Effect of Mengovirus Replication on Choline Metabolism and Membrane Formation in Novikoff Hepatoma Cells

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Infection of Novikoff rat hepatoma cells (subline NISI-67) with mengovirus resulted in a two- to threefold increase in the rate of choline incorporation into membrane phosphatidylcholine at about 3 hr after infection, without affecting the rate of transport of choline into the cell or its phosphorylation. The time course of virus-stimulated phosphatidylcholine synthesis was compared with the time courses of other virus-induced processes during a single cycle of replication. The formation of viral ribonucleic acid (RNA) polymerase and of viral RNA commenced about 1 hr earlier than the virus-stimulated choline incorporation. Further, isopycnic centrifugation of cytoplasmic extracts indicated that the excess of phosphatidylcholine synthesized by infected cells is not located in the membrane structures associated with the viral RNA replication complex, but with structures of a lower density (1.08 to 1.14 g/cc). These membrane structures probably represent the smooth vesicles which accumulate in the cytoplasm of infected cells during the period of increased phosphatidylcholine synthesis between 3 and 5 hr after infection. They are formed with both newly synthesized phosphatidylcholine and phosphatidylcholine present prior to infection. However, concomitant protein synthesis is not required for the stimulated synthesis of membranes; the effect was not inhibited by treating the cells with inhibitors of protein synthesis at 3 hr after infection, although virus production was inhibited about 90% and virus-induced cell degeneration was markedly reduced and delayed. Production of mature virus began normally at about the same time as the stimulation of phosphatidylcholine synthesis. Treatment of infected cells with puromycin at 2 hr, on the other hand, completely inhibited the stimulation of phosphatidylcholine synthesis.

The virus-induced ribonucleic acid (RNA) polymerase activity of picornavirus-infected mammalian cells and the newly synthesized viral RNA are associated with large membranous cytoplasmic structures (2, 3, 8, 19, 27). Solubilization of the lipid portion of the membranous structures from mengovirus-infected Novikoff cells by treatment with deoxycholate results in the release of the viral RNA polymerase-RNA template complex, along with newly synthesized viral RNA in a particulate form sedimenting slightly more rapidly than the bulk of the cellular polyribosomes (27). A similar, but slower-sedimenting, particle has been isolated from poliovirus-infected HeLa cells (12) and foot-and-mouth disease virus-infected baby hamster kidney cells (2), and has been referred to as viral RNA replication complex (12). Little is known about the nature of the membrane components which are normally associated with the RNA replication complex or what

function, if any, the membranes may have in the synthesis of viral RNA. Protection of the endogenous RNA template may be one function, as it has been shown that the membrane-associated viral RNA polymerase activity from mengovirus-infected cells is highly resistant to inactivation by ribonuclease, but is rapidly destroyed by ribonuclease after treatment with deoxycholate (27). Membranes may have additional functions in viral RNA replication, since the main *in vitro* product of the detergent-solubilized viral RNA polymerase-RNA template complex is viral double-stranded RNA, whereas relatively little single-stranded viral RNA is made (2, 10, 29). In addition, the viral polyribosomes from poliovirus-infected HeLa cells and mature virus are also associated with the large cytoplasmic membranous structures (19). It is, therefore, of interest that infection with picornaviruses stimulates cellular phospholipid synthesis (1, 4, 18) and causes

the accumulation of smooth vesicles (cisternae) in the cytoplasm of the infected cell (1, 5, 6, 17, 31); however, it is uncertain whether there exists any relationship between these two processes or between them and the membrane structures involved in viral replication.

To gain further insight into the relationship between these processes, we have determined in a single cycle of replication of mengovirus in Novikoff rat hepatoma cells the time courses for virus-stimulated phosphatidylcholine synthesis, for the production of viral RNA polymerase, viral RNA, and mature virus, and for the virus-induced formation of clusters of vesicles and cell degeneration. We also investigated the physical nature of the RNA replication complex-containing membrane structures and the membrane structures formed in response to viral infection. A preliminary report of this work has been presented (Plagemann, *Bacteriol. Proc.*, p. 168, 1968).

MATERIALS AND METHODS

Materials. Materials were obtained as follows: ^3H -5-uridine, ^{14}C -methyl- and ^3H -methyl-choline from New England Nuclear Corp., Boston, Mass.; puromycin from Nutritional Biochemicals Corp., Cleveland, Ohio; cycloheximide from Calbiochem, Los Angeles, Calif.; and ^3H -guanosine triphosphate (GTP) and unlabeled nucleoside triphosphates from Schwarz BioResearch, Orangeburg, N.Y. Actinomycin D was a gift from Merck Sharp and Dohme. B11 was 0.1 M phosphate buffer containing 45 mg of sucrose per ml. Sources of other materials have been stated (28).

