg/ml; albumin, 2.5 mg/ml. Serum-free control cultures were treated similarly but incubated without fatty acid complex in the final 8-hr period. Serum control cultures, the third group, were rinsed with Eagle's minimal essential medium and reincubated each time in 20% fetal calf serum.

Fatty Acid Complexes. *Trans*-18:1 (Mann; Analabs) or 19:0 (Sigma) fatty acid was complexed with fatty acid-poor bovine serum albumin (Miles) in 1:1 molar ratios. An aliquot of a $CHCl_3$ solution containing 500 μg of fatty acid was dried in an acid-washed screw-capped tube and heated in N_2 atmosphere with a slight excess of 0.015 M KOH. A solution of defatted bovine serum albumin (125 mg) prepared in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline was added to the K^+ salt of the fatty acid, and the tube was flushed with N_2, capped, and placed on a shaker at 37° for 24–48 hr. After the salt was entirely dissolved, the pH of the solution was adjusted to 7.2 with KOH and the volume was brought to 5.0 ml with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. Aliquots of the fatty acid–albumin stock solutions were diluted with 10 volumes of serumless medium and membrane-filtered immediately before being added to cultures.

Labeling of Macrophage Lipids. Cultures were labeled with [^{14}C]acetate 1 hr prior to harvest by adding 12 μCi of sterile [*1*-^{14}C]acetate (60.1 μCi/mol) to each dish containing 2 ml of culture medium. Separate cultures were labeled and harvested

Abbreviations: HRP, horseradish peroxidase; E(IgG), antibody-coated erythrocytes; E_{act}, activation energy.

at three different stages during the final 8-hr cultivation period. Cultures to be labeled with [*1*-^{14}C]*trans*-18:1 fatty acid (55.7 μCi/μmol) were incubated continuously with fatty acid–albumin complex, 1 μCi/ml, for 1–24 hr in medium containing a total of 10 μg of *trans*-18:1 fatty acid per ml. The radioactivity in phospholipids and in neutral lipids was determined for all labeled samples.

Lipid Analysis. At the end of the cultivation period, cells to be analyzed for fatty acid content were rinsed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and once with isotonic saline and then scraped into isotonic saline for extraction at 0° by the method of Bligh and Dyer (10), as modified by Ames (11), using redistilled solvents. Neutral and phospholipids were separated on small columns of Supelcosil (Supelco, Inc.). The fatty acyl moieties of each fraction were transesterified by heating samples for 16 hr at 80° in 6% HCl in CH_3OH under N_2. The methyl esters were extracted into hexane, purified by thin-layer chromatography on silica gel G by development in hexane/ether/acetic acid, 90:10:1 (vol/vol), and analyzed with a Varian model 2740 gas chromatograph. Samples containing odd-numbered long-chain fatty acid methyl esters were chromatographed on a 10 ft × 1/8 in. (305 cm × 0.32 cm) column of 10% SP-2330 on Chromosorb W/AW (Supelco, Inc.) at 180° with carrier gas flow at 40 ml/min which resolved odd-chain saturates from even-chain unsaturates. Samples containing methyl esters of *trans*-18:1 fatty acid were chromatographed on a 20 ft × 1/8 in. (610 cm × 0.32 cm) column of 15% OV-275 on Chromosorb P 100/120 AW/DMCS (Supelco, Inc.) at 220° with carrier gas flow at 10 ml/min, which effected base-line separation of *cis* and *trans* isomers of 18:1 fatty acid. Other samples were analyzed on either or both columns.

