JOURNAL OF VIROLOGY, Nov. 1970, p. 637-643

Vol. 6, No. 5
Printed in U.S.A.

Phospholipid Synthesis in Sindbis Virus-Infected Cells

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Received for publication 4 August 1970

We investigated the metabolic requirements for the decrease in phospholipid synthesis previously observed by Pfefferkorn and Hunter in primary cultures of chick embryo fibroblasts infected with Sindbis virus. The incorporation of $^{32}PO_4$ into all classes of phospholipids was found to decline at the same rate and to the same extent; thus, incorporation of ^{14}C-choline into acid-precipitable form provided a convenient measure of phospholipid synthesis that was used in subsequent experiments. Experiments with temperature-sensitive mutants suggested that some viral ribonucleic acid (RNA) synthesis was essential for the inhibition of choline incorporation, but that functional viral structural proteins were not required. The reduction in phospholipid synthesis was probably a secondary effect of infection resulting from viral inhibition of the cellular RNA and protein synthesis. All three inhibitory effects required about the same amount of viral RNA synthesis; the inhibition of host RNA and protein synthesis began sooner than the decline in phospholipid synthesis; and both actinomycin D and cycloheximide inhibited ^{14}C-choline incorporation in uninfected cells. In contrast, incorporation of ^{14}C-choline into BHK-21 cells was not decreased by 10 hr of exposure to actinomycin D and declined only slowly after cycloheximide treatment. Growth of Sindbis virus in BHK cells did not cause the marked stimulation of phospholipid synthesis seen in picornavirus infections of other mammalian cells; however, inhibition was seen only late in infection.

Sindbis virus and the picornaviruses apparently have opposite effects on the synthesis of phospholipids in infected cells. Poliovirus infection of HeLa cells stimulated the incorporation of $^{32}PO_4$ (5) and of ^{14}C-choline (10) into cellular phospholipids, and mengovirus had a similar effect in L cells (2). It was suggested (2, 10) that this increase might reflect the formation of membranous structures involved in picornavirus growth. Arbovirus replication is known to cause the development of several types of cytoplasmic vesicles (7). In addition, the virion acquires its membranous outer coat by budding through either the cell membrane (1) or the membrane of cytoplasmic vacuoles (1, 8). Despite this extensive involvement of membranous structures, Sindbis virus infection of primary cultures of chick embryo fibroblasts (CEF) causes a decrease in the incorporation of $^{32}PO_4$ into phospholipid that cannot be explained by removal of part of the membrane by maturing virions (12).

The contrast between these results could arise from basic differences between the mechanisms of replication of these two groups of small ribonucleic acid (RNA) viruses, or from differences in the cells involved. We therefore decided to study further the effect of Sindbis virus infection on phospholipid synthesis in two types of cells: CEF and BHK. BHK cells (derived from hamster kidneys) are, like HeLa and L cells, a continuous mammalian cell line, but one in which Sindbis grows well. We employed temperature-sensitive (*ts*) mutants of Sindbis virus and various antimetabolites in an attempt to elucidate the mechanism of viral inhibition of phospholipid synthesis. A preliminary report of this work has already appeared (M. R. F. Waite and E. R. Pfefferkorn, Bacteriol. Proc., p. 190, 1970).

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of primary CEF were prepared as described previously (11) and incubated before use for 48 to 72 hr in Eagle' medium containing 3% calf serum and the following antibiotics: penicillin (50 units/ml), streptomycin (50 μg/ml), neomycin (100 μg/ml), and mycostatin (50 units/ml). The BHK-21 (C-13) cell line was obtained from the American Type Culture Collection, Rockville, Md. Cultures were grown in Eagle' medium supplemented with 10% Tryptose phosphate broth (Difco) and 10% calf serum. The isolation and char-

acterization of the *ts* mutants of Sindbis virus and the heat-resistant (HR) stock from which they were derived were previously described (3, 4).

Confluent monolayer cultures of BHK or CEF cells were infected by exposing them for 1 hr at 23 C to 10 plaque-forming units (PFU) per cell of the appropriate virus stock in Hanks balanced salt solution containing 5% dialyzed rabbit serum. At zero time, the inoculum was replaced by Eagle' medium containing 3% dialyzed rabbit serum at the appropriate temperature. Actinomycin D (1 μg/ml) or cycloheximide (10 μg/ml) was added as indicated. All experiments described here were completed before any cytopathic effect or detachment of cells from the monolayer became apparent, unless otherwise noted.

