Modification of the Lipid Composition of Normal and Rous Sarcoma Virus-infected Cells

EFFECTS ON TRANSFORMATION-ASSOCIATED MEMBRANE PROPERTIES*

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Procedures are described for modification of the phospholipid polar head group and fatty acid composition of normal and Rous sarcoma virus-infected chicken embryo fibroblasts. Lipid modification was carried out by growth of cells in delipidated medium containing either polar head group analogues or specific fatty acids. Normal and infected cells displayed similar kinetics of lipid alteration, and the modification was 50% complete in approximately 10 h. Since this is faster than can be accounted for by growth and dilution, extensive turnover of polar head groups and fatty acids must occur in this system.

Supplementation with linoleate (18:2) had little or no effect on the growth of cells. Supplementation with the choline analogues N-methylethanolamine and N,N-dimethylethanolamine caused some growth inhibition, but still allowed substantial cellular multiplication. Supplementation with 1,2-amino-1-butanol or ethanolamine, or the absence of any choline analogue, significantly inhibited cell growth. Rous sarcoma virus-infected cells showed increased sensitivity to growth inhibition by the supplements and began to detach from the dish after a growth plateau was reached. Growth inhibition could be reversed in all cases by changing to standard medium without lipid supplements. The production of infectious virus in the cells with modified polar head groups was similar to the control value except for the cases of supplementation with 1,2-amino-1-butanol, which caused a reduction in virus production to approximately 40%.

The effects of lipid modification on various parameters of transformation were examined. Lipid modification had little or no effect on the rate of hexose transport by normal or Rous sarcoma virus-transformed cells, except for ethanolamine which caused a slight drop in transport in the normal cultures. Lipid modification had a variety of effects on the adherence of cells to their substrate, the most dramatic being a decreased adherence in normal cultures without any choline analogue or supplemented with 1,2-amino-1-butanol, ethanolamine, or N-methylethanolamine. Accompanying the decreased adherence in the 1,2-amino-1-butanol-supplemented cultures was a profound change in morphology: the cells became round and refractile, and their surface displayed numerous blebs, ruffles, and microvilli (as revealed by scanning electron microscopy). Thus, 1,2-amino-1-butanol supplementation converted the normal cells into partial phenocopies of transformed cells with respect to their adhesiveness and their morphology.

It is widely suspected that cellular membranes play an important role in the regulation of cellular physiology, and are especially important in the process of malignant transformation by tumor viruses (see Ref. 1 for a recent review). The lipid composition of biological membranes has been shown to affect a number of membrane-associated activities in a variety of systems, including sugar transport in bacteria (2) and (Na/K)ATPase in animal cells (3, 4). In addition, alterations in membrane lipids have been reported by several workers to accompany malignant transformation. An increased ratio of oleic to arachidonic acid occurs in rat hepatomas (5), SV40-transformed WI-38 (6) and 3T3 cells (7), and Rous sarcoma virus-transformed chicken embryo fibroblasts (8-10). These changes in lipid composition seem in most cases to be accompanied by alterations in the biophysical properties of the membrane bilayer (10-12). Thus, it is possible that membrane lipids could also participate in the expression of transformation-associated alterations in membrane function and structure, such as changes in hexose transport (13, 14), adhesiveness (15), surface proteins (16-18), protease secretion (19), and cellular morphology (20, 21).

One approach that has been used to study the role lipids play in cellular activities has been to manipulate the lipid composition of cells growing under defined conditions (22). The majority of this work has concentrated on the alteration of the fatty acid composition in bacterial systems. Recently, however, several papers have been published in which the fatty acid composition of animal cells growing in tissue culture has been manipulated (7, 23-26) and studies have been carried out on their surface properties (7, 27-29). In contrast to the work on fatty acid composition, relatively little has been done with respect to the biosynthetic modification of the polar head

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group composition of membrane phospholipids. It is possible to alter the phospholipid composition in several different types of organisms (30-36). In animal cells Glaser et al. have developed techniques to systematically manipulate the phospholipid composition of LM cells growing in culture (37). Cells grown in medium which contain choline analogues such as N-methyl-
ethanolamine or 1-2-amino-1-butanol, will incorporate the analogues into membrane phospholipids (37, 38). The polar head group composition can be modified either independently or in conjunction with the fatty acid composition (25). The polar head group analogues are incorporated into all membrane fractions of the cell (39). Changes in the polar head group composition in these cells have been shown to cause changes in membrane viscosity (40) and adenylate cyclase activity (39). The binding characteristics of β-paranic acid and 8-anilino-1-naphthalenesulfonylic acid were also changed, but these probes did not reveal any changes in the physical properties of the membranes (41). In addition, the incorporation of specific choline analogues makes possible studies on membrane structure and function (42).

