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# Effect of Fatty Acids on Growth of Japanese Encephalitis Virus Cultivated in BHK-21 Cells and Phospholipid Metabolism of the Infected Cells

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Growth of Japanese encephalitis virus (JEV) in BHK-21 cells was stimulated in the presence of 20 to 40 μg of the sodium salt of oleic acid (*cis*-9-octadecenoic acid, 9-18:1) per ml supplemented in Waymouth medium. The stimulatory effect of the salt was highest when 9-18:1 was added after adsorption of the virus. Study of the effect of other fatty acids on growth of JEV showed the following results: the longer the chain length of the saturated fatty acid salt, the higher the stimulatory effect on viral growth. In contrast, polyunsaturated fatty acids had an inhibitory effect on viral growth. The effect of isomeric *cis*-octadecenoic acids on viral growth was variable, depending upon the position of the double bond. The *cis*-6-octadecenoic acid had the highest inhibitory effect on growth of JEV compared to other isomeric octadecenoic acids. The sodium salt of [1-$^{14}$C]*cis*-9-octadecenoic acid (9-18:1, 20 μg/ml) was rapidly incorporated into control and JEV-infected cells. Specific radioactivity in phosphatidylcholine dropped 12 to 24 h after virus inoculation, whereas synthesis of phosphatidylethanolamine increased 12 to 24 h after virus inoculation in infected cells compared to uninfected cells. Results from these studies suggest that phospholipid metabolism of infected cells is markedly changed, which can be associated with altered fatty acid metabolism when using labeled 9-18:1 fatty acid as a marker.

The association of lipids as a viral structural component in Japanese encephalitis virus (JEV) or other related group B arboviruses has long been suspected since lipid solvents (2, 19, 50), lipolytic enzymes (2, 44, 50), and deoxycholate (45) inactivate these viruses. Several reports have been published on lipids of group A arboviruses, including Sindbis virus (28), Semliki Forest virus (11, 30) and Venezuelan equine encephalitis virus (17; F. P. Heydrick, R. F. Wachter, and E. H. Ludwig, Bacteriol. Proc., p. 168, 1968; and F. P. Heydrick, J. F. Comer, and R. F. Wachter, Bacteriol. Proc., p. 162, 1969). Lipid composition of group B arboviruses has not been fully examined by direct analysis of purified virus particles (27). Grossberg and O'Leary and Grossberg et al. showed occurrence of hyperlipemia in chicken embryos after JEV infection (14, 15).

These reports suggest that lipids may have a fundamental role in arbovirus infection; therefore, effect of lipids on the infectious cycle is of particular interest. However, the effect of lipids on arbovirus biosynthesis, as well as the effect on lipid metabolism of the infected host cell, has not been well documented.

Recently, attempts have been made to elucidate the function of lipids in growth of tissue culture cells (1, 4–6, 20, 22, 39, 47), and extensive reviews of lipid metabolism in cultured cells have been presented (31, 32). The results in these studies show that, under appropriate conditions, exogenous fatty acid can readily be incorporated into mammalian cells and be metabolized.

The present investigation is a report on (i) the effect of exogenous fatty acids on multiplication of JEV in hamster kidney cells (BHK-21 cells) cultivated in Waymouth medium as a first step to study lipid metabolism of arboviruses in vitro and (ii) the uptake of the radioactive salt of 9-18:1 in JEV-infected and normal BHK-21 cells to determine the complex lipids which may change during infection.

The results show that the presence of sodium oleate (sodium salt of 9-18:1) in Waymouth medium stimulates growth of JEV in BHK-21 cells, whereas the salts of other fatty acids have varying effects on viral growth.

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## MATERIALS AND METHODS

**Preparation of BHK-21 cells.** BHK-21 cells (42) were obtained from T. B. Stim, Yale Arbovirus Research Unit, Yale University, New Haven, Conn., and were cultivated in Eagle minimum essential medium (10) supplemented with 5% newborn calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (MEM $Ca_s$). The cells were maintained in 32-oz (0.95-liter) prescription bottles as follows. The cell monolayer was washed twice with Hank balanced salt solution without calcium and magnesium ions (GKNP) and trypsinized (0.05% trypsin; Difco, Detroit, Mich.) at 37 C for 5 min. Fresh medium (35 ml) was added to the bottle. The cells were transferred every 3 to 4 days and were usually split 1:4. Newborn calf serum, vitamins, and essential amino acids were obtained from Grand Island Biological Co. (Grand Island, N.Y.). Glutamine was obtained from Calbiochem (Los Angeles, Calif.).

**Uninfected BHK-21 cell growth.** Triplicate screw-cap Leighton tubes were used for the study of cell growth. A 1-ml amount of MEM $Ca_s$ containing about $3 \times 10^4$ cells was added into each tube, and the tubes were incubated for 48 h at 37 C. Cells were washed twice with 2 ml of GKNP. Waymouth medium (1 ml) (49; Waymouth dry powder media tissue culture medium, Schwarz BioResearch, Inc., Orangeburg, N.Y.), containing 2 mg of bovine albumin per ml (fatty acid-free; Pentex, Inc., Kankakee, Ill.), and different sodium salts of fatty acids were added to each tube. The cells were further incubated as described above. A hemocytometer was used to measure cell growth by taking quadruplicate samples from each of three tubes using the procedure of Jenkin and Anderson (20).

**Preparation of the sodium salt of fatty acids.** Saturated fatty acids 12:0 through 22:0 and unsaturated fatty acids 9-18:1; 9,12-18:2; 9,12,15-18:3; and 5,8,11,14-20:4 were purchased from the Lipids Preparation Laboratory, The Hormel Institute, Austin, Minn. Isomeric *cis*-octadecenoic acids 2-18:1 through 17-18:1 were obtained from Gunstone and Ismail (16) and R. T. Holman (The Hormel Institute). All acids were tested for purity by mass spectrophotometry and ozonolysis. The acids were converted to the sodium salt according to Jenkin and Anderson (20) and stored at −20 C. An appropriate amount of the salt was dissolved in Waymouth medium just prior to use. Two milligrams of bovine albumin per ml (fatty acid free) were present in all medium unless otherwise stated.