Pinocytosis Assay. Macrophage cultures (35-mm dishes) were rinsed three times with serumless medium and placed on ice until assayed. Separate cultures were incubated for 30 min in 2.0 ml of serumless medium containing horseradish peroxidase (HRP) (type II, Sigma), 1 mg/ml, at temperatures between 15° and 37° in a small water-jacketed chamber gassed with 5% CO_2/95% air. Temperature was controlled by using a constant-temperature circulating water bath; maximum temperature fluctuation within the chamber during the assay period was ±0.1°. The uptake of HRP by macrophages was linear for at least 2 hr at 37° with an enzyme concentration of 1 mg/ml in the culture medium. After exposure to HRP, monolayers of macrophages were rinsed thoroughly five times with phosphate-buffered saline and twice with 0.9% NaCl and then solubilized in 0.05% Triton X-100. Control cultures not exposed to HRP during incubation were processed similarly to measure endogenous peroxidase activity. Control dishes containing no cells but incubated with HRP were routinely processed to measure the amount of HRP bound to the plastic dish surface during the assay period. Aliquots of the detergent-solubilized cells were assayed for peroxidase activity with *o*-dianisidine (Sigma) according to the method of Steinman *et al.* (5).

Preparation of Antibody-Coated Erythrocytes. Sheep blood was drawn and diluted 1:2 with Alsever's anticoagulant at least 5 days prior to preparation of antibody-coated erythrocytes [E(IgG)]. Erythrocytes were washed three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and coated with rabbit anti-sheep IgG (Cordis Labs) as described (12).

Phagocytosis Assay. Culture dishes containing monolayers of macrophages on cover slips were rinsed three times with serumless medium and placed on ice. Fifteen to 20 min later, a 0.25% suspension of E(IgG) (or 5 × 10^7 erythrocytes) in serumless medium was added to each dish containing duplicate cover slips. The particles were allowed to attach to the macrophages for 1 hr at 0°, during which period no interiorization of the particles occurred. The dishes were then thoroughly rinsed with serumless medium to remove all nonattached erythrocytes. Cultures were incubated for 30 min at temperatures between 5° and 37° in serumless medium in the same apparatus described under *Pinocytosis Assay*. The interiorization of attached particles by the macrophages was halted at the end of the incubation period by replacing the incubation medium in the dishes with a cold phosphate-buffered saline/H_2O mixture, 1:4 (vol/vol), which lysed the extracellular erythrocytes without disturbing the integrity of the macrophages or the internalized erythrocytes. Within 30 sec, the lysis mixture was removed and the monolayers were fixed immediately in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4). The number of erythrocytes ingested per 100 macrophages was counted in random fields by viewing mounted cover slips at ×1250 magnification. At temperatures between 34° and 37°, the larger number of erythrocytes ingested sometimes rendered microscopic quantitation difficult so that only data at 32° or lower are presented. The phagocytic rates were linear for 30 min and longer in the various cultures at these temperatures.

RESULTS

Culture Conditions and Cell Viability. Initially, a number of supplementation methods were used for modifying the fatty acid composition of the macrophages. The method of choice for these experiments, which allowed excellent cell viability and normal architecture, included the use of Neuman–Tytell serumless medium with or without a fatty acid-albumin complex. Cells cultured under these conditions with either 19:0 or *trans*-18:1 fatty acid at 10 μg/ml for 8 hr consistently maintained a viability of >95% and demonstrated significant incorporation of the fatty acids into phospholipids. The use of serum that was extracted with organic solvents or treated with charcoal was less satisfactory.

Incorporation of [^{14}C]Acetate and Long-Chain Fatty Acids into Cellular Lipids. The relative rates of *de novo* fatty acid synthesis, as compared to the incorporation of long-chain fatty acids, was examined under several conditions of culture. Cultures incubated in 20% fetal calf serum containing [*1*-^{14}C]-acetate (5.7 μCi/ml, 60.1 μCi/μmol) incorporated 0.06 nmol of [^{14}C]acetate/hr per mg of protein into total cell phospholipids. Serum-free cultures incorporated 6 times as much label (0.41 nmol/hr per mg of protein) into phospholipids whereas *trans*-18:1 fatty acid-enriched cultures incorporated 0.04 nmol/hr per mg of protein. The incorporation of [*1*-^{14}C] *trans*-18:1 fatty acid was more than 10 to 100-fold greater (8.8 nmol/hr per mg of protein with 1 μCi/ml in the culture medium) than the incorporation of [^{14}C]acetate into macrophage phospholipids. It seems, therefore, that *de novo* synthesis does not play an important role in modifying phosphatide composition under these conditions and in the absence of inhibitors of endogenous synthesis.