Cell culture and virus infection. Novikoff rat hepatoma cells (subline N1S1-67) were propagated in suspension culture in Swim's medium 67 as described previously (28, 33). Cells were harvested from cultures in the exponential phase of growth at about 2×10^6 cells per ml. The cells were infected (22), except where indicated otherwise, with 10 to 30 plaque-forming units (PFU) of mengovirus per cell in basal medium (BM42; 28) and were further incubated on a gyratory shaker at 37 C. Suspensions of uninfected cells were prepared and incubated in an identical manner, except that virus was omitted. The time of addition of virus to the cells was designated 0 hr in all experiments. Methods for the enumeration of cells, vital staining with trypan blue, and the assay of virus concentration by hemagglutination titration have been described previously (28, 33).

Incorporation of choline. Suspensions of cells in BM42 were supplemented with labeled choline as indicated in the appropriate experiments. Replicate 0.5-ml samples of suspension were analyzed for total radioactivity associated with cells and radioactivity in acid-insoluble material. For determination of cell-associated radioactivity, two samples were centrifuged at $600 \times g$ for 2 min at 0 C. The cells were rapidly washed in 5 ml of balanced salt solution (23) and suspended in 0.2 ml 0.5 N trichloroacetic acid. These mixtures were heated at 70 C for 30 min and then

analyzed for radioactivity. For determination of radioactivity in acid-insoluble material, another two samples were quickly frozen in a bath of solid CO_2 in ethyl alcohol; these samples were later analyzed for radioactivity in acid-insoluble material as described previously (23).

Extraction of acid-soluble material and lipids and their analysis by thin-layer chromatography. Choline-labeled cells were extracted with perchloric acid, and the lipids were extracted from the acid-insoluble residue by the method of Folch as described previously (23). Perchloric acid extracts were chromatographed on layers of cellulose with a mixture of *n*-butyl alcohol-ethyl alcohol-acetic acid-water (8:2:1:3, v/v); lipid extracts were chromatographed on silica-coated sheets with a mixture of chloroform-methanol-water (65:25:4, v/v) as described previously (23).

Cell fractionation and sucrose density gradient analysis. Cells were fractionated in a solution composed of 0.45 M sucrose and 10 mM sodium ethylenediaminetetraacetate (Na_2EDTA), and the postnuclear fraction was analyzed by isopycnic centrifugation in sucrose density gradients as described previously (23).

Enzyme assays. Virus-induced RNA polymerase activity was measured as described previously (22). Infected cells were lysed by treatment with sodium deoxycholate in the presence of actinomycin D. The lysate from 10^7 cells (0.5 ml) or 1-ml fractions from sucrose density gradients were mixed with 0.4 ml of a solution containing (in micromoles); magnesium acetate, 5; tris(hydroxymethyl)aminomethane (Tris)-chloride (pH 8.2), 50; adenosine, cytidine, and uridine triphosphates, 0.06 each; ^3H -GTP (1,000 $\mu\text{Ci}/\mu\text{mole}$), 0.001; sodium phosphoenol pyruvate, 4; and 8 μg of pyruvate kinase. The mixtures were incubated at 27 C for 2 hr and then analyzed for radioactivity in acid-insoluble material.

Fractions from sucrose density gradients were assayed for the activities of cytochrome oxidase, acid phosphatase, and total adenosine triphosphatase (Mg^{++} - plus Na^+K^+ -activated adenosine triphosphatase) as described previously (23).

Determination of radioactivity. Samples were mixed with 15 ml of a scintillator-solvent mixture which has been described (28). Radioactivity was measured with a Packard liquid scintillation spectrometer (model 2002). The counting efficiency for ^3H was approximately 12%. Simultaneous counting of ^3H and ^{14}C was conducted as described previously (25).

Electron microscopy. Cells were collected by centrifugation, washed in balanced salt solution, and then fixed with 6.2% (w/v) glutaraldehyde in B11 followed by 1% (w/v) OsO_4 in B11. The fixed cells were washed three times with water and then treated with 2% (w/v) uranyl acetate in 10 mM CaCl_2 for 1 hr at room temperature. The cell pellets were dehydrated with ethyl alcohol and propylene oxide and then embedded in epoxy resin (Epon 812). Sections were cut with a glass knife in an LKB Ultratome microtome and then mounted on copper grids covered with a carbon-coated Parlodion film. The sections were stained with 2% (w/v) uranyl acetate followed by Reynold's lead citrate and were examined with a Siemens Elmiskop electron microscope.