**Virus.** JEV strain M5/596 (23) from the 10th suckling mouse brain passage was kindly supplied by S. Grossberg, Medical College of Wisconsin, Milwaukee, Wis. Virus seed was prepared by infecting BHK-21 cells with JEV in 32-oz prescription bottles as described below. Infected cells were incubated at 37 C in MEM $Ca_s$. Forty-eight hours after infection, the supernatant fluid was removed and centrifuged at $12,000 \times g$ for 30 min. Newborn calf serum was added to the supernatant fluid (final concentration, 20%). The virus was stored at −70 C in sealed ampoules.

**Plaque assay.** Plaque assays of JEV were performed according to Makino et al. (24) on monolayers of BHK-21 cells in 6-unit plastic plates, 10 by 35 mm (Linbro Chemical Co., Inc., New Haven, Conn.). Methyl cellulose (1%; 4,000 centipoise) was obtained from Dow Chemical Co., Midland, Mich. The monolayer was stained with crystal violet, and the plaques were counted according to the method of Holland and McLaren (18). PFU were determined from the average count of triplicate plates from each of triplicate tubes.

**Stability of JEV.** Stability of JEV in Waymouth medium containing various supplements was studied. Stock inoculum of JEV was diluted at least 100-fold (to approximately $10^6$ PFU/ml) with each medium to be tested. The mixture was held at 37 C in vials. At the end of each incubation period, samples of the mixture were removed and further diluted 100-fold with 0.1% bovine plasma albumin (fraction V; Pentex, Inc., Kankakee, Ill.) in phosphate buffer (pH 7.2). The infectivity was determined by plaque assay. Percentage of surviving virus was calculated from the ratio of PFU at the time of sampling over PFU at zero time, multiplied by 100.

**Virus infection.** BHK-21 cell monolayers in Leighton tubes were prepared as described for uninfected cells. Cell monolayers were incubated in unsupplemented Waymouth medium for 24 h prior to infection. The cell number per tube was determined in control tubes. The cells were infected with JEV (0.1 ml/tube) with a multiplicity of infection of 10 PFU/cell. Virus adsorption was performed at room temperature for 90 min while agitating the tubes on a rocking platform (Bellco Glass Co., Vineland, N.J.). At the end of the adsorption period, the monolayer was washed twice with 2 ml of GKNP, and then 1 ml of Waymouth medium containing various supplements of the sodium salt of fatty acids was added to each tube. At each interval of sampling, infected medium was removed and centrifuged at $600 \times g$ for 10 min to remove cell debris. The supernatant fluid was removed and stored at −70 C. Loss of infectivity did not occur at this storage temperature. Virus infectivity was determined using the plaque assay described above. Infectivity of cell-associated virus was titrated after quick freezing at −70 C and thawing at 37 C three times in the buffer system of Schulze and Schlesinger (36). A low-speed centrifugation ($600 \times g$ for 10 min) was performed, and the supernatant fluid was removed and stored at −70 C for a plaque assay.

**Virus infection studies for incorporation of radioactive 9-18:1.** The cell monolayers were prepared in 2-oz (ca. 60-ml) prescription bottles ($10^6$ cells/bottle) and used in the study of general distribution of radioactivity in medium, cells, or $CO_2$. After virus adsorption, 3 ml of Waymouth medium supplemented with 20 μg of labeled sodium oleate per ml (0.17 μCi/ml) and 2 mg of bovine albumin per ml was added to each bottle. Bottles were plugged with a double-sealed rubber stopper constructed with a central well (Kontes Glass Co., Vineland, N.J.). Three bottles were sampled after appropriate incubation times by trapping $CO_2$ as described below, removing the supernatant fluid, and trypsinizing the cells to measure the

radioactivity of the $CO_2$ and cells. Triplicate radioisotope counts were made from each of the three bottles. Variations of counts were less than 15%.

Cell monolayers were prepared in 8-oz (ca. 0.24-liter) bottles (approximately $4 \times 10^6$ cells/bottle) to study the lipids of control and JEV-infected cells. A 15-ml amount of the Waymouth medium, supplemented as described above, was added to each bottle. Cells were collected from four bottles for each set of samples with the aid of trypsinization followed by low-speed centrifugation ($600 \times g$ for 10 min). Uninfected BHK-21 cells were treated as described above.

**Collection of $^{14}CO_2$.** $^{14}CO_2$ was collected by the modification of Snyder's procedure (38). At each interval of sampling after virus inoculation, 0.2 ml of hyamine and 0.2 ml of 0.1 N HCl were injected through the rubber stopper into the center well and medium, respectively. All bottles were agitated in a water bath shaker at 37 C for 60 min. At the end of the incubation period, the rubber stopper was removed, and hyamine containing trapped $^{14}CO_2$ was transferred directly into the scintillation vial by cutting the neck of the center well.

**Extraction and separation of lipid from cells.** Total lipid was extracted by the method of Bligh and Dyer (7). Cell lipid (0.5 to 1 mg) was further fractionated into neutral lipid, phospholipid, and glycolipid according to the method of Rouser et al. (33). Activated silicic acid (400 mg) (100 mesh; Mallinckrodt Chemical Works, New York, N.Y.) was packed into a chromatographic tube (0.5 by 10 cm). Lipids were successively eluted as follows: neutral lipid with 10 ml of chloroform, glycolipid with 20 ml of acetone, and phospholipid with 10 ml of methanol. Individual lipid classes were separated and identified as described elsewhere (25). Recovery of radioactivity was determined at each step of the experiment.