Fatty Acid Analyses. Fatty acid analyses for the phospholipids of peritoneal macrophages cultured in each of four media are presented in Table 1. The cultivation of macrophages in serumless medium increases the saturated/unsaturated fatty acid ratio of the phospholipids 26% over that of cells maintained in serum-containing medium. In the phospholipids of cells incubated with *trans*-18:1 fatty acid (mp 44.5°), 25.4% of the fatty acyl moieties were *trans*-18:1. The inclusion of *trans*-18:1 fatty acid with saturated species rather than *cis*-unsaturated species in the determination of the effective saturated/unsaturated fatty acid ratio yielded a value of 3.6 which is 1.9-fold

Table 1. Fatty acid composition of total cell phospholipids

Culture conditions*	Fatty acid composition, mol %										
	15:0	16:0	17:0	18:0	19:0	*trans*-18:1	18:1	18:2	20:4	Other	Ratio†
20% FCS	0.7	29.9	1.0	28.2			13.9	2.9	15.0	8.4	1.9
Serum-free	0.8	34.6	1.1	27.1			10.5	3.3	12.5	10.0	2.4
trans-18:1–albumin		24.5		22.2		25.4	9.8	2.9	9.5	5.8	3.6
19:0–albumin	1.3	25.3	7.5	18.3	20.7		8.3	3.1	7.3	8.2	3.9

Data presented are the means of analyses from two or three experiments.
* FCS, fetal calf serum; albumin, bovine serum albumin.
† Ratio = (% saturated fatty acids + % *trans*-18:1)/% *cis* unsaturated fatty acids.

higher than that for serum-cultured cells. In cultures supplemented with 19:0 fatty acid (mp 68.6°), 29.5% of the phospholipid fatty acids were odd-numbered long-chain saturated fatty acids. Desaturated metabolites of 19:0 fatty acid were not detectable. The phospholipid saturated/unsaturated fatty acid ratio in 19:0 fatty acid-supplemented cultures was 2-fold higher than in serum control cultures and 1.6 times higher than in serum-free control cultures. Comparable incorporation of the supplements into neutral lipid was observed.

Fluid-Phase Pinocytosis. The level of fluid-phase pinocytosis in macrophages after culture in the four media was determined by measuring the rate of uptake of the soluble macromolecule HRP. Significant amounts of HRP were interiorized within 30 min at 15° and warmer. Data obtained at 37° for each type of culture are presented in Table 2.

At all temperatures examined between 15° and 37°, the pinocytic rate was greatest in the cells cultured with serum. Macrophages cultivated in serumless medium pinocytized at a rate 70% that of the serum-cultured cells. Macrophages enriched with *trans*-18:1 fatty acid pinocytized at an even lower rate (50% of that of serum-cultured cells). Macrophages that had incorporated 19:0 fatty acid from the culture medium pinocytized at the lowest rate (40% of that of serum-cultured cells). Arrhenius plots of pinocytic activity at temperatures between 15° and 37° were linear within the temperature range examined (Fig. 1); the corresponding calculated activation energies were 17–25 kcal/mol (Table 2). The activation energy (E_{act}) for fibroblasts cultured in 5% fetal calf serum had previously been determined to be 18 kcal/mol (5).