Radioisotopes and labeling procedures. ^{14}C-choline (19 mCi/mmole), ^{3}H-leucine (58 Ci/mmole), and ^{3}H-uridine (20 Ci/mmole) were obtained from Schwarz BioResearch Inc. Their incorporation into trichloroacetic acid-precipitable material (phospholipid, protein, RNA) was determined as described previously (16).

Cultures to be labeled with $^{32}PO_4$ were incubated after infection in medium containing one-tenth the usual amount of inorganic phosphate, and carrier-free phosphate (2.5 μCi/ml) obtained from New England Nuclear Corp., Boston, Mass. A hot chloroform-methanol extract was prepared as described (11) and evaporated to dryness. The residue was dissolved in 3 ml of methanol, and duplicate samples (0.5 ml) were removed for determination of total $^{32}PO_4$ in the phospholipid. The remaining material was concentrated by evaporation and analyzed by chromatography on silicic acid-impregnated paper (11). Rhodamine G staining revealed three major phospholipid spots that contained more than 90% of the radioactivity; these were previously identified (11) as sphingomyelin, lecithin, and phosphatidylethanolamine. The fractional distribution of radioactivity among these three molecular species was multiplied by the total incorporation to determine the rate of synthesis of the individual phospholipids.

RESULTS

Kinetics of inhibition. Sindbis virus has been shown to decrease the incorporation of $^{32}PO_4$ into the chloroform-methanol extractable fraction in CEF (12). Since this determination is cumbersome for multiple samples, we wished to use a procedure similar to the simple phospholipid assay employed by Penman (10) who measured the incorporation of ^{14}C-choline into acid-precipitable form. However, because only certain phospholipids contain choline, it was first essential to determine whether the reduced incorporation of $^{32}PO_4$ reflected a generalized supression of the synthesis of all classes of phospholipids or a more specific effect.

Incorporation of $^{32}PO_4$ (Fig. 1) into lecithin and sphingomyelin (both of which contain choline) was inhibited at the same rate and to approximately the same extent as its appearance in

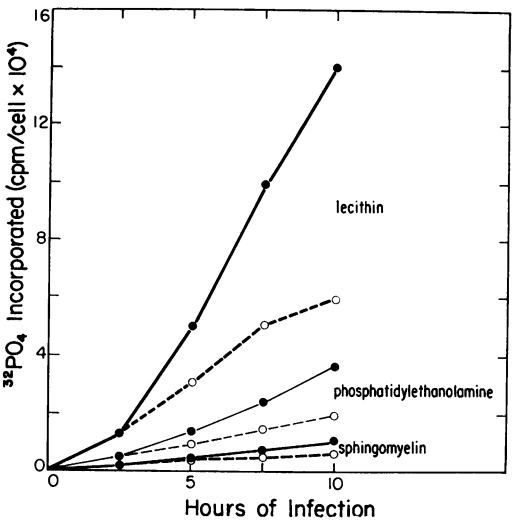

FIG. 1. *Effect of infection on $^{32}PO_4$ incorporation into the three major phospholipids of chick embryo fibroblasts. Replicate infected* (○) *and uninfected* (●) *cultures were incubated at 37 C in medium containing $^{32}PO_4$ (2.5 μCi/ml). At intervals of 2.5 hr, duplicate cultures of each series were removed to determine the total $^{32}PO_4$ incorporation into the phospholipid fraction and the distribution of this radioactivity among the three principal phospholipids, lecithin, sphingomyelin, and phosphatidylethanolamine, which comprised (in each case) about 90% of the total.*

phosphatidylethanolamine. Thus, we concluded that incorporation of ^{14}C-choline into acid-precipitable material would be a valid measurement of the effect of viral infection on phospholipid synthesis. This conclusion is reinforced by the similar effects of viral infection on chloroform-methanol-extractable $^{32}PO_4$ and acid-precipitable ^{14}C-choline (compare Fig. 1 with Fig. 2, this report, or Fig. 3, reference 16).