RESULTS

Effect of viral infection on choline incorporation by N1S1-67 cells. Penman (18) used ^{14}C -choline as a precursor to measure the poliovirus-induced stimulation of phospholipid synthesis in HeLa cells. We have shown previously (23, 25, 26) that labeled choline is rapidly taken up by N1S1-67 cells and converted to phosphorylcholine which accumulates intracellularly and serves as a precursor for the synthesis of membrane phosphatidylcholine, probably via the Kennedy pathway (14), but that significant amounts of cytidine diphosphate-choline do not accumulate in the cells. A direct incorporation of choline into lecithin by base exchange (32) does not seem to play a major role in the incorporation of choline into phosphatidylcholine by N1S1-67 in suspension culture. Label in phosphorylcholine is readily distinguished from that in phosphatidylcholine. Phosphorylcholine, although not removed by washing cells with balanced salt solution, is solubilized by extraction of the cells with perchloric acid, whereas the phosphatidylcholine remains in the acid-insoluble residue (23). The results in Fig. 1B illustrate that mengovirus infection resulted in a

sudden two- to threefold increase in the rate of choline incorporation into acid-insoluble material at about 3 hr after infection. Incorporation by infected cells ceased between 5 and 6 hr, concomitant with the appearance of trypan blue-stainable cells in the culture (see Fig. 5). Results from thin-layer chromatography of lipid extracts (Table 1) show that in both infected cells and uninfected cells (23) about 95% of the incorporated choline was located in phosphatidylcholine. The remainder was present in sphingomyelin and lysophosphatidylcholine. Further, the results in Fig. 1B demonstrate that the effect on phosphatidylcholine synthesis was the same whether the cells were infected with 6, 75, or 500 PFU per cell. The stimulation of phosphatidylcholine synthesis was not observed when the cells were treated with ultraviolet light-inactivated mengovirus.

It also has been demonstrated previously (26) that the rate-limiting step in the conversion of choline to phosphorylcholine and its incorporation into membrane phosphatidylcholine is the transport of choline into the cell. It was possible, therefore, that the virus-induced increase in choline incorporation into phosphatidylcholine could

FIG. 1. Choline incorporation into total cell material (A) and acid-soluble material (B) by uninfected N1S1-67 cells and cells infected with mengovirus at various multiplicities of infection (m.o.i.). Cells were infected (0 hr) with 6, 75, or 500 PFU per cell. Suspensions of infected and uninfected cells in BM42 (2×10^6 cells/ml) were supplemented with $0.25 \mu\text{Ci}$ (62,000 counts/min) of ^3H -methyl-choline ($100 \mu\text{Ci}/\mu\text{mole}$) per ml at 0.5 hr and then incubated at 37°C on a gyratory shaker. Duplicate samples of 0.5 ml of suspension were analyzed for total radioactivity associated with the cells (A) or for radioactivity in acid-insoluble material (B) as described in Materials and Methods. For clarity, the values from cells infected with 75 PFU per cell have been omitted from A. Points in B represent averages of the duplicate analyses.

TABLE 1. *Thin-layer chromatography of lipids extracted from uninfected and mengovirus-infected cells as a function of time of labeling with ^3H -choline^a*

Fraction	^3H /fraction (counts/min)					
	Uninfected			Mengovirus-infected		
	2.5 hr ^b	3.5 hr	5.0 hr	2.5 hr	3.5 hr	5.0 hr
Above phosphatidylethanolamine.....	0	0	0	19	2	5
Phosphatidylethanolamine.....	1	0	7	0	5	3
Phosphatidylcholine.....	610	1,330	2,430	566	1,844	3,150
Sphingomyelin.....	1	6	56	2	10	19
Lysophosphatidylcholine.....	5	56	106	7	36	89
Below lysophosphatidylcholine.....	0	6	13	0	2	8

^a Cells were labeled with ^3H -choline as described in the legend to Fig. 1. Lipids were extracted from the cells and chromatographed on silica gel-coated sheets (solvent: chloroform-methanol-water, 65:24:4, v/v) as described in Materials and Methods.

^b Time of labeling.

have resulted from an increased rate of transport of choline into the cell. The results in Fig. 1A, however, show that this was not the case, since infection had no effect on the time course of incorporation of choline into total cell material (acid-soluble plus acid-insoluble material) until about 4 hr after infection. Thin-layer chromatography of acid-soluble extracts from choline-labeled infected cells showed that over 95% of the label was associated with phosphorylcholine and the remainder with choline. This is similar to uninfected cells (23). Labeling of additional acid-soluble components from choline, as has been reported for poliovirus-infected HeLa cells (21), has not been observed with either uninfected (23) or infected NISI-67 cells.