**Radioactivity assay.** A Packard Tri-Carb scintillation counter was used for estimating the radioactivity. Radioactivity was determined in scintillation fluid which consisted of 5.5 g of Packard Permablend 1 (Packard Instrument Co., Inc., Warrenville, Ill.) in 1 liter of toluene for lipid samples or Bray's scintillation fluid (8) for samples in aqueous solutions. Lipids were separated by thin-layer chromatography (25), and the silica gel was directly scraped into scintillation vials. Radioactivity was determined by using the dioxane-water system of Snyder (37). Counts per minute were converted to degradations per minute (dpm) by correcting for quenching, using the automatic external standardization of the counter (35).

**Fatty acid methylation and gas-liquid chromatography of the esters.** Lipids were transesterified with 5% HCl in anhydrous methanol (26) and were analyzed by gas-liquid chromatography using a Packard gas chromatograph equipped with an argon ionization detector containing a radium-D foil as a radiation source. A glass column (1.8 m long, 0.32 cm outer diameter) packed with 15% ethylene glycol succinate plus 2% phosphoric acid on Gas Chrom P (80 to 100 mesh) was used isothermally at 180 C to separate the methyl esters. The methyl esters were identified as previously described (25) and were directly collected into scintillation vials using the method of Schlenk and Sand (34).

**Lipid quantitation.** The amounts of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were estimated according to the method of Townsend et al. (46). The peak area of the methyl esters on gas-liquid chromatography was compared with that of a known amount of internal standard (21:0 ester), and the molar concentration of the sample was calculated.

## RESULTS

**Effect of fatty acids on uninfected BHK-21 cells.** Under the conditions described in Materials and Methods, infected cells continued growing normally for 2 additional days after the medium was changed to Waymouth medium containing different amounts of 9-18:1 (Fig. 1). Cell growth was at least twofold higher in Waymouth medium supplemented with 5% calf serum ($WCa_5$) than that in the medium containing the 9-18:1 without serum. Different amounts of 9-18:1 (10 to 40 $\mu$g/ml) had little effect on the cell growth. Inhibition of cell growth was observed in the presence of 80 $\mu$g of 9-18:1 per ml. Cells could not be serially passed in Waymouth medium containing bovine albumin and sodium oleate without the presence of calf serum.

A series of saturated and other unsaturated fatty acids (see Materials and Methods) supplemented in Waymouth medium also did not permit serial passage of BHK-21 cells, but some cell growth occurred after cells adhered to glass as reported for 9-18:1.

**Stability of JEV in Waymouth medium containing different supplements incubated at 37 C.** Table 1 shows that viral infectivity decreased three- to tenfold in the presence of saturated, polyunsaturated, and octadecenoic fatty acids after a 24-h incubation at 37 C. A further decrease in infectivity was observed after 48-h incubation, depending upon the fatty acid present in the medium. Virus incubated in $WCa_5$ or Waymouth medium supplemented with bovine albumin medium and maintained under the same conditions described above showed a 51 and 38% reduction in infectivity, respectively, after 24 h of incubation at 37 C and about a 50% further decrease in infectivity was observed after 48 h of incubation. Virus held in Waymouth medium not supplemented with bovine albumin or fatty acids showed no detectable infectivity after a 24-h incubation period at 37 C. In general, the presence of fatty acid supplements in medium containing extracellular virus decreased viral infectivity with prolonged incubation at 37 C more rapidly than

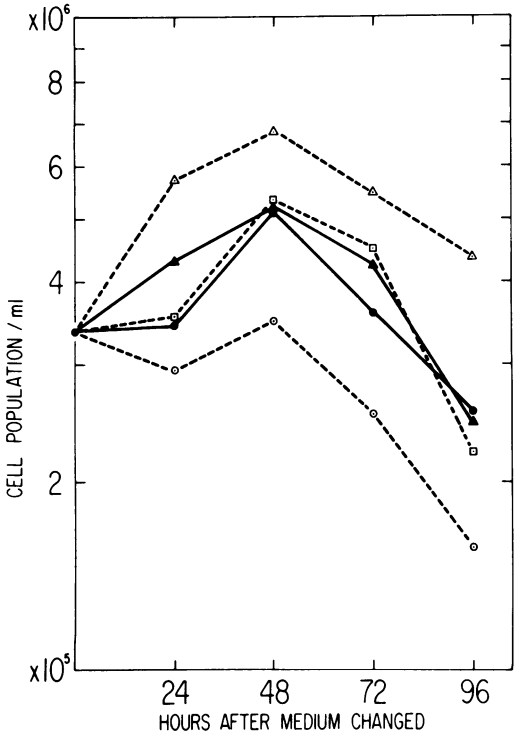


FIG. 1. *Effect of different concentrations of sodium oleate on BHK-21 cell growth. After BHK-21 cells were grown in Eagle MEM with 5% calf serum for 48 h at 37 C, the cells were washed two times with Hanks balanced salt solution without calcium and magnesium. Fresh Waymouth medium was added to the cells, supplemented with 5% calf serum or 2 mg of fatty acid-poor bovine albumin per ml combined with various concentrations of sodium oleate. Cells were further incubated at 37 C, and cell counts were determined at 24-h intervals with the aid of a hemocytometer. Symbols: ⊡, 10 μg of oleate per ml; ▲, 20 μg of oleate per ml; ●, 40 μg of oleate per ml; ⊙, 80 μg of oleate per ml; △, 5% calf serum.*

in control $WCa_5$ and Waymouth medium supplemented with bovine albumin media.