We do not feel that the inhibition we observed can be explained by reduced transport of the labeled precursors into the cell. During infection the incorporation of labeled inorganic phosphate into the acid-soluble nucleotide pool proceeds at a normal rate for the first 10 hr (12). The uptake of ^{14}C-choline into infected cells is slightly reduced, but only after the viral inhibition of phospholipid synthesis is well established. Even then, the ratio of acid-soluble to acid-precipitable label is greater in infected than in uninfected cells (*unpublished data*). Thus inhibition of choline uptake cannot explain the reduced phospholipid synthesis.

Effect of ts mutants. To determine which step in viral growth was responsible for the inhibition of phospholipid synthesis, we surveyed representative *ts* mutants of Sindbis virus. These mutants

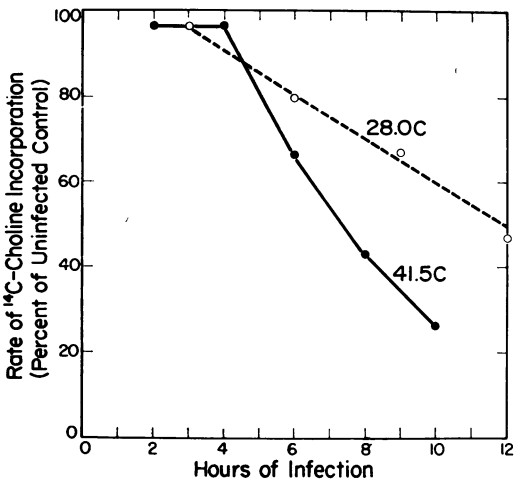

FIG. 2. *Comparison of the effect of viral infection on ¹⁴C-choline incorporation at 28 and 41.5 C. Infected cultures and mock-infected controls were incubated at 28 or 41.5 C. At various times after infection, triplicate cultures were exposed for 2 hr to medium containing ¹⁴C-choline (0.02 μCi/ml) and assayed for acid-precipitable radioactivity. Incorporation into infected cultures at 28 C (○) and 41.5 C (●) was expressed as the percentage of that incorporated by the appropriate uninfected control cultures.*

grow well at 28 C, but, due to the malfunction of a mutant protein at the nonpermissive temperature, each of these mutants is unable to carry out one of the several steps necessary to produce progeny and therefore cannot replicate at 41.5 C.

Before using these mutants, it was essential to show that infection with the parental HR strain, which grows well at 28 and 41.5 C, reduced choline incorporation at both temperatures (Fig. 2). The more rapid inhibition of ¹⁴C-choline incorporation at 41.5 C probably reflected the faster growth of the virus at this temperature.

On the basis of this experiment, we examined the effect of mutant infections on ¹⁴C-choline incorporation between 4 and 6 hr at 41.5 C and between 7 and 10 hr at 28 C. All of the mutants (Table 1) inhibited phospholipid synthesis at 28 C where their growth is normal. At 41.5 C all of the RNA⁺ mutants (that can make viral RNA, but have defects in one of the viral structural proteins) also inhibited choline incorporation. Thus neither the nucleocapsid nor the envelope protein of Sindbis virus need be functional for the virus-caused inhibition of phospholipid synthesis to occur.

In contrast to the results obtained with the RNA⁺ mutants, infection with several RNA⁻ mutants caused no reduction in phospholipid synthesis at the nonpermissive temperature. Since these mutants are incapable of viral RNA synthesis at 41.5 C, the amount of viral RNA introduced into the cells by the adsorption of about 10 plaque-forming units per cell was apparently insufficient to affect phospholipid synthesis; some viral RNA synthesis was needed.

TABLE 1. *Incorporation of ¹⁴C-choline by cultures infected with ts-mutants of Sindbis virus at 28 and 41.5 C*[a]

Virus	Defect at 41.5 C	Incorporation at 28 C[b] (%)	Incorporation at 41.5 C[c] (%)
HR	None	48	61
ts-2	Defective nucleocapsid protein	27	48
ts-5		48	56
ts-10	Defective membrane protein	34	53
ts-23		36	57
ts-7	Unable to synthesize viral RNA	53	93
ts-24		51	110

[a] All values determined in triplicate.
[b] Exposed to ¹⁴C-choline (0.04 μCi/ml) between 7 and 10 hr after infection. Incorporation expressed as the percentage of the uninfected controls which averaged 403 counts per min per sample.
[c] Exposed to the same medium between 4 and 6 hr after infection and expressed similarly. The appropriate controls averaged 802 counts/min.