The loss of phosphorylcholine from infected cells beginning between 4.5 and 5 hr after infection (Fig. 1A) was probably a result of virus-induced cell lysis, as it coincided with an increase in the number of trypan blue-stainable cells in the culture (see Fig. 5). At about the same time, infected cells begin to lose their pool of nucleotides (Shea and Plagemann, *in preparation*) and to release mature virus (28). Until 4.5 hr after infection, however, infected cells behave like uninfected cells (23) in that they do not leak phosphorylated intermediates. This has been demonstrated by results from pulse-chase experiments with choline, similar to those previously performed with uninfected cells (23). Infected cells were labeled with ^3H -choline from 0.75 to 1.5 hr after infection and then were incubated in fresh medium containing unlabeled choline until 6 hr. Throughout the pulse and chase periods, samples of suspension were analyzed for radioactivity in total cell material and in acid-insoluble material.

As with uninfected cells (23), about 90% of the total radioactivity associated with the infected cells after 45 min of labeling was located in phosphorylcholine (see Fig. 1). During the chase, about 30% of the labeled phosphorylcholine accumulated intracellularly was incorporated into acid-insoluble material, whereas the total amount of radioactivity associated with the cells remained constant until trypan blue-stainable cells began to appear in the culture at 4.5 hr. Subsequently, the remaining intracellular phosphorylcholine was rapidly lost from the cells.

In the following experiment, the effect of inhibition of protein synthesis on the virus-induced stimulation of phosphatidylcholine synthesis was investigated. Suspensions of uninfected and infected cells were supplemented with ^3H -choline at 0.5 hr and with puromycin at 0.5, 2, or 3 hr, and samples of suspension were analyzed for radioactivity in acid-soluble material (Fig. 2A). Uninfected control cultures were included for each treatment, since treatment with puromycin itself results in a progressive decrease in the rate of choline incorporation (23; see Fig. 2A).

The results in Fig. 2A demonstrate that treatment of the cells with puromycin at 0.5 or 2 hr after infection almost completely inhibited the virus-induced stimulation of phosphatidylcholine synthesis. This treatment also inhibited completely the production of mengovirus and virus-induced cell degeneration as measured by vital staining with trypan blue (Table 2). In contrast, addition of puromycin at 3 hr had little effect on virus-stimulated choline incorporation (Fig. 2A), although virus production was still inhibited about 90% and cell degeneration was markedly delayed and low (Table 2). The latter observation

FIG. 2. Effect of (A) puromycin (PM) and (B) actinomycin D on virus-stimulated choline incorporation. (A) Suspensions of infected and uninfected cells in BM42 (2×10^6 cells/ml) were supplemented with 0.25 μCi (62,000 counts/min) of ^3H -methyl-choline (100 $\mu\text{Ci}/\mu\text{mole}$) per ml at 0.5 hr; at various times thereafter, duplicate 0.5-ml samples of suspension were analyzed for radioactivity in acid-insoluble material (—PM). At 0.5, 2, and 3 hr, portions of both suspensions were supplemented with puromycin to 50 μg per ml and further monitored for radioactivity in acid-insoluble material. (B) Suspensions of infected and uninfected cells in BM42 (2×10^6 cells/ml) were supplemented with actinomycin D to 2 μg per ml at 0.5 hr, and portions thereof received 0.25 μCi (62,000 counts/min) of ^3H -methyl-choline (100 $\mu\text{Ci}/\mu\text{mole}$) per ml at 1 and 3.5 hr. Samples of 0.5 ml of each suspension were analyzed for radioactivity in acid-insoluble material. All points represent averages of duplicate samples.

TABLE 2. Effect of addition of puromycin at various times after infection on virus yield and virus-induced cell death^a

Time of puromycin addition (hr after infection)	Hemagglutinin (units/ml)		Trypan blue-stainable cells (% of total)	
	1 hr	8 hr	6 hr	8 hr
None added	160	5,120	70	95
0.5	160	160	<5	<5
2	—	160	<5	<5
3	—	640	5–10	20
4	—	2,560	50	80
5	160	5,120	70	95

^a The details of the experiment are described in the legend to Fig. 2A. Samples of a suspension of infected cells were supplemented with 50 μg of puromycin per ml at the indicated times after infection. The suspensions were assayed for hemagglutination activity and percentage of cells stainable by trypan blue at the indicated times after infection as described in Materials and Methods.

probably explains the finding that infected cells treated with puromycin at 3 hr after infection continued to incorporate choline until the termination of the experiment at 8 hr (Fig. 2A),

whereas choline incorporation ceased in infected cells not treated with puromycin when the cells became trypan blue stainable between 5 and 6 hr after infection (Fig. 1 and 2). Similar results were obtained when the cells were treated with cycloheximide (100 $\mu\text{g}/\text{ml}$), but the results were more difficult to evaluate because cycloheximide causes a rapid but transitory slight stimulation of choline incorporation into phosphatidylcholine without affecting its uptake or phosphorylation (23). Independent experiments showed that treatment of infected or uninfected cells with 50 μg of puromycin or 100 μg of cycloheximide per ml of suspension inhibited amino acid incorporation into protein over 95% within 5 min of their addition.