In order to investigate the role of membrane lipids in transformation-associated membrane changes, we have chosen to analyze the effects of lipid modification on cell surface-related properties of chicken embryo fibroblasts, either normal or infected with Rous sarcoma virus. This system has a number of advantages for an investigation of this type. First, the cells are diploid, only a few passages removed from the animal, and (when unaffected) are still sensitive to normal regulatory controls, such as density-dependent inhibition of growth and the growth rate contingent changes in membrane transport (43). Secondly, 100% of a culture can rapidly be transformed by Rous sarcoma virus, minimizing problems of specific cell selection inherent in most other systems. The availability of temperature-sensitive transformation mutants further increases this advantage. Finally, transformation-associated cell surface changes have been analyzed in great detail in this system over the past several years (44). In this report, we describe the methodology for modifying both the polar head group and fatty acid composition of normal and Rous-infected chicken embryo fibroblasts, and the effects of this modification on a variety of transformation-associated cell surface changes.

MATERIALS AND METHODS

Cell Culture and Lipid Modification—Primary cell culture and virus infection were carried out as described (14). After 3 days as primary cultures, normal or Rous-infected cells were trypsinized with 0.05% crystalline trypsin (Miles Serva, Grade I) in Tris-buffered saline, pH 7.4, and replated as secondary cultures at 3.5 × 10⁶ cells/cm² for the normal cells, and 5.0 × 10⁶ cells/cm² for the infected cells. Because a slightly lower percentage of the infected cells attach and grow, these plating densities result in similar cell densities in the two types of cultures. These densities were chosen so as to allow optimal growth of both cell types throughout the experiments. Freshly plated infected cultures did not fully display the transformed phenotype under our conditions until approximately 24 to 36 h after plating. However, in order to maximize the modification of the phospholipid composition without transferring the cells, it was necessary to begin the polar head group supplementation shortly after cell plating. Thus, in transformation-associated cultures, properties between supplemented and unsupplemented Rous-infected cells could be due either to reversal of an aspect of transformation or inhibition of its appearance. In this communication the term "transformed" will be used to apply only to those cultures which have been infected long enough to display either the morphology, adhesiveness, or kexosé transport characteristic of transformed cells. Other cultures will be termed "infected."

Secondary cultures were plated either in standard medium (Dulbecco's modified Eagle's medium containing choline (GIBCO) and supplemented with 4% calf, 1% chicken serum, and bovine serum albumin or in delipidated medium (Dulbecco's modified Eagle's medium without choline and supplemented with delipidated calf and chicken serum and bovine serum albumin). For linoleate supplementation, secondary cultures were plated in medium with bovine serum albumin at a concentration of 10 μg/ml. This concentration of bovine serum albumin was found to allow sufficient growth without blocking the fatty acid modifications. Supplementation was performed by adding the fatty acid (20 μg/ml) final concentration complexed to bovine serum albumin (Pentex, fatty acid free) directly to the growth medium. The method of Spector and Hoak (45) was used to complex the fatty acid to the bovine serum albumin. Linoleate was added 36 h after plating of secondary cultures, and the cells were generally harvested 24 h later.

Deplidation of Serum—Removal of lipid from calf and chick serum was accomplished by multiple alcohol and ether extractions essentially as described by Horwitz et al. (7). The delipidation procedure removed total fatty acids (determined by total lipid extraction, hydrolysis, and titration) and cholesterol (46), free choline (48) to less than 1 to 2% of the starting value. After delipidation, the serum was reconstituted with buffer (0.16 NaCl, 4.9 mm KCl, 0.12 mm MgSO₄, and 16 mm NaPO₄, pH 7.4) to a protein concentration identical with that obtained prior to delipidation.