**JEV growth in Waymouth medium with addition of 9-18:1 and/or other supplements.** Various supplements, including serum albumin and/or the sodium salt of fatty acids, were added to Waymouth medium immediately after virus adsorption. Each point in Fig. 2 represents infectivity of released virus at different sampling intervals. Virus growth was highest in $WCa_5$. When JEV was cultivated in Waymouth medium without any supplements, the maximum infectivity was $1.2 \times 10^4$ PFU/ml during a 48-h period of incubation. Addition of bovine albumin (2 mg/ml) to the Waymouth medium increased the infectivity to $1.3 \times 10^5$ PFU/ml. Addition of 9-18:1 (20 μg/ml) further increased the infectivity to $10^7$ PFU/ml, which was a 10-fold lower infectivity of virus than the maximum titer obtained in $WCa_5$ (Fig. 2). Infectivity of cell-associated virus was always 10- to 15-fold lower than that of released virus. A virus-specific cytopathogenic effect occurred 48 h after cell infection. The drop in infectivity observed after 48 h could be attributed to extracellular viral lability at 37 C.

**Effect on JEV growth by 9-18:1 when added at different intervals of time.** Since it was evident that 9-18:1 had a stimulatory effect on JEV growth, an infectivity study was performed with 9-18:1 (20 μg/ml) added to the medium at different intervals of time during the course of infection. Growth of JEV was always higher when the 9-18:1 was added to the medium than in the control medium without supplementation with bovine albumin (Fig. 3). Higher viral infectivity appeared 12 h earlier when 9-18:1 was added to the medium 24 h before the virus inoculation, compared to addition of 9-18:1 after the infection started. However, the peak of virus infectivity, which normally occurred 48 h after the inoculation, was higher when the 9-18:1 was added immediately

TABLE 1. *Stability of JEV in cell-free Waymouth medium supplemented with the sodium salt of various fatty acids (20 mg/ml)[a] at 37 C*

| Fatty acids | Incubation period (h) | |
|---|---|---|
| | 24 | 48 |
| Saturated | | |
| 12:0 | 20.2[b] | 2.8 |
| 14:0 | 21.7 | 2.9 |
| 16:0 | 26.7 | 5.2 |
| 18:0 | 26.4 | 3.7 |
| 20:0 | 27.6 | 4.2 |
| 22:0 | 31.5 | 7.1 |
| Polyunsaturated | | |
| 18:2 | 21.2 | 1.4 |
| 18:3 | 14.1 | 2.4 |
| 20:4 | 14.1 | 1.7 |
| Isomeric octadecenoic acids | | |
| 3-18:1 | 20.9 | 0.4 |
| 4-18:1 | 10.0 | 2.1 |
| 6-18:1 | 12.9 | 3.7 |
| 9-18:1 | 25.0 | 2.8 |
| 9-18:1[c] | 21.0 | 0.2 |
| 11-18:1 | 18.7 | 0.2 |
| Control | | |
| Waymouth medium, no supplements | <0.01 | <0.01 |
| Waymouth medium + 2 mg of bovine albumin per ml | 49.0 | 24 |
| Waymouth medium + 5% newborn calf serum | 62.0 | 29 |

[a] Bovine albumin (2 mg/ml) was present in the medium.
[b] Values are percent of surviving virus (ratio of PFU at each incubation period over PFU at zero time).
[c] 9-18:1 (80 μg/ml).

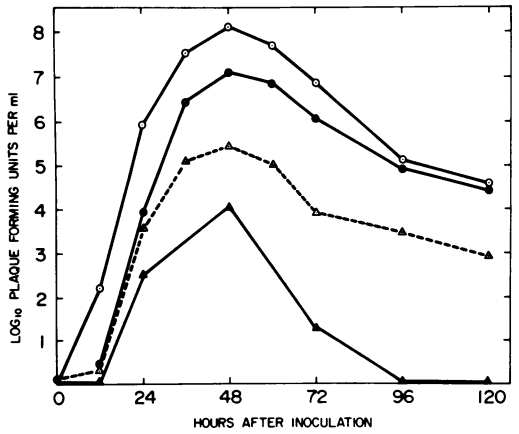


FIG. 2. *Effect of Waymouth medium containing various concentrations of sodium oleate on growth of JEV cultivated in BHK-21 cells. BHK-21 cells were grown in MEM with 5% calf serum. When the cell monolayer was formed, the medium was changed to Waymouth medium without supplements and the monolayer was incubated at 37 C for 24 h. The medium was removed, and the cells were infected with JEV for 90 min at room temperature. Infectivity of released virus was determined by plaque assay at various intervals of time after virus inoculation, using BHK-21 for the assay. Symbols: ⊙, 5% calf serum; ◬, 2 mg of bovine albumin per ml; ●, 2 mg of bovine albumin per ml plus 20 μg of oleate per ml; ▲, no supplements.*

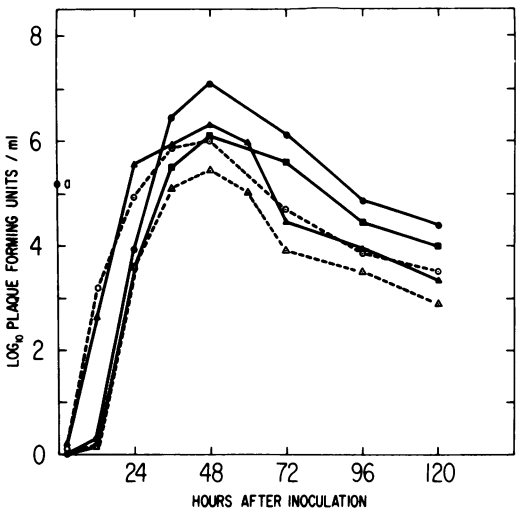


FIG. 3. *Effect of sodium oleate (20 μg/ml) added at different intervals of time on JEV growth. The conditions for cell cultivation, virus infection, and assay of infectivity are described in Fig. 2 and Materials and Methods. Symbols: ▲, oleate present from 24 h before virus inoculation to just before absorption; ⊙, oleate present after absorption to 24 h after infection; ●, oleate present from 24 to 48 h after infection; ◬, no oleate present throughout experiment; a, input virus infectivity.*

after viral adsorption to 24 h after infection than when added before the infection or maintained throughout the experiment. Even when the fatty acid salt was added to the medium 24 h after the infection started, the peak virus titer was threefold higher than that in the medium without the salt (Fig. 3).