Quantitation of the requirement for viral RNA synthesis. To determine how much viral RNA synthesis was required, we took advantage of a property of the RNA⁻ mutant *ts*-6. When cells infected by this mutant at 28 C are shifted to 41.5 C, viral RNA synthesis rapidly ceases, even if it has been going on at a maximal rate. This was the case in the experiment shown in Fig. 3, when the shift from permissive to nonpermissive conditions took place at 7.5 hr after infection. Since the incorporation during the 30-min pulse of labeled precursor was plotted at the time when the labeling period ended, it is clear that no detectable viral RNA was synthesized after 65 min at 41.5 C and that the rate of incorporation between 35 and 65 min after the temperature shift averaged only 11% of the control at 28 C. When cells infected with HR were treated similarly, the rate of viral RNA synthesis increased markedly (13).

Any activity that is expressed after cultures infected with *ts*-6 are shifted from 28 to 41.5 C must depend primarily on RNA synthesized before the temperature is raised; a temperature-shift experiment allows determination of the amount required (Fig. 4). We shifted cultures infected with *ts*-6 to the nonpermissive temperature at various times to determine how much viral RNA synthesis was required to permit the de-

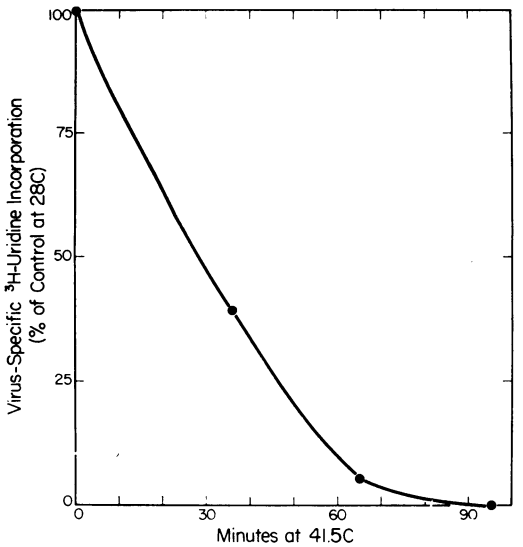

FIG. 3. *Rate of decay of ts-6-specific viral RNA synthesis at 41.5 C. Replicate cultures, infected with ts-6 or mock-infected, were incubated at 28 C for 7.5 hr in the presence of actinomycin D. Triplicate infected and uninfected cultures were labeled for 30 min by exposure to ³H-uridine (3.3 μCi/ml) in the continuous presence of actinomycin D, and then processed to determine acid-precipitable radioactivity. Remaining cultures were placed in a 41.5 C water bath for 5 min, and then transferred to a 41.5 C incubator. After 30-min labeling periods, ending at the times indicated on the graph, these cultures were also processed. Virus-specific RNA synthesis was determined by subtracting the radioactivity incorporated by the uninfected controls. Virus-specific incorporation at 41.5 C was then expressed as the percentage of the initial value at 28 C.*

velopment of the viral inhibitory effect on the synthesis of phospholipid, protein, and cellular RNA.

Figure 4A shows the kinetics of *ts*-6 specific viral RNA synthesis at 28 C. During the first 3 hr of infection, very little viral RNA synthesis could be detected, although it obviously must have been taking place at a low rate; thereafter, it increased markedly.

At each of the times indicated on the upper graph, other infected cultures were shifted from the permissive to the nonpermissive temperature. Between 7.5 and 9 hr after infection, all cultures were labeled with ¹⁴C-choline at 41.5 C. Figure 4B shows the amount of choline incorporated between 7.5 and 9 hr after infection plotted against the amount of time the cultures were incubated at 28 C before transfer to 41.5 C. Only a few hours at 28 C, during which little viral RNA was made, were necessary for a significant degree of inhibition. The other two curves show the effect of *ts*-6 infection on host-specific RNA synthesis and