Fc Receptor-Mediated Phagocytosis. The receptor-mediated phagocytic capacity of control cultures and fatty acid-supplemented cultures was determined with E(IgG). The opsonized erythrocytes were bound equally well at 0° by all cultures, indicating that the binding capacity of the Fc receptor is unaltered by fatty acid supplementation. The bound erythrocytes were not interiorized at 0° in any of the cultures. The lowest temperature at which internalized bound erythrocytes were detected was 17–20° in serum-cultured cells, 20°–21° in serum-free cultures, and 24°–25° in cells supplemented with 19:0 or *trans*-18:1 fatty acid. At all temperatures up to 37°, more particles were interiorized by serum-cultured cells than by cells in serum-free cultures or fatty acid-enriched cultures. These distinctions in the phagocytic capability of the various cultures reflect differences in both the relative percentage of macrophages per culture involved in ingesting erythrocytes and the average number of particles ingested per phagocytizing macrophage (Table 2). At 30°, cells cultured under serum-free conditions phagocytized at 90% of the rate demonstrated by serum-cultured cells, and *trans*-18:1 and 19:0 fatty acid-supplemented cells phagocytized at 50% and 30% of the rate of serum-cultured cells, respectively.

Table 2. Pinocytosis and phagocytosis

Culture conditions*	Pinocytosis		Phagocytosis	
	Pinocytic activity† at 37°	E_{act},‡ kcal/mol	Phagocytic activity§ at 30°	E_{act},‡ kcal/mol
20% FCS	1.01	25	142	54
Serum-free	0.69	25	128	65
trans-18:1–albumin	0.51	23	70	75
19:0–albumin	0.39	17	44	90

* Abbreviations as in Table 1.
† As ng of HRP pinocytized per μg of cell protein per 30 min; mean of three or four experiments.
‡ Calculated from slope of Arrhenius plot constructed by using linear regression analysis of normalized data.
§ As number of erythrocytes phagocytized per 100 macrophages per 30 min; mean of three to five experiments.

The Arrhenius plots of receptor-mediated phagocytosis are presented in Fig. 1. The E_{act} (Table 2) for receptor-mediated phagocytosis calculated for the serum-free cultures was 1.2 times that of the serum-cultured cells, and for the *trans*-18:1 and 19:0 fatty acid-supplemented cultures it was 1.4 and 1.7 times that of the serum-cultured cells, respectively. These values are 2–5 times greater than the corresponding E_{act} values of fluid-phase pinocytosis.

DISCUSSION

Although the major emphasis of the study was an examination of the role of fatty acid substitution on the process of endocytosis, it also represents a careful comparison of the temperature-dependence of fluid-phase pinocytosis and receptor-mediated phagocytosis. Both sets of data can be described by the Arrhenius equation yielding a straight line without deflections. In the case of pinocytosis, the calculated E_{act} values of 17–25 kcal/mol are similar to the value found by Steinman *et al.* (5) in the mouse L-cell fibroblast for the uptake of HRP and higher than the values found for cholesterol exchange in macrophages (12 kcal/mol) (13) and for the transport of water across the toad bladder (11.7 kcal/mol) (14).

In contrast, the E_{act} for receptor-mediated phagocytosis in serum cultures is 54 kcal/mol, considerably higher than E_{act} values ascribed to other biological phenomena. It should be pointed out, however, that "activation energy," as used to describe complex biological systems such as endocytosis, represents a composite energy barrier that may reflect various events. These could include the requirement for high-energy phosphate compounds, the activation of the contractile reticulum, membrane movements, and the fusion of membranes to form the endocytic vesicle. In any event, the two forms of endocytosis differ strikingly in this parameter and this may be related to particle size, the amount of membrane interiorized during each discrete endocytic event, the total movement of membrane, or