**Effect of different concentrations of 9-18:1 on JEV growth.** Figure 4 illustrates the effect of different concentrations of 9-18:1 added immediately after viral adsorption. Forty-eight hours after the virus inoculation, infectivity of released and cell-associated virus was determined. The highest virus infectivity was observed in the presence of 20 to 40 μg of 9-18:1 per ml. When 80 μg of 9-18:1 per ml was present in the medium, no virus infectivity was detected. Infectivity of cell-associated virus was always approximately 1 $\log_{10}$ lower than that of released virus.

**Effect of sodium salts of other fatty acids on JEV growth.** The sodium salts of polyunsaturated fatty acids (2.5 to 80 μg/ml), saturated fatty acids (20 μg/ml), the 3-, 6-, 9-, and 11-octadecenoic acids (2.5 to 80 μg/ml), and all other isomeric octadecenoic acids (20 μg/ml) were added to Waymouth medium after viral adsorption. Infectivity was determined 48 h after infection. In the presence of 10 μg of polyunsaturated fatty acids (9,12-18:2; 9,12,15-

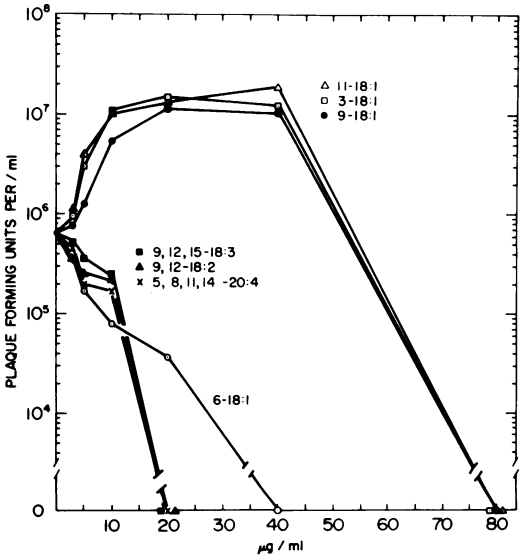


FIG. 4. *Effect of different concentrations of various fatty acid salts on growth of JEV. The conditions for cell cultivation, virus infection, and assay of infectivity 48 h after infection are described in Fig. 2 and Materials and Methods. Symbols: ⊡, 3-18:1; ⊙, 6-18:1; ●, 9-18:1; ◬, 11-18:1; △, 9,12-18:2; ■, 9,12,15-18:3; and ×, 5,8,11,14-20:4.*

18:3; and 5,8,11,14-20:4) per ml, viral growth was partially inhibited and was completely inhibited in the presence of 20 μg/ml (Fig. 4). Viral growth was stimulated in the presence of 2.5 to 40 μg of 3-, 9-, and 11-18:1 isomers per ml and completely inhibited at 80 μg/ml. The 6-18:1 isomer was partially inhibitory at 2.5 μg/ml, increasingly inhibitory to 20 μg/ml, and completely inhibitory at 40 μg/ml (Fig. 4).

The longer the chain length of the saturated fatty acids (12:0 to 22:0) in the medium, the higher the stimulatory effect on viral growth (Fig. 5). Below 15:0 no release of infectious virus was detected, whereas below 18:0 no cell-associated infectious virus was found (Fig. 5).

Addition of salts of isomeric *cis*-octadecenoic acids, 2-18:1 through 17-18:1, to the medium had different effects on JEV growth, depending upon the position of double bond (Fig. 6). When double bonds were at Δ3, Δ11, or Δ13 position, the acids were highly stimulatory for JEV growth, whereas the Δ6 positional isomer was markedly inhibitory. In general, acids with double bonds at odd-numbered positions from the Δ end showed a higher stimulatory effect

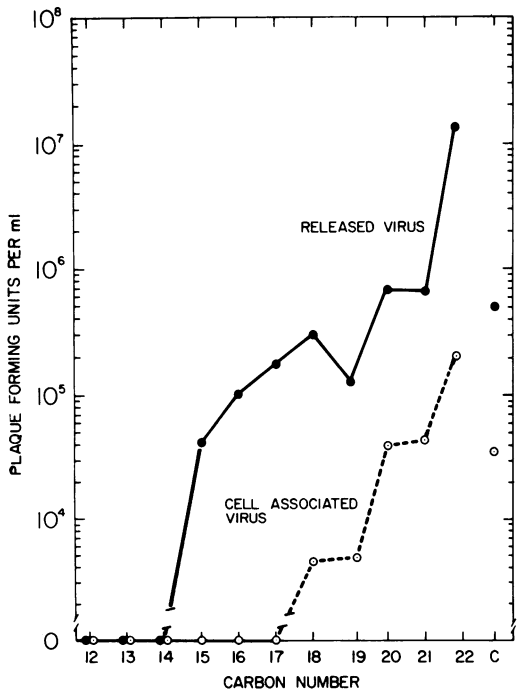


FIG. 5. *Effect of sodium salts of saturated fatty acids (20 μg/ml) on growth of JEV. The conditions for cell cultivation, virus infection, and assay of infectivity 48 h after infection are described in Fig. 2 and Materials and Methods. Symbols: ●, infectivity of released virus; ⊙, infectivity of cell associated virus; c, bovine albumin control.*