Lipid Analysis—Cells harvested by scraping were washed in phosphate-buffered saline by centrifugation and extracted using the method of Bligh and Dyer (50) as described by Ames (51). Phospholipids were separated by two-dimensional thin layer chromatography on silica gel G (250 μm, Analtech, Inc.) as previously described (37). Spots were visualized either by I₂ vapors or alternatively by autoradiography (37). Spots were then scraped and counted in 5 ml of a scintillation mixture consisting of xylene (2400 mCi), Toluene X-114 (800 ml), and 2,5-diphenyloxazole (9.16 g). Fatty acids were prepared from phospholipids obtained by extraction of cellular material followed by separation of phospholipids and neutral lipids on a 2-inch Unisil column (25). Methyl esters were prepared in sodium methoxide/methanol (Applied Science) and chromatographed isothermally at 180 °C on a column (1.5 m × 0.317 cm) of cyanosilicone SP-2340 on 80 to 100 mesh chromosorb W-AW (Supelco, Inc.). A Hewlett-Packard 5830A equipped with a flame ionization detector and peak integrator was used to conduct the gas-liquid chromatographic analysis.

Transformation Assays—Transport of 3-O-methylglucose (14), adenylate measurements (40), and preparation of cells for scanning electron microscopy (21) have all been described elsewhere. The fixation procedures used for scanning electron microscopy provided optimum preservation of cell surface morphology. The virus titer in the growth medium was determined by the casein plate method of Goldberg (53).

RESULTS

Polar Head Group Supplementation—The phospholipid composition of chicken embryo fibroblasts grown either in standard medium or in delipidated medium with various polar head group supplements is shown in Table I. For cells grown in standard medium, small differences were consistently detected (seven experiments) between normal and transformed

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Secondary cultures were plated in medium with a biotin concentration of 1 μg/ml. Choline or its analogues were added as supplements to 3 to 6 h after plating at a concentration of 40 μg/ml N,N-Dimethylethanolamine (b.p. 133-134°), N-methylethanolamine (m.p. -4.5 to -3.5°) and ethanolamine (99%) were obtained from Eastman Kodak. 1-2-Amine-1-butanol (99%) was obtained from Aldrich.

For linoleate supplementation, secondary cultures were plated in medium with bovine serum albumin at a concentration of 10 μg/ml. This concentration of bovine serum albumin was found to allow sufficient growth without blocking the fatty acid modifications. Supplementation was performed by adding the fatty acid (20 μg/ml) final concentration complexed to bovine serum albumin (Pentex, fatty acid free) directly to the growth medium. The method of Spector and Hoak (45) was used to complex the fatty acid to the bovine serum albumin. Linoleate was added 36 h after plating of secondary cultures, and the cells were generally harvested 24 h later.

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cells in the ratio of phosphatidylcholine/phosphatidylethanolamine. Normal cells had an average phosphatidylcholine/phosphatidylethanolamine ratio of 2.31, whereas the transformed cells had an average phosphatidylcholine/phosphatidylethanolamine ratio of 1.29. This finding is contrary to the conclusion of Quigley et al. that there is no alteration in the phospholipid composition caused by Rous sarcoma virus transformation of chicken embryo fibroblasts (52, 54). However, close inspection of their data for whole cells does indicate a small difference in the phosphatidylcholine/phosphatidylethanolamine ratio. No other differences in the phospholipid composition were found between normal and transformed cultures.

Normal cultures grown in delipidated medium plus choline (40 μg/ml) exhibited a phospholipid composition not significantly different from that displayed by the cultures grown in standard medium. However, transformed cells grown in delipidated medium plus choline did not exhibit the decreased ratio of phosphatidylcholine/phosphatidylethanolamine seen when they were grown in standard medium. Aside from this, there were no other alterations in phospholipid composition induced by growth in delipidated medium plus choline. When cells were grown in delipidated medium without any analogue the amount of phosphatidylethanolamine decreased substantially. The decrease in phosphatidylcholine was mostly compensated for by an increase in phosphatidylethanolamine.

When cells were grown in delipidated medium containing choline analogues, new spots appeared on the autoradiograms due to the synthesis of phospholipids with new polar head groups. These new spots were identified by comparing their chromatographic behavior to that of synthetic phospholipid standards on two-dimensional thin layer chromatography using two different solvent systems (37). Supplementation of either normal or transformed cultures with N,N-dimethylethanolamine for 40 and 34.5 h, respectively, resulted in over 50% of the cellular phospholipid becoming phosphatidyl(dimethylethanolamine) (Table I). Nearly as good phospholipid substitution was accomplished with N-methyllethanolamine. Supplementation with 2-amino-1-butanol resulted in slightly less incorporation. Phosphatidylethanolamine comprised 35.2% of the normal cell phospholipid and 30.5% of the transformed cell phospholipid after maximal substitution. Appearance of the new phospholipids was accompanied by a decrease in the levels of both phosphatidylcholine and phosphatidylethanolamine. No consistent analogue-induced alterations in phosphatidylinositol + phosphatidylserine, sphingomyelin, or cardiolipin were seen. The appearance of small amounts of phosphatidyl(dimethylethanolamine) following N-methyllethanolamine supplementation can be accounted for by small amounts of N,N-dimethylethanolamine contaminating the N-methyllethanolamine solution.