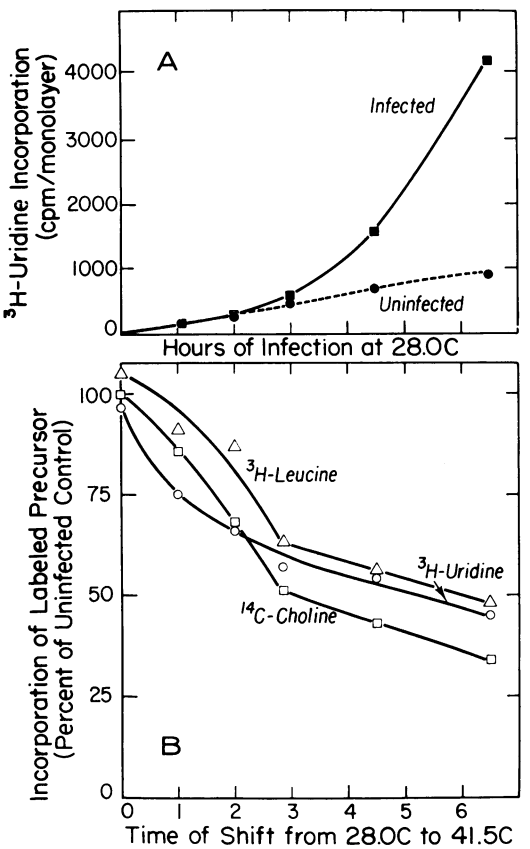

FIG. 4. *Determination of the amount of viral RNA synthesis required for inhibition of phospholipid, protein, and RNA synthesis. A, Cumulative ts-6-specific RNA synthesis. Replicate cultures were pretreated with actinomycin D and infected (■) or mock-infected (●). To triplicate cultures from each series, medium containing ³H-uridine (0.5 μCi/ml) and actinomycin D was added. At the first point indicated on the graph, these cultures were processed and an additional set was labeled. This was repeated at each time interval. B, Inhibition of macromolecular synthesis. At each time indicated on the upper graph replicate cultures, infected and uninfected, which had not been exposed to actinomycin D were transferred from 28 to 41.5 C. Between 7.5 and 9 hr after infection, all cultures were labeled with ¹⁴C-choline (□), 0.02 μCi/ml, and either ³H-leucine (△), 1.3 μCi/ml, or ³H-uridine (○), 0.33 μCi/ml. All cultures were then processed to determine trichloroacetic acid-precipitable radioactivity, and results were expressed as the percentage of the label incorporated by the uninfected control. Protein and RNA points were determined in triplicate; since double labeling was used, the ¹⁴C-choline points are averages of six determinations. Actinomycin D-resistant RNA synthesis, determined at the same time in infected cultures shifted from 28 to 41.5 C at 6 hr after infection, did not differ significantly from the amount synthesized by similar uninfected controls (unpublished data), as would be expected from the data presented in Fig. 3.*

overall protein synthesis determined in the same manner. The amount of time at 28 C required to allow these inhibitions to develop was clearly similar to that required for reduction of choline incorporation.

These results do not suggest that any inhibition of macromolecular synthesis could be detected at 1 hr after infection, nor that the virus-directed inhibition of phospholipid, protein, or host RNA synthesis necessarily appeared at the same time. They do suggest that a similar, small amount of viral RNA synthesis will permit the subsequent inhibition of the synthesis of three major classes of macromolecules.

Temporal relationship between the inhibition of RNA, protein, and phospholipid synthesis. Although the requirement for a similar amount of viral RNA to establish each of these inhibitory effects could be coincidental, the phenomena could also be interdependent. Specifically, we were interested in the possibility that the decline in phospholipid synthesis could result from the effects of arboviruses on cellular protein (14) and RNA (15) synthesis.

Before such a suggestion can be made, one must show that the viral inhibition of the synthesis of these macromolecules occurs before inhibition of ^{14}C-choline incorporation. Figure 5 shows the amount of labeled precursor incorporated into phospholipid, protein, and host RNA during 2-hr intervals at various times after infection. Host RNA synthesis in infected cells was obtained by correcting the total RNA synthesis in infected cells for virus-specific, actinomycin D-resistant RNA synthesis. No such correction was possible in the case of protein synthesis; that curve therefore represents protein synthesis coded for by both host and viral RNA. Since, by 3 hr after infection, 90% of the protein synthesis is viral (14), the host protein synthesis certainly declines faster than the graph indicates.