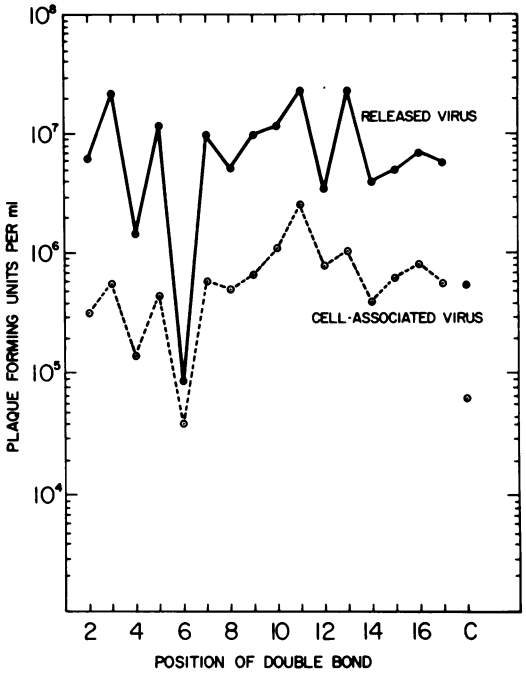


FIG. 6. *Effect of sodium salts of cis-octadecenoic acids (20 μg/ml) on JEV growth. The conditions for cell cultivation, virus infection, and assay of infectivity 48 h after infection are described in Fig. 2 and Materials and Methods. Symbols: ●, infectivity of released virus; ⊙, infectivity of cell associated virus; c, bovine albumin control.*

than neighboring acids with double bonds at even-numbered positions, with the exception of Δ10 > Δ9 and Δ16 > Δ15 and Δ17.

Differences in the effect between acids with double bonds at odd- and even-numbered positions on viral growth tended to be larger when the double bonds were close to the Δ end of the chain than when they were at the ω end (Fig. 6).

**Incorporation of [1-$^{14}$C]oleate into lipids of JEV-infected and uninfected cells.** [1-$^{14}$C]sodium oleate was rapidly incorporated into the total lipids of JEV-infected and control cells (Fig. 7). The labeled oleate was rapidly incorporated into the total lipid of infected and uninfected cells during the first 12 h of incubation, and incorporation remained constant throughout the rest of the experiment. A slightly higher increase of incorporation was observed in infected cells in the first 12 h than in uninfected cells. A concomitant decrease in radioactivity was observed in the supernatant fluid of infected and uninfected cells.

The amount of $^{14}CO_2$ released was 15% of the total radioactivity. The $^{14}CO_2$ evolved, gradually increased up to 24 h after virus inoculation (a.v.i.), and remained constant thereafter.

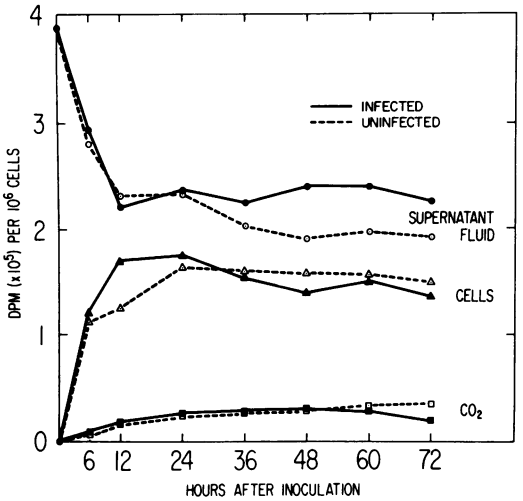


FIG. 7. *Incorporation of [$^{14}$C]oleate into the total lipid of uninfected and JEV-infected BHK-21 cells and $CO_2$. Replicate cultures of infected and uninfected cells in 2-oz bottles were incubated, each bottle containing 3 ml of Waymouth's medium supplemented with a mixture containing 20 μg of [1-$^{14}$C]oleate (0.17 μCi/ml) per ml and 2 mg of bovine albumin per ml. At various intervals of time, samples from triplicate bottles were analyzed for radioactivity in the cell-associated total lipid, lipids of the supernatant culture fluid, and $CO_2$. Symbols: cell lipid (▲, infected; △, control); supernatant fluid (●, infected; ○, control), and $CO_2$ (■, infected cells; □, control cells).*

There was no difference in infected and control cells in the amount of $^{14}CO_2$ produced.

The amount of radioactivity from the nonlipid fraction of infected and control cells, which was recovered from the methanol-water layer of the Bligh-Dyer extraction, was similar to the $^{14}CO_2$ data shown in Fig. 7. However, radioactivity from infected cells was 20 to 30% higher than control cells.

**Incorporation of [1-$^{14}$C]oleate into lipid classes of the cells.** Figure 8 illustrates the results of incorporation of [1-$^{14}$C]oleate into PC, PE, and triglyceride in JEV-infected and control cells. Radioactivity of PC from infected cells suddenly dropped between 12 to 24 h a.v.i. ($1.04 \times 10^6$ to $0.44 \times 10^6$ dpm/$10^7$ cells), whereas the PC of the control had the highest activity ($1.79 \times 10^6$ dpm/$10^7$ cells) at 24 h a.v.i. Rapid increase of radioactivity in PE of infected cells ($1.4 \times 10^6$ dpm/$10^7$ cells) was observed 24 h after inoculation. Radioactivity of PE from control cells remained low ($0.09 \times 10^6$ to $0.37 \times 10^6$ counts/min per $10^7$ cells) during the 48-h observation period. The amount and pattern of radioactivity of triglyceride was the same for infected and control cells during the course of the experiment.

**Changes of PC and PE content.** Changes of PC and PE content between JEV-infected and control cells showed a similar pattern during infection (Fig. 9). However, changes in the amount of PC and PE (micromoles/$10^7$ cells) during 12 to 24 h a.v.i. were quantitatively different between infected and normal cells. PC decreased from 0.16 to 0.05 μmol in infected cells and from 0.21 to 0.13 μmol in control cells, but PE increased from 0.16 to 0.82 μmol in infected cells and from 0.08 to 0.12 μmol in control cells.

**Specific activity of PC and PE of the cells.** Calculation of the specific activity showed some changes in the patterns of PC and PE (Fig. 10). Specific activity of PC was always higher than that of PE (Fig. 10). Radioactivity of PC from infected cells had a lower specific activity (592 dpm/μmol) than that of PC in control cells (959 dpm/μmol) at 24 h a.v.i. Specific activity of PE was shown to be 341 dpm/μmol in infected cells and 201 dpm/μmol in control cells after 24 h a.v.i.