Supplementation with ethanolamine in normal and transformed cells resulted in increasing the phosphatidyl ethanolamine content of the cells to over 30%. The phospholipid composition was similar to cells grown in delipidated medium without any analogue except that normal cells supplemented with ethanolamine contained more phosphatidylethanolamine.

In Fig. 1 is shown the time course of polar head group modification. When cells were supplemented with choline analogues, alterations in the lipid composition began without apparent lag and were complete within 40 h. Only small differences between normal and Rous-infected cultures were seen in the kinetics of modification. When cells were grown without any choline analogue, the changes in the phospholipid composition were slower and could be accounted for by dilution of phosphatidylcholine by growth.

To further characterize the lipid composition of cells supplemented with polar head group analogues, the fatty acid compositions of these cells were analyzed. Transformed cells grown in standard medium showed a drop of 204 and a corresponding increase in 18:1 compared to normal cells as previously reported (8–10) (Tables II and III). However, both normal
and transformed cells grown in the presence of delipidated medium plus choline exhibited an increase in 18:1 and 16:1 and a decrease in 20:4, long chain fatty acids (>18), and polyunsaturated fatty acids relative to the corresponding cells grown in standard medium. Moreover, the transformation-associated change in the ratio of 18:1/20:4 was no longer detectable, although now the transformed cells seemed to show a change in the percentage of saturated fatty acids relative to normal cells. There were also some changes in the fatty acid compositions of cells supplemented with the choline analogues.

The effects of the lipid supplementation procedure on cell growth are shown in Fig. 2. Supplementation with choline provided the best growth for normal cells, comparable to that obtained in standard medium (containing choline). When cells were grown in delipidated medium without any choline analogue, the cells stopped growth at a lower saturation density and eventually began to detach from the plate. Growth in N,N-dimethylethanolamine was not quite as good as in choline, and N-methylethanolamine was slightly worse. Ethanolamine and l-2-amino-1-butanol caused the greatest growth inhibition. In general, the infected cells showed a greater sensitivity to growth inhibition than did the normal cells. When either cell type was supplemented with N,N-dimethylethanolamine, N-methylethanolamine, and l-2-amino-1-butanol, the addition of choline along with the polar head group analogue increased growth (data for l-2-amino-1-butanol plus choline shown in Fig. 2, C and F) and also blocked the lipid modification (data not shown). Addition of choline along with ethanolamine did not enhance growth (Fig. 2, C and F), but it did prevent the incorporation of ethanolamine to give increased phosphatidylethanolamine levels (data not shown). In all cases after supplementation had occurred, if the culture was changed back into standard medium, growth resumed and the lipid composition returned to control values (data not shown).

Modification of Fatty Acid Composition—In order to establish conditions for the modification of the acyl group composition of chicken embryo fibroblasts, the effects of supplementation with linoleate (18:2) were investigated. The addition of 10^{-6} \mu g/ml of biotin to delipidated medium plus choline along with the fatty acid was necessary for adequate growth and supplementation to occur. Lower concentrations (lo^{-8} pg/ml) of biotin prevented the incorporation of ethanolamine to give increased phosphatidylethanolamine levels (data not shown). In all cases after supplementation had occurred, if the culture was changed back into standard medium, growth resumed and the lipid composition returned to control values (data not shown).