Actinomycin D also causes a progressive decrease in choline incorporation (23), but did not inhibit the virus-induced stimulation of phosphatidylcholine synthesis (Fig. 2B).

Vesiculation of infected cells. Results from a number of studies (1, 5, 6, 17, 31) have shown that infection of mammalian cells with picornaviruses results in the accumulation of clusters of vesicles (cisternae) in the cytoplasm of infected cells. Electron microscopy showed that similar vesicular bodies accumulate in mengovirus-infected NISI-67 cells. Examination of sections of samples of cells

taken at 1-hr intervals after infection indicated that extensive vesiculation occurred between 3 and 4 hr after infection (Fig. 3), resulting in the displacement of the nucleus to one side of the cell. There was little vesicle formation until 3 hr, and the cells did not look significantly different from uninfected controls. Uninfected NISI-67 cells contain only small amounts of endoplasmic reticulum, and other types of vesicles or vacuoles, except for the Golgi apparatus, are almost completely absent from exponentially growing cells (23). The vesicles accumulating in infected cells were bounded by a single membrane (Fig. 4), as has been reported for other systems (1, 5, 6, 17, 31). Cells at 5 hr after infection looked similar to those at 4 hr, with a somewhat greater degree of vesiculation and distortion of the nucleus (see Fig. 3). The results indicated that the formation of foci of vesicles was coincidental with the virus-stimulated synthesis of phosphatidylcholine between 3 and 5 hr (see Figs. 1B and 5). A separation of the nuclear membrane as observed in HeLa cells early after infection with poliovirus (17) was not observed in mengovirus-infected NISI-67 cells, nor were distinct foci of "viroplasm" or crystals of virus particles detectable in any of the sections examined. The failure of NISI-67 cells to accumulate virus crystals might be related to the early lysis and release of virus from the infected cells (6).

Comparison of time courses of various processes in infected cells. In the following experiment, samples of infected cells were analyzed as a function of time after infection for virus-induced RNA polymerase activity, for the synthesis of viral RNA and protein and of mature virus, for the virus-stimulated synthesis of phosphatidylcholine, for the leakage of cytoplasmic protein, and for cell degeneration. Virus-stimulated phosphatidylcholine synthesis was expressed as the amount of choline incorporated into acid-insoluble material by infected cells minus the amount incorporated by an equivalent number of uninfected cells. The results presented in Fig. 5 demonstrate that the formation of viral RNA polymerase and the synthesis of viral RNA began at 2 hr after infection, that is, approximately 1 hr before any virus-stimulated synthesis of phosphatidylcholine became apparent. Almost all of the RNA polymerase and about 50% of the total viral RNA made in infected cells had been formed before the virus-stimulated phosphatidylcholine and vesicle formation began. The secondary increase in polymerase activity occurring coincidental with cell degeneration between 5 and 7 hr after infection is not inhibited by inhibition of protein synthesis, and thus does not represent *de novo* synthesis of polymerase (24). During the same

period (5 to 7 hr), the rate of protein synthesis decreased rapidly (not shown, *see* 28) concomitant with a loss of polyribosomes (22), and the cells lost cytoplasmic proteins and RNA (not shown, *see* 28). Virus production, as measured by the incorporation of ^3H -uridine into virus particles, began at about the same time as the virus-stimulated phosphatidylcholine synthesis (3 hr after infection, Fig. 5), indicating that the synthesis of capsid proteins must have begun prior to that time.

Turnover of phosphatidylcholine in infected cells and isopycnic centrifugation of cytoplasmic extracts. The following type of experiment was undertaken to investigate further the nature of the membrane components that are synthesized by NISI-67 cells in response to viral infection. Cells were grown in the presence of ^3H -choline for 2 days. Then a portion of the cells was infected, treated with actinomycin D, and labeled with ^{14}C -choline beginning at 3 hr after infection. Uninfected cells were treated and labeled identically. Samples of both suspensions were analyzed for ^3H and ^{14}C in acid-insoluble material (Fig. 6A). Results from previous experiments have shown that after 2 days of growth with labeled choline the intracellular pool of labeled phosphorylcholine is relatively small compared with the amount of choline incorporated into membrane phosphatidylcholine, and that there is no significant further incorporation of label into phosphatidylcholine during a subsequent incubation of the cells in fresh medium (see Fig. 6A). Thus, during the chase the rate of turnover of the choline moiety of phosphatidylcholine can be estimated (25). The data in Fig. 6A show that virus infection had no effect on the turnover of ^3H -labeled phosphatidylcholine, whereas it caused a stimulation of the incorporation of ^{14}C -choline into phosphatidylcholine. As indicated by results from thin-layer chromatography of lipid extracts from the labeled cells, over 90% of the ^3H or ^{14}C was located in phosphatidylcholine throughout the period of investigation.