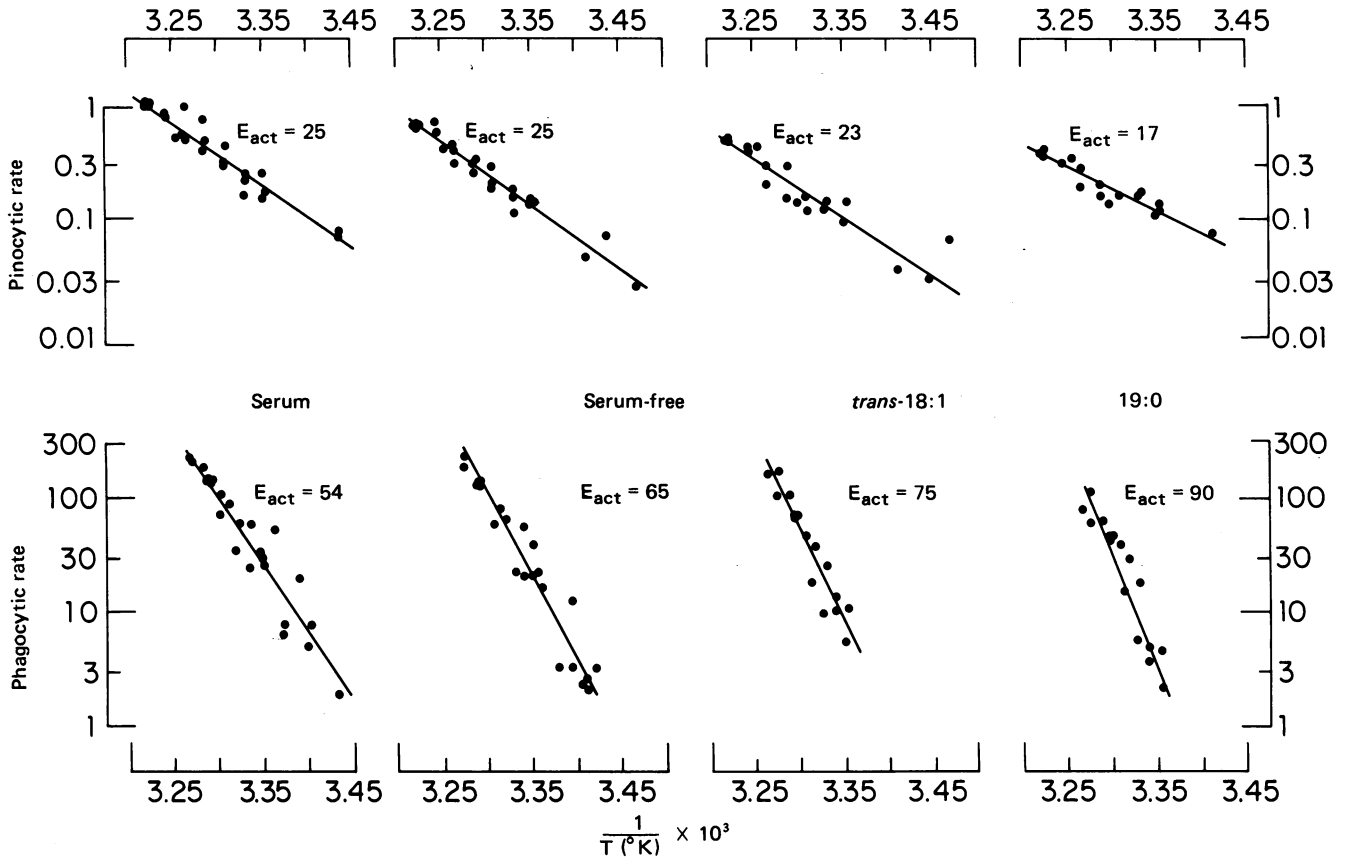

FIG. 1. Arrhenius plots of fluid-phase pinocytosis (*Upper*) and receptor-mediated phagocytosis (*Lower*). Data were obtained in three to five experiments, normalized at 37° (*Upper*) or 30° (*Lower*), and submitted to linear regression analysis to determine the line of best fit. E_{act} values were calculated from the corresponding slopes. (*Upper*) Pinocytic rate is plotted as ng of HRP interiorized per μg of cell protein per 30 min. The correlation coefficients for a straight-line fit are: serum, 0.96; serum-free, 0.98; *trans*-18:1, 0.95; and 19:0, 0.95. (*Lower*) Phagocytic rate is plotted as number of erythrocytes ingested per 100 macrophages per 30 min. The correlation coefficients for a straight-line fit are: serum, 0.91; serum-free, 0.96; *trans*-18:1, 0.95; and 19:0, 0.93.

the recruitment of other systems in phagocytosis that do not play significant roles in pinocytosis.