**Table II**

Fatty acid composition of normal and transformed cells grown in medium containing different analogues of choline

<table>
<thead>
<tr>
<th>Conditions of the experiment were the same as in Table I.</th>
<th>Medium</th>
<th>Fatty acid compositions</th>
<th>%</th>
<th>Fatty acid</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformed cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>16.0</td>
<td>16.1</td>
<td>18.0</td>
<td>18.1</td>
<td>18.2</td>
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<tr>
<td>Delipidated</td>
<td>15.8</td>
<td>5.2</td>
<td>16.0</td>
<td>25.2</td>
<td>9.0</td>
</tr>
<tr>
<td>+ Choline</td>
<td>16.7</td>
<td>8.2</td>
<td>15.8</td>
<td>33.7</td>
<td>6.3</td>
</tr>
<tr>
<td>+ DMEA</td>
<td>16.7</td>
<td>9.2</td>
<td>15.7</td>
<td>35.6</td>
<td>4.2</td>
</tr>
<tr>
<td>+ MEA</td>
<td>16.7</td>
<td>9.2</td>
<td>15.7</td>
<td>35.6</td>
<td>4.2</td>
</tr>
<tr>
<td>+ Ethanolamine</td>
<td>16.7</td>
<td>9.2</td>
<td>15.7</td>
<td>35.6</td>
<td>4.2</td>
</tr>
<tr>
<td>+ MEA*</td>
<td>16.3</td>
<td>8.2</td>
<td>15.7</td>
<td>35.1</td>
<td>3.7</td>
</tr>
<tr>
<td>+ l-2-Amino-1-butanol</td>
<td>17.5</td>
<td>11.2</td>
<td>11.2</td>
<td>32.1</td>
<td>4.0</td>
</tr>
<tr>
<td>+ Ethanolamine</td>
<td>16.8</td>
<td>12.1</td>
<td>11.8</td>
<td>30.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Normal cells</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Standard</td>
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<td>5.2</td>
<td>18.0</td>
<td>18.9</td>
<td>9.3</td>
</tr>
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<td>Delipidated</td>
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<td>8.4</td>
<td>16.2</td>
<td>31.0</td>
<td>4.0</td>
</tr>
<tr>
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<td>13.8</td>
<td>10.2</td>
<td>13.7</td>
<td>34.7</td>
<td>3.4</td>
</tr>
<tr>
<td>+ DMEA*</td>
<td>14.3</td>
<td>10.4</td>
<td>14.8</td>
<td>34.5</td>
<td>2.2</td>
</tr>
<tr>
<td>+ MEA*</td>
<td>13.5</td>
<td>11.1</td>
<td>18.2</td>
<td>32.2</td>
<td>2.8</td>
</tr>
<tr>
<td>+ Ethanolamine</td>
<td>15.9</td>
<td>11.1</td>
<td>15.2</td>
<td>26.7</td>
<td>4.5</td>
</tr>
<tr>
<td>+ l-2-Amino-1-butanol</td>
<td>15.8</td>
<td>12.1</td>
<td>17.3</td>
<td>28.6</td>
<td>4.0</td>
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</table>

* DMEA, N,N-dimethylethanolamine.
* MEA, N-methylethanolamine.
In Fig. 3 is shown the concentration dependence of linoleate supplementation. The 20 μg/ml of fatty acid in the medium were adequate to give maximum modification of the lipids of both normal and transformed cells. The time course of the modification (Fig. 4) demonstrated that modification was complete by 16 h for both cell types. The higher the cell density at which supplementation was carried out, the less complete was the modification of the lipid composition (Fig. 5).

The acyl group composition of total cellular lipids from normal and transformed cells with or without linoleate supplementation is shown in Table III. Cells grown in delipidated medium plus linoleate did not contain high levels of 18:1 and 16:1, but rather incorporated extensive amounts of the exogenously supplied fatty acid. Transformation-associated differences in the acyl group compositions of these cells were marginal at best. The fatty acid supplement had little effect on the growth of either normal or transformed cells (Fig. 6).

Supplementation and Virus Production - To determine the effect of lipid supplementation on virus production, growth medium was removed from the cells 11 h after plating (6 h after supplementation was initiated), and then fresh medium, with the appropriate supplement, was readed for 22 h or, medium was collected 43 h after supplementation, with no

**Fig. 2.** Growth of normal and Rous-infected cells in standard medium (SM) and in delipidated medium (DM) containing choline (C) and/or choline analogues. At zero time supplements were added to secondary cultures in 35-mm diameter tissue culture dishes. At various times the cells were collected and counted as described under “Materials and Methods.” Normal cells (A, B, and C) and Rous-infected cells (D, E, and F) were plated in either standard medium, delipidated medium without any analogue, or in delipidated medium with various analogues as indicated. DMEA, N,N-dimethyl-ethanolamine; MEA, N-methyl-ethanolamine; BA, 1,2-amino-1-butanol, E, ethanolamine.