At 4.5 hr, samples of uninfected and infected labeled cells were disrupted in a solution composed of 0.45 M sucrose and 10 mM Na_3EDTA , the nuclei were removed by low-speed centrifugation, and the cytoplasmic fractions were analyzed by isopycnic centrifugation in sucrose density gradients (Fig. 6B and C). We have shown previously (23) that mitochondria from cells incubated in BM42 equilibrate in this type of gradient at approximately 1.18 g/cc (fractions 23–25, Fig. 6B) as indicated by the distribution of cytochrome oxidase. Fragments of the plasma membrane equilibrate at about 1.15 g/cc (fractions 19–20, Fig. 6B) as indicated by peaks of Na^+K^+

FIG. 3. Electron micrograph of a section from a 4-hr infected N1S1-67 cell. Sections were prepared and examined as described in Materials and Methods. $\times 12,000$. N = nucleus, M = mitochondria, VB = vesicular bodies, ER = endoplasmic reticulum.

FIG. 4. *Enlargement of a focus of vesicles in a 4-hr infected cell. $\times 78,000$.*

activated adenosine triphosphatase and acid phosphatase. Fractionation of cells in the sucrose-EDTA solution ensures the release of ribosomes from the membranes and their dissociation, and

prevents nonspecific binding of cytoplasmic components to mitochondria and other membranes (*see 23*). Most of the phosphatidylcholine synthesized by uninfected cells is associated with the

FIG. 5. Time courses of virus-stimulated phosphatidylcholine synthesis, formation of virus-induced RNA polymerase, production of viral RNA and virus, and virus-induced cell degeneration. Suspensions of infected and uninfected cells in BM42 (2×10^6 cells/ml) were supplemented with actinomycin D (AcD) to $2 \mu\text{g}$ per ml at 0.5 hr. To a portion of each suspension, ^3H -methyl-choline ($100 \mu\text{Ci}/\mu\text{mole}$) was added to $0.25 \mu\text{Ci}$ per ml at 2 hr, and the suspensions were monitored for radioactivity in acid-insoluble material. Virus-stimulated choline incorporation ($\bullet$) was expressed as the difference between the radioactivity in infected cells and that in uninfected cells (see Fig. 1B). Other samples of the suspension of infected cells were assayed for viral RNA polymerase activity ($\circ$) and for trypan blue-stainable cells ($\times$). Another portion of the suspension of actinomycin-treated infected cells was supplemented with 0.01 mM ^3H -5-uridine ($100 \mu\text{Ci}/\mu\text{mole}$) at 1 hr after infection; at various times thereafter, duplicate 0.5-ml samples were analyzed for radioactivity in acid-insoluble material (Δ) or for radioactivity in virus particles ($\blacktriangle$) as described previously (24).

mitochondria and plasma membrane fragments; relatively little is associated with membrane structures of lower density (23; see Fig. 6B) which seem to represent smooth endoplasmic reticulum. The profiles in Fig. 6B also show that the distribution of phosphatidylcholine among the various membrane components was the same whether the cells were labeled during 2 days of growth (^3H) or during a 75-min subsequent incubation in

BM42 (^{14}C). A comparison of the profiles in Fig. 6B and C, however, shows that in virus-infected cells the ^{14}C -choline was largely incorporated into membrane structures of relatively low density (1.08 to 1.14 g/cc, fractions 8–18). The latter probably are the single-membrane vesicles shown by electron microscopy to be formed between 3 and 5 hr after infection, since the profiles of cytochrome oxidase, adenosine triphosphatase, and acid phosphatase were very similar in cytoplasmic fractions from infected and uninfected cells (Fig. 6B and C). It will also be noted that the low-density membrane structures were composed of both newly synthesized phosphatidylcholine (^{14}C -labeled) and phosphatidylcholine synthesized prior to infection (^3H -labeled), although the relative proportion of new to old phosphatidylcholine was higher in this fraction than in the mitochondrial peak (fractions 22–28).

The results in Fig. 7A demonstrate that most of the viral RNA polymerase activity was not associated with the low-density membrane particles. Cells were fractionated at 3.5 hr after infection in the sucrose-EDTA solution, the cytoplasmic fraction was subjected to isopycnic centrifugation, and the fractions from the gradient were analyzed for viral RNA polymerase and cytochrome oxidase activities. Most of the viral RNA polymerase activity was associated with the mitochondria or membrane structures of similar density, as indicated by the coincidence of the polymerase and cytochrome oxidase activity profiles in fractions 20–30. About 50 to 100% more RNA polymerase activity was recovered from infected cells when the cells were fractionated in the sucrose-EDTA solution than when fractionated in a solution employed previously (10 mM Tris-chloride, pH 7.4, 10 mM NaCl, and 1.5 mM MgCl_2 ; 27). About 40% of the polymerase activity placed on the gradient was recovered in the gradient fractions after centrifugation (Fig. 7A). Fractions from a gradient of a cytoplasmic fraction from uninfected cells exhibited little RNA polymerase activity (Fig. 7B). Some activity coincided with that of cytochrome oxidase and some was recovered in the upper 6 ml of the gradient. Results from additional studies have shown that the polymerase activity in fractions 22–24 was partially inhibited by actinomycin D and thus probably represents mitochondrial deoxyribonucleic acid-dependent RNA polymerase. The incorporation of ^3H -GTP by fractions 1–6 was stimulated approximately threefold by omission of uridine, cytidine, and adenosine triphosphates from the reaction mixture, and thus was probably due to the synthesis of polyguanylic acid.