## DISCUSSION

There have been several reports that fatty acids stimulated cell growth in vitro (31). Jenkin and Anderson showed that the sodium salt

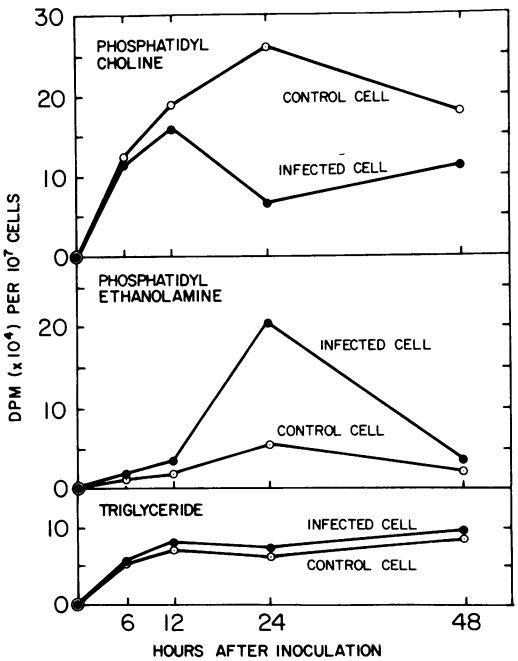


FIG. 8. *Uptake of [$^{14}$C]oleate into phospholipids and triglycerides of uninfected and JEV-infected BHK-21 cells. See Materials and Methods and legend to Fig. 1. Symbols: dpm (× $10^4$) per $10^7$ cells of PC, PE, and triglyceride (●, infected cells; ○, uninfected cells).*

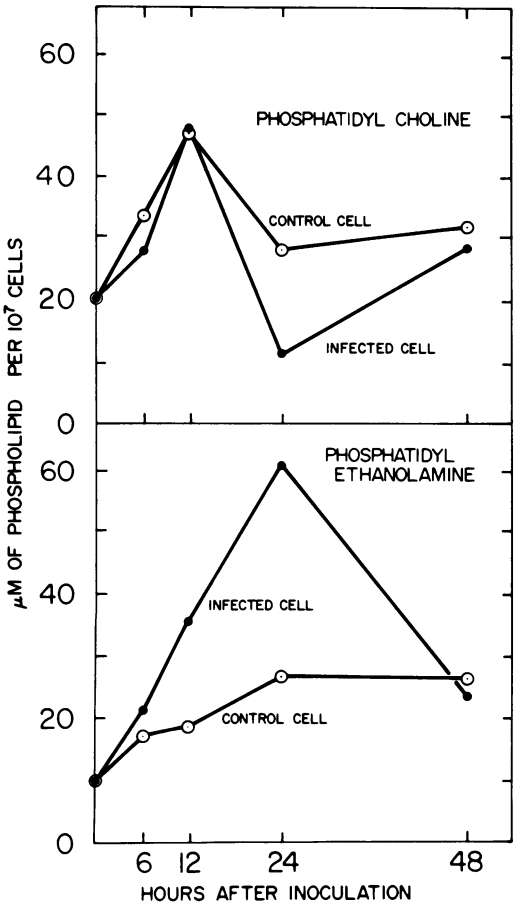


FIG. 9. *Changes in PC and PE in uninfected and JEV-infected BHK-21 cells. The conditions for labeling and infecting the cells are the same as in Fig. 1. The methods for lipid analysis are described in Materials and Methods. Symbols: micromoles of phospholipid per $10^7$ cells of PC and PE (●, infected cells; ⊙, control cells).*

of oleic acid could substitute for calf serum in the growth of LLC-$MK_2$ cells (20), once the cells were attached to the glass. Therefore, failure to grow BHK-21 cells in the medium with the sodium oleate suggests that the nutritional requirements of BHK-21 cells are more critical than that of LLC-$MK_2$ cells or a long adaptation period may be required for growth to occur readily. The almost twofold increase in cell number after the serum-containing medium was changed to medium containing 9–18:1 could be associated with the presence of residual calf serum, since a similar pattern was observed in the same medium without the 9–18:1 (Fig. 1). This observation was used to an advantage, since the fatty acid had little effect on normal cell growth and thereby permitted an investigation of the effect of the fatty acids on viral growth without background interference from the cell.

Bovine albumin in the medium aided in maintenance of viability of the virus to about the same level as observed in medium containing calf serum; therefore, bovine albumin was supplemented in all the experimental studies.

The stimulatory effect of sodium oleate on JEV growth is clearly shown in Fig. 2, where viral infectivity was almost 50-fold higher in the presence of 9–18:1 (20 μg/ml) than in control medium (Waymouth medium with bovine albumin, 2 mg/ml). JEV growth in Waymouth medium supplemented only with 9–18:1 could not be studied because the salt was not well solubilized in the medium without bovine albumin.

Goodman (13) and Spector et al. (40) reported that albumin had several binding sites for fatty acid anions, and Spector et al. (41) demonstrated that uptake of free fatty acid by Ehrlich ascites cells was dependent upon the