**Fig. 3.** Concentration dependence of linoleate incorporation into normal and transformed cells. Identical plates of cells were plated in delipidated medium containing choline and 10⁻¹ μg/ml of biotin. Various concentrations of linoleate were added 36 h after plating and the percentage of linoleate in phospholipids was determined 24 h later as described under “Materials and Methods.”
The virus titer in the medium was then determined by the casein plate method of Goldberg (53). It can be seen that at either time, supplementation had only a slight depressing effect on virus production, with L-2-amino-1-butanol exerting the maximum effect by inhibiting virus production nearly 2.5-fold.

Adherence to Substrate—Alterations in cell-substrate adherence as a function of time after the addition of a polar head group supplement are shown in Fig. 7. Adherence of a normal noninfected culture grown in standard medium (Fig. 7A) was substantially greater than cells which were transformed and grown in standard medium (Fig. 7C) consistent with previous results (15). As can be seen (Fig. 7, C and D) the Rous-infected cells became increasingly less adherent with time as this parameter of transformation became expressed. Normal cells grown in delipidated medium plus choline or N,N-dimethyl-ethanolamine (Fig. 7B) had a low level of detachability, as did normal cells grown in standard medium. There was, however, a substantial difference in the adherence of normal cells grown without any analogue or in ethanolamine-, L-2-amino-1-butanol-, or N-methyl ethanolamine-supplemented media (Fig. 7, A and B). Normal cells with no analogue or supplemented with ethanolamine or L-2-amino-1-butanol showed a steady reduction in adherence. L-2-Amino-1-butanol-supplemented cells (Fig. 7A) showed the most rapid change and approached 80% detachability—a marked difference occurred within 40 h after supplementation was begun. N-Methylethanolamine-supplemented normal cells (Fig. 7B) showed little difference from control cells until 75 h after the beginning of supplementation. At this point, a substantial rise in the percentage of cells detachable occurred. Infected cells behaved in a similar manner. These cells with no analogue or supplemented with N-methylethanolamine, L-2-amino-1-butanol, or ethanolamine were significantly less adherent than control cells or cells supplemented with N,N-dimethylethanolamine.

Hexose Uptake Rate—Transformed cells transport hexoses at a much higher rate than normal cells (13, 14). Transformed cells grown in standard medium or in delipidated medium plus choline transported 3-O-methylglucose at approximately 10 times the rate of normal cells grown in delipidated medium plus choline (Table V). The transport rate of transformed cells was only marginally affected by supplementation with any of the choline analogues. Normal cells supplemented with ethanolamine transported hexose at a substantially reduced rate compared to choline-supplemented cells, but were not greatly affected by supplementation with the other analogues. No significant difference in the hexose uptake rate was seen for transformed or normal cells supplemented with linoleate when compared to cells grown in standard medium (data not shown).

Cell Morphology and Cell Surface Morphology—One of the most dramatic changes on supplementation with choline analogues was a change in cell morphology. Phase micrographs of

Table III

| Fatty acid compositions of normal and transformed cells grown in medium containing linoleate | Linoleate was added to cells 31 h after plating. The cells were harvested approximately 24 h later at which time the composition represented the maximum incorporation of the fatty acids into phospholipids. |

<table>
<thead>
<tr>
<th>Medium</th>
<th>Fatty acid compositions</th>
<th>&gt;C18 18:1/20:4</th>
<th>Fatty acids</th>
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<tr>
<td></td>
<td></td>
<td>Saturated</td>
<td>Polyunsaturated</td>
</tr>
<tr>
<td>Transformed cells</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>16.1 18.1 18.1 24.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delipidated + choline</td>
<td>16.8 22.5 22.5 22.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delipidated + choline + 18:2</td>
<td>16.8 22.5 22.5 22.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal cells</td>
<td>15.8 22.5 22.5 22.5</td>
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</tr>
<tr>
<td>Delipidated + choline</td>
<td>14.2 22.5 22.5 22.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delipidated + choline + 18:2</td>
<td>16.1 22.5 22.5 22.5</td>
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typical transformed and normal cultures in standard medium and delipidated medium plus choline are shown in Fig. 8. No significant differences in morphology were seen in transformed cells grown without any analogue or supplemented with either I-2-amino-1-butanol, ethanolamine, N-methyl ethanolamine, or N,N-dimethyl ethanolamine. Normal cells also showed no significant differences grown without any analogue or supplemented with ethanolamine or N,N-dimethyl ethanolamine. However, normal cells grown in the presence of N-methylethanolamine displayed a distinct change in shape as a result of the presence of the choline analogue (Fig. 8B). The typically narrow and elongated shape of normal chicken embryo fibroblasts (Fig. 8A) became shortened and broader in the presence of N-methylethanolamine. Normal cells grown in the presence of I-2-amino-1-butanol displayed a dramatic change in morphology going from elongated and flattened (Fig. 8C) to round and refractile (Fig. 8F) — not unlike the morphology of transformed cells (Fig. 8D) or transformed cells grown in the presence of I-2-amino-1-butanol (Fig. 8E). Normal and transformed cells supplemented with linoleate did not display significant changes in cell morphology (data not shown).