FIG. 6. Turnover of phosphatidylcholine in uninfected and infected cells (A) and isopycnic centrifugation of cytoplasmic extracts from uninfected (B) and infected (C) choline-labeled cells. A culture of NISI-67 cells was initiated by inoculating medium 67 with 2×10^4 cells per ml and supplemented with $0.25 \mu\text{Ci}$ of ^3H -methyl-choline ($100 \mu\text{Ci}/\mu\text{mole}$) per ml. After 2 days of growth (at 2.2×10^6 cells/ml), the cells were collected by centrifugation and washed once in warm (37°C) BM42. One half of the cells were infected in BM42 with 25 PFU of mengovirus per cell (0 hr) and the other half were suspended in BM42 in the absence of virus. Both suspensions were supplemented with actinomycin D to $2 \mu\text{g}$ per ml at 0.5 hr and with $0.02 \mu\text{Ci}$ of ^{14}C -methyl-choline ($7.6 \mu\text{Ci}/\mu\text{mole}$) at 3.25 hr. Both suspensions were monitored for ^3H ($\circ$, $\bullet$) and ^{14}C (Δ , $\blacktriangle$) in acid-insoluble material (A). At 4.5 hr, 1.5×10^6 cells from each suspension were fractionated in 3 ml of a solution composed of 0.45 M sucrose and 10 mM Na_3EDTA , and the postnuclear fractions were each layered over 28 ml of a linear 0.5 to 1.6 M gradient of sucrose in 1 mM Na_3EDTA . The gradients were centrifuged in a SW25.1 rotor at 20,000 rev/min (model L-2 Beckman ultracentrifuge) for 12 hr at 4°C (B and C). Fractions of 1 ml were collected from the gradients by means of an ISCO gradient fractionator (22), and these were analyzed as follows: (i) 0.02 to 0.05 ml for cytochrome oxidase activity ($\circ$); (ii) 0.2 ml for acid-phosphatase activity (Δ); (iii) 0.2 ml for total adenosine triphosphatase (ATPase) activity (Mg^{++} -plus Na^+K^+ -activated ATPase) (∇); and (iv) the remainder of each fraction for ^3H ($\bullet$) and ^{14}C ($\blacktriangle$) in acid-insoluble material.

DISCUSSION

The present investigation confirms reports by other investigators (1, 18, 21) that infection of mammalian cells with picornaviruses stimulates the incorporation of choline into phospholipids by the cells. Further, it is shown that this stimulation does not simply reflect an increase in the transport of choline into the cells or its phosphorylation, and thus seems to reflect an increase in the *de novo* synthesis of phosphatidylcholine. The former possibility had to be considered, since transport is the rate-limiting step in the conversion of choline to phosphorylcholine and its incorporation into phosphatidylcholine (26).

In NISI-67 cells, phosphatidylcholine is the only phospholipid that becomes labeled from choline in the medium, except for small amounts of label in sphingomyelin and lysophosphatidylcholine (23; Table 1). The labeling pattern is about the same whether the cells are incubated with labeled choline for 1 or 6 hr and is not affected by mengovirus replication. These results are similar to those obtained with poliovirus-infected HeLa cells (21), but in contrast to the results of Amako and Dales (1), who reported that up to 40% of the total choline incorporated by L cells is recovered in six phospholipid fractions distinct from phosphatidylcholine and that the labeling

FIG. 7. Isopycnic centrifugation of cytoplasmic fractions from infected (A) and uninfected (B) cells for RNA polymerase activity. Suspensions of infected and uninfected cells in BM42 (2×10^6 cells/ml) were incubated on a gyratory shaker for 3.5 hr. Then samples of 1.6×10^8 cells were disrupted and the postnuclear fractions were centrifuged through sucrose density gradients as described in the legend to Fig. 6, except that the gradients were centrifuged for 4 hr rather than 12 hr. After centrifugation, 0.02 to 0.05 ml of each fraction was assayed for cytochrome oxidase activity (○), and the remainder, for viral RNA polymerase activity (●) as described in Materials and Methods.

pattern varied markedly with the length of time of labeling.