A clear-cut sequential relationship is not apparent in this experiment, but the protein and host RNA synthesis appear to decline more rapidly than ^{14}C-choline incorporation. Thus, it is possible that the decrease in phospholipid synthesis is a secondary effect of the viral inhibition of cellular RNA and protein synthesis. We attempted to test this hypothesis by the use of inhibitors of protein and cellular RNA synthesis.

Effect of inhibitors. Pastan and Friedman reported that actinomycin D, an inhibitor of deoxyribonucleic acid-primed RNA synthesis, depressed phospholipid synthesis in CEF (9), but Penman reported that it had no effect on the ^{14}C-choline incorporation of HeLa cells (10). We therefore examined the effects of actinomycin D in CEF and in a continuous line of mammalian cells (BHK) that supported the growth of Sindbis virus (Fig. 6).

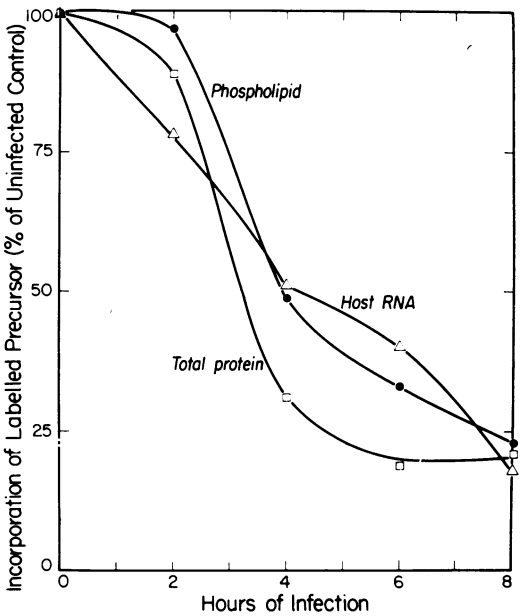

FIG. 5. *Time course of inhibition of macromolecular synthesis by HR virus at 37 C. Infected and mock-infected cultures were incubated and pulse labeled in triplicate with ^{3}H-leucine (□), 0.5 μCi/ml, or ^{14}C-choline (●), 0.5 μCi/ml, for 2-hr intervals ending at the times indicated on the graph, and then processed. Rate of synthesis was expressed as the percentage of the incorporation in uninfected controls. To determine host-specific RNA synthesis in virus-infected cells (△), four kinds of cultures were utilized for each point: infected and mock-infected cultures that had been exposed to actinomycin D for 1 hr before infection and continuously thereafter, and replicate infected and mock-infected cultures that had not been exposed to the drug. Triplicate cultures from each series were pulse-labeled with ^{3}H-uridine (0.5 μCi/ml) for 2-hr periods ending at the times indicated on the graph. They were then processed to determine acid-precipitable radioactivity. Virus-specific RNA synthesis was determined by subtracting from the amount of ^{3}H-uridine incorporation occurring in virus-infected, actinomycin D-treated cells, the amount that occurred in uninfected cells in the presence of the drug. Virus-specific incorporation was then subtracted from the total incorporation in untreated, infected cells to yield the host-specific incorporation in infected cells. This was plotted as the percentage of the normal control value.*

In agreement with Pastan and Friedman (9), we found that actinomycin D rapidly inhibited choline incorporation into uninfected CEF; however, it had no effect on phospholipid synthesis in BHK cells for 10 hr, although RNA synthesis was inhibited more than 98% (*unpublished data*).

Cycloheximide, an inhibitor of protein synthesis, rapidly reduced choline incorporation in CEF.