In order to evaluate the effects of polar head group and linoleate supplementation on cell morphology in more detail,
the cell surface morphology was examined by scanning electron microscopy. The typical surface morphology of normal exponentially growing chicken embryo fibroblasts was smooth with few microvilli, blebs, or ruffles (Fig. 9A). Transformed cells, however, displayed a significant number of microvilli, with some blebs and ruffles (Fig. 9D). When normal cells were grown in the presence of N-methylethanolamine their shape changed, but their surface features remained the same (Fig. 9C).
FIG. 9. Scanning electron micrographs of normal and transformed cells supplemented with choline analogues. Cells were plated on glass coverslips under the same conditions as Fig. 2. The coverslips were removed and fixed as described under “Materials and Methods.” Normal cells were grown in: A, delipidated medium plus choline for 50 h (× 1200); B, delipidated medium plus N-methyl ethanolamine for 50 h (× 1000); C, E, and F, delipidated medium plus L-2-amino-1-butanol for 50 h (× 7800, × 3000, and × 8000, respectively). D, transformed cells were grown in delipidated medium plus choline for 50 h (× 2000).
exception for ethanolamine appeared to exert their effect on cells to standard medium. Consequently, all the analogues lipid modification could be readily reversed by changing the nolamine into cellular membranes. Growth inhibition and thus, supplementation with ethanolamine appears to have lipid composition but it did not reverse the effects on growth. This suggests that turnover of membrane findings of Hirschberg* who observed substantial turnover of way for the incorporation of lipids. This is consistent with the kinetics of lipid modification by both hnoleate and the composition and growth control, the technology to modify both the phosopholipid polar head group and the fatty acid composition on normal and transformed cultures. Modification of the head group composition induced only small changes in the acyl group composition, in particular a decrease in the percentage of polyunsaturated fatty acids. However, this change seemed to be primarily a consequence of growth in delipidated medium rather than a consequence of directed head group modification. That is the absence of polyunsaturated fatty acids in the delipidated medium resulted in the decreased percentage of polyunsaturated fatty acids in the cellular phospholipids.

The kinetics of lipid modification by both linoleate and the choline analogues occurred more rapidly than could be accounted for by growth. In fact, in the absence of biotin, good incorporation of linoleate could be achieved in the near absence of growth. This suggests that turnover of membrane lipids occurs rapidly in this system and is a substantial pathway for the incorporation of lipids. This is consistent with the findings of Hirschberg2 who observed substantial turnover of the glycerol backbone of phosphatidylcholine in chicken embry fibroblasts.

The effects of the supplementation on cellular growth were interesting since there are numerous growth-dependent changes in cell behavior which occur following malignant transformation. The infected cells showed an increased sensitivity to all the supplements, and began to detach from the dish after a growth plateau was reached. Choline competed with N,N-dimethylglycerolamine, N,N-dimethylglycerolamine, and l-2-amino-1-butanol for incorporation into cellular phospholipids and could reverse the effects of these analogues on growth. In these cases inhibition of growth occurred only when incorporation of the head group analogue occurred. However, choline was able to block the effects of ethanolamine on the lipid composition but it did not reverse the effects on growth. Thus, supplementation with ethanolamine appears to have some inhibitory effects independent of incorporation of etha nolamine into cellular membranes. Growth inhibition and lipid modification could be readily reversed by changing the cells to standard medium. Consequently, all the analogues except for ethanolamine appeared to exert their effect on cellular properties through alterations of the phospholipid composition.