The results of Cornatzer et al. (4) suggest that the synthesis of all phospholipid fractions is stimulated by picornaviruses, with the possible exception of phosphatidylserine, but the mechanism by which infection exerts this effect is still uncertain. It seems to be a late effect and not related to the formation of membranes associated with the viral RNA replication complex, since viral RNA synthesis begins at least 1 hr prior to the virus-stimulated synthesis of phosphatidylcholine (Fig. 5; 20) and since the viral RNA polymerase activity is associated with a membrane fraction that can be separated by isopycnic centrifugation from the membrane vesicles formed in response to viral infection. It also does not seem to be a consequence of an increased turnover of phosphatidylcholine in infected cells. It seems more likely that the increased phospholipid synthesis is related to the virus-induced formation of clusters of smooth vesicles in the cytoplasm. This conclusion is suggested by the close temporal relationship between the increase in phospholipid synthesis and the appearance of clusters of vesicles between 3 and 5 hr after infection, as well as by the finding that the infected cells incorporate

choline largely into membrane structures with a low density. It seems that in poliovirus-infected HeLa cells, too, the increase in phospholipid synthesis (18, 20) and the proliferation of smooth vesicles (5, 17) first become detectable around 3 hr after infection, but the data are not directly comparable because they were obtained in different laboratories and probably not under identical experimental conditions. The results from the autoradiographic study of choline-labeled mengovirus-infected L cells by Amako and Dales (1) are consistent with the conclusion that the stimulated phospholipid synthesis is related to the proliferation of cisternae, since a large proportion of the grains were recovered in the foci of newly formed vesicles. But temporally, the two processes did not correlate in infected L cells. Whereas a stimulation of choline incorporation was observed as early as 2 hr after infection, rapidly proliferating cisternae became evident in the centrosphere region only between 5 and 6 hr (1). The autoradiographic study also indicated that the cisternae are formed with both newly synthesized phosphatidylcholine and phosphatidylcholine that was present prior to infection (1). This conclusion has been confirmed by the present biochemical analyses.

Amako and Dales also reported (1) that the mengovirus-induced enhancement of choline incorporation by L cells is demonstrable only when the cells are treated with actinomycin D. In NISI-67 cells, on the other hand, the viral effect was equally observed whether or not the cells were treated with actinomycin D. Further, it was found that L cells continue to incorporate choline into phosphatidylcholine for several hours after the virus-induced rupture of the cell membrane (1). In contrast, NISI-67 cells regularly start losing their pool of accumulated phosphorylcholine and cease to incorporate choline into phosphatidylcholine when they become stainable by trypan blue.

The increased synthesis of phospholipids and of cytoplasmic vesicles is probably a general host response to a viral product, since a similar cytoplasmic membrane formation is observed in cells after infection with other viruses or treatment with India ink or carcinogenic dyes (see 7). It probably does not involve a virus-induced synthesis of a cellular protein(s), since the enhancement of phosphatidylcholine synthesis is not inhibited by actinomycin D, and ultraviolet light-inactivated virus is inactive. It might involve an inactivation of the phospholipid-synthesizing system or an interference with its normal regulation. The production of the responsible viral product requires protein synthesis between 2 and 3 hr after infection, since the increase in choline incorporation in

poliovirus-infected HeLa cells (18) or mengovirus-infected NISI-67 cells (Fig. 2) is inhibited by treating the cells with inhibitors of protein synthesis at 2 hr, whereas the addition of the inhibitors at 3 hr has no effect. It cannot be decided at present which of the viral products is responsible for the effect. Any of the virus-induced proteins could be the responsible toxic principle. But it could also be the viral RNA itself, since treatment of cells with inhibitors of protein synthesis prior to 2 hr after infection inhibits the formation of viral RNA polymerase and thus viral RNA (27), whereas at 3 hr a large proportion of the total yield of viral RNA has already been produced (Fig. 5).

If one of the capsid proteins is the toxic agent, small amounts of it must be sufficient to induce a maximum stimulation of phosphatidylcholine synthesis. This is indicated by the fact that inhibition of protein synthesis at 3 hr after infection has no effect on the virus-induced choline incorporation (Fig. 2A), whereas it almost completely inhibits virus production (Table 2), probably by inhibiting the synthesis of capsid protein. The synthesis of capsid proteins probably begins in mengovirus-infected NISI-67 cells shortly before 3 hr after infection, since it has been shown that in many systems the synthesis of capsid proteins begins just prior to the formation of mature virus particles (9, 11, 13, 15, 16, 28, 30). Further, the increased phosphatidylcholine synthesis is probably not directly related to virus-induced cell degeneration, since the latter, like virus production, is largely prevented by treating the infected NISI-67 cells with puromycin at 3 hr after infection.

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