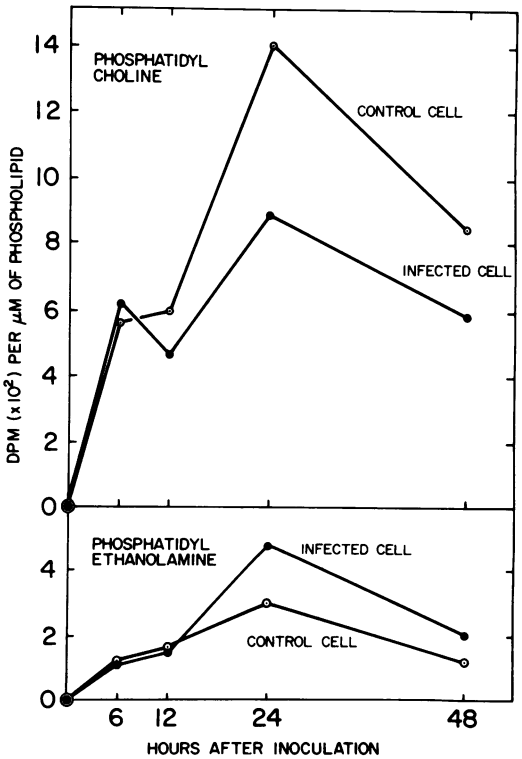


FIG. 10. *Changes in specific activity of PC and PE in uninfected and JEV-infected BHK-21 cells. The specific activity was obtained by dividing the radioactivity (shown in Fig. 2) by the concentration of lipid in each phospholipid (Fig. 3). Symbols: PC and PE (●, infected cells; ⊙, control cells).*

molar ratio of fatty acid to albumin. The same ratio in the present experiment was found for sodium oleate, which meant the fatty acids were readily bound within the binding capacity of the albumin. This fatty acid-albumin binding seems to have provided the higher solubility of fatty acids in the medium and better availability of 9–18:1 for the cells. This hypothesis could account for the increase of JEV growth in the presence of bovine albumin with 9–18:1 in the medium, in addition to the virus protective effect of the albumin.

The finding that the effect of the 9–18:1 on JEV growth was highest when the 9–18:1 was added 0 to 24 h after the virus inoculation suggests that there is a high requirement for fatty acids during virus multiplication. To reduce the effect of residual calf serum on virus growth, the cell monolayer was maintained in Waymouth medium containing bovine albumin alone for 24 h prior to infection. Therefore, the cells were probably in a fasting state at the time of infection so that uptake of 9–18:1 increased when the fatty acid was added to the medium after the infection started.

The reason for the higher yield of virus in the presence of 22:0 is not well understood at this time. Presence of the monoene 9–18:1 seemed to be favored over saturated fatty acids for utilization by the cells or at least for the virus growth (Fig. 5 and 6). Isomeric *cis*-octadecenoic acids have various effects on JEV growth, depending on the location of the double bond. The pattern of the effect of isomers of JEV growth is quite different from the pattern shown for acyltransferase of rat liver microsomes (29), a bacterium, *Leptospira interrogans* serotype *patoc* (21), or mammalian cells, LLC-$MK_2$ cells (22). It appears that each biological system has its own pattern of utilization of octadecenoic acids associated with the presence of an array of different enzyme systems.

It was shown by Stoffel (42) that different enzymatic systems were involved in $\beta$-oxidation of fatty acids with double bonds at even- or odd-numbered positions. This result may suggest that a different pattern for utilization of isomeric octadecenoic acids is present during virus multiplication. A marked inhibitory effect of *cis*-6-octadecenoic acid is of particular interest, though presently there is no reasonable explanation for the observation.

All of the polyunsaturated fatty acids tested showed inhibitory effect on virus growth (Fig. 4). This effect may be associated with the position of a common bond on all of these acids, i.e., $\Delta 12$ in 18:2 and 18:3. Although 9–18:1 shows a stimulatory effect on virus growth, 9, 12–18:2 and 9, 12, 15–18:3 acids are highly inhibitory.

Fatty acid-free bovine albumin contains trace amounts of phospholipid. Therefore, contribution of fatty acids derived from this fraction cannot be excluded from the present results. However, the results illustrate that a more highly defined system than has been previously available allows for more critical studies of viral lipid metabolism.

The results obtained from the study of [1-$^{14}$C]9–18:1 incorporation suggested that little $\beta$-oxidation occurred and that the oleate was mainly incorporated into lipids with little degradation to acetate, or else labeled $^{14}CO_2$ was recycled extremely efficiently in the pool after oxidation.

Apparently incorporation of [$^{14}$C]oleate into PE was stimulated by viral infection between 0 to 24 h a.v.i., whereas incorporation of the isotope into PC was relatively suppressed. It was of particular interest that Waite and Pfefferkorn (48) reported that Sindbis virus infection suppressed [$^{14}$C]choline, as well as $^{32}PO_4$ incorporation, into phospholipid of primary chicken embryo fibroblast but not in BHK-21 cells. Also, the same authors reported that in cells infected with Sindbis virus both showed an increase in incorporation of $^{32}PO_4$ in PE and PC, which was not observed in both phospholipids of BHK-21 cells infected with JEV from 0 to 12 h after infection in our studies.

Decrease of radioactivity in PC of infected cells 24 h a.v.i. was associated with a lower specific activity, whereas increase of radioactivity of PE in infected cells was a reflection of a higher amount of PE synthesis. It was recently reported that phosphoglyceride acyltransferase activity of chorioallantoic membrane of the embryonated egg was inhibited by influenza infection (W. R. Smith and H. A. Blough, Bacteriol. Proc., p. 157, 1970). A similar association could be made in these experiments; i.e., decrease of specific activity of PC was found in infected cells. Also, the results from these observations suggest that incorporation of [$^{14}$C]oleate into PC was suppressed by infection which, in turn, stimulated PE synthesis. PE may have accumulated because of a blockade of the methylation step. Biosynthesis of PC by serial methylation of PE was reported in rat liver microsomes by Bremer and Greenberg (9) and by others (3, 12).

It was concluded from the studies thus far carried out that the effect of JEV infection on infected cells seemed to be a suppression of PC metabolism associated with increase of incorporation of oleate in the PE. Apparent decrease of

fatty acid metabolism was also found in infected cells. However, these findings could be simply the result of rapid utilization of lipid as an energy and/or carbon source in infected cells. Investigation of radioactivity incorporated into released virus, as well as radioactivity remaining or excreted into the medium and specific isotopic tracing of labeled fatty acid precursors into the fatty acids of complex lipids, is necessary to further elucidate the mechanism of the effect of oleate on viral growth.

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