Role of Lipids in Transformation—Previous work demonstrated that transformed cells grown in standard medium displayed an increased ratio of 18:1/20:4 in cellular phospholipids and a decrease in the unsaturation index (the average number of double bonds per acyl group) (8-10). In addition, the present work has demonstrated a small but reproducible drop in the ratio of phosphatidylcholine/phosphatidylethanolamine in transformed cells grown in standard medium.

It now seems probable from the results obtained with the lipid-supplemented cultures that these alterations in lipid composition have little functional significance, since they can largely be abolished without abolishing the transformation-associated differences in morphology, adhesiveness, and hexose transport. For example, both normal and transformed cells grown in delipidated medium plus choline had the same ratio of phosphatidylcholine/phosphatidylethanolamine. Similarly, both normal and transformed cells grown in delipidated medium displayed an increased ratio of 18:1/20:4 in cellular phospholipids, but the two cell types no longer differed from each other in this parameter. It is possible that the transformation-associated difference in the ratio of 18:1/20:4 detected when cells were grown in serum-containing medium was due to a difference in uptake of unsaturated fatty acids between the normal and transformed cells.

In general, the modifications of the lipid composition that were observed did not greatly affect the production of Rous sarcoma virus by infected cells within experimental error. The one exception was cells supplemented with l-2-amino-1-butanol, which displayed a 2- to 3-fold decrease in virus titer. It remains to be determined whether this decrease is due to a decrease in the percentage of the cells producing virus, the yield of virus per cell, or the specific infectivity of the virus. The fact that supplemented cells are able to produce high titers of virus will be important for future studies on the role of lipids in the structure and assembly of these enveloped virions.

The easiest example of an alteration in membrane function in transformed cells is the increased rate of hexose transport displayed by a variety of transformed cell types (13, 14). Lipid modification had only a small effect on the rate of hexose transport and did not stimulate the transport rate in normal cultures in any case. Ethanolamine supplementation caused a drop in hexose transport in normal cells but did not affect the rate of transport in transformed cells. The drop in hexose transport in normal cells supplemented with ethanolamine is most likely due to the toxic properties of ethanolamine since growth of normal cells without any analogue resulted in a similar increase in phosphatidylethanolamine but showed no decrease in hexose transport.

Decreased adherence to the substrate is among the most dramatic early manifestations of transformation by Rous sarcoma virus (15). Modification of the lipid composition had a variety of effects on cellular detachability: ethanolamine, l-2-amino-1-butanol, or N-methyl ethanolamine-supplemented normal cells were much less adherent than their controls and infected cells grown in delipidated medium plus choline were somewhat more adherent than their counterparts grown in standard medium. The alterations in adherence induced by supplementation could be due to lipid-dependent changes in the number or arrangement of cell surface adhesion proteins, to changes in the deformability of the lipid bilayer, or to changes in the surface charge.

* C. B. Hirschberg, personal communication.
Lipid Composition and Transformation-associated Properties

Accompanying the changes in adherence and hexose transport during the course of malignant transformation are striking alterations in cell morphology. Whereas modification of the lipid composition had little effect on the morphology of transformed cultures, supplementation of normal cells caused dramatic and specific changes. Normal cells supplemented with N-methylethanolamine became more epithelial in appearance, taking on a broader and less elongated appearance than control fibroblasts. Normal cells supplemented with L-2-amino-1-butanol appeared very much like transformed cells: rounded, with microvilli, blebs, and ruffles on the surface. It is worth pointing out that these cells also showed the most dramatic alterations in cell substrate adherence. Since there is substantial correlational evidence that microfilaments and microtubules play a role in the control of cell shape (55), it seems likely that supplementation with L-2-amino-1-butanol and perhaps with N-methylethanolamine has altered the organization of cytoskeletal elements, perhaps by affecting their sites of membrane attachment.

In correlating the lipid modification with effects on cell behavior, it is important to point out that many of the changes described above (such as the morphological alterations) appeared after the majority of the change in phospholipid composition had taken place. It is possible that the state of the membrane was changing continuously and uniformly as the new analogue was being incorporated, but changes in a particular membrane property did not become apparent until a large change in the membrane had taken place. Alternatively, the analysis of the lipid composition of the whole cell that was carried out may not reflect the composition of the plasma membrane. Also, a critical membrane protein could be surrounded by boundary lipid or be localized in a particular membrane region that was altered more slowly than the total membrane phospholipid.

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REFERENCES