The methods described in this article provide a simple and rapid means to alter the fatty acid composition of cellular phosphatides and still maintain viability. Although applied to saturated fatty acids in this study they are equally effective with unsaturated fatty acids and under conditions in which there is no appreciable endogenous synthesis. Merely removing serum with its abundant fatty acids and maintaining the cells in fatty acid-free tissue culture medium leads to an increase in the degree of saturation of cellular fatty acids. This is progressively enhanced with the addition of *trans*-18:1 or 19:0 fatty acid, respectively, and leads to discrete and different effects on the processes of pinocytosis and phagocytosis.

As the saturated/unsaturated fatty acid ratio increases, the levels of both phagocytosis and pinocytosis decrease at all temperatures; the minimum temperature at which interiorization of erythrocytes is observed in phagocytosis increases, and the E_{act} of phagocytosis increases. The data presented for phagocytosis may reflect relative degrees of total plasma membrane fluidity not manifested in the measurements of pinocytosis. Because the binding capacity of the surface Fc receptors in serum-free and enriched cultures is not altered by the culture conditions used, our observations indicate that increased saturation in phospholipids reduces the macrophage's ability to interiorize particles attached to the Fc receptor and increases the "energy barrier" for a phagocytic event. It is possible that, although the total number of Fc receptors is unchanged in enriched cultures, the number of receptors with bound erythrocytes residing in portions of membrane retaining the correct degree of fluidity for effective ingestion may be reduced in comparison with serum cultures.

The data obtained for pinocytosis after enrichment do not reveal increased E_{act} or minimum temperatures at which activity is expressed, as observed in the case of phagocytosis. In fact, the E_{act} for pinocytosis in 19:0 fatty acid-enriched cultures is, unexpectedly, somewhat lower than that measured for the other cultures. If this is not within experimental error, it could be viewed as the result of heterogeneity within the lipid phase of the plasma membrane where regions containing a preponderance of saturated fatty acids interiorize little or no HRP and regions relatively rich in unsaturated lipid interiorize HRP quite readily and therefore with a decreased E_{act}. In either case, the reduction in the uptake of HRP at all temperatures after enrichment is in complete accord with the reductions in the ingesting phase of phagocytosis and indicates that the saturated fatty acid content of cellular lipids influences both pinocytosis and phagocytosis.

It is apparent that striking transition points are not seen within the Arrhenius plot for either form of endocytosis. It would not be surprising if the obvious breaks in Arrhenius plots observed in transport functions or enzymatic activities of bacterial mutants are not duplicated in certain complicated processes of higher organisms. In addition, subtle changes in the slopes of the Arrhenius plots of endocytosis would be obscured because the nature of the assays used in these studies precludes

obtaining measurements as precise as those for typical bacterial membrane functions. We believe, as a working hypothesis, that this is the result of modifications in plasma membrane fluidity. It should be noted, however, that modifications also occur in neutral lipids and in the phospholipids of other cellular compartments, and these may also influence endocytosis.

The phospholipids of other cultured mammalian cell types have been enriched for saturated or unsaturated fatty acids by various techniques (15–17), with effects on such membrane properties as concanavalin A binding (18) and lectin-induced cell agglutination (16) which correlated with differences induced in saturated fatty acid content. Similarly, a report concerning nonreceptor-mediated phagocytosis in thioglycollate-stimulated peritoneal macrophages incubated for 4 days in medium containing individual fatty acids appeared during the course of our work and showed reduced particle uptake at 37° (19).

In conclusion, our results on the relationship between lipid composition and temperature-dependent expression of two important membrane-mediated processes in a cultured mammalian cell indicate that the degree of saturation in macrophage phospholipids is a significant factor in plasma membrane internalization.

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