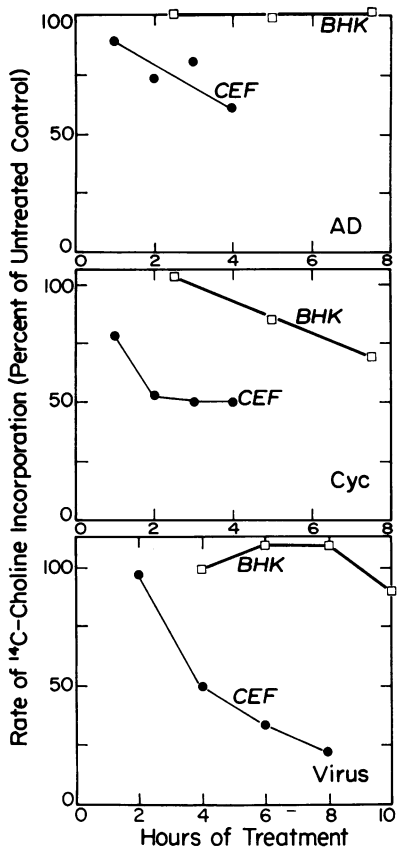

FIG. 6. *Comparison of the effect of actinomycin D, cycloheximide, and virus infection on ^{14}C-choline incorporation in chick embryo fibroblast (CEF) and BHK cells at 37 C. At zero time, confluent monolayer cultures of CEF (●) or BHK (□) were exposed to actinomycin D (AD) or cycloheximide (Cyc). CEF cultures were labeled for 1-hr periods (0.09 μCi/ml) and the BHK monolayers during 2.5-hr intervals (0.01 μCi/ml) in the continuous presence of the appropriate inhibitor. At the times indicated, pulses were terminated and the cultures were processed to determine how much label had been incorporated. Appropriate untreated controls were included in each experiment. Results, which are the average of triplicate determinations, are expressed as the percentage of incorporation in the uninfected controls. Effect of virus infection (Virus) was determined in the same way, except that both CEF (●) and BHK (□) cultures were labeled for 2 hr at various times after infection with ^{14}C-choline (0.5 and 0.1 μCi/ml, respectively).*

It also affected phospholipid synthesis in BHK cells, but here the inhibitory effect was slow to develop and less extensive. In the BHK system, a slight cytopathic effect was apparent after 7 hr of exposure to the drug.

Since phospholipid synthesis in BHK cells appeared to be less sensitive to metabolic inhibitors, it was of interest to examine the effect of Sindbis virus infection in these cells. The growth of this virus in BHK and CEF is virtually identical with respect to both the time course of virus production and the amount of virus produced (*unpublished data*). Moreover, BHK cellular protein synthesis is markedly inhibited after infection, much as it is in chick cells (14). Despite these similarities, infection with Sindbis virus caused no decrease in phospholipid synthesis until between 8 and 10 hr after infection. We suspect that the slight decrease may be real because we observed it several times, but we did not carry the experiment further because of the development of a pronounced viral cytopathic effect during the next 2 hr. The time of appearance of the decrease is consistent with the idea that it might result from the viral inhibition of protein synthesis followed by a slow decline in phospholipid synthesis as was seen after cycloheximide. It is clear that the phospholipid synthesis of BHK cells is more resistant than that of CEF to both metabolic inhibitors and Sindbis infection.

DISCUSSION

The reduction of phospholipid synthesis that occurs in primary CEF cultures infected by Sindbis virus cannot be attributed to removal of part of the cell membrane by released virus. It did not occur in BHK cells which released the same amount of virus, but normally took place in infected CEF cells which were treated in a variety of ways to prevent virus release. Interferon pretreatment (12), incubation in medium of lowered ionic strength (16), or incubation of cultures infected with RNA$^+$ mutants at the nonpermissive temperature allowed normal inhibition of phospholipid synthesis although viral yields were reduced 98 to 99.9%.

Evidence presented in this report suggests that the virus-directed depression of either host protein or RNA synthesis might account for the inhibition of phospholipid synthesis in infected CEF. Inhibitors of RNA or protein synthesis caused a reduction of ^{14}C-choline incorporation into uninfected CEF. In addition, experiments with the RNA$^-$ mutant *ts*-6 showed that all three viral inhibitory effects required approximately the same small amount of viral RNA synthesis. The temptation to call those "early" viral functions, in the sense that is used with the T-even bacteriophages or the pox viruses, should be resisted until more is known about the control mechanisms, if any, that are active in RNA-containing animal viruses. It seems likely that the inhibition of RNA, protein, and phospholipid synthesis requires rela-

tively few active molecules of virus-specific protein, and that these few can be synthesized from a small amount of RNA.

If the reduced phospholipid synthesis seen after Sindbis infection were a consequence of the viral effect on only one macromolecular species, the experiments presented here would not permit us to conclude with certainty whether inhibition of RNA or protein synthesis was responsible. If we were to choose between them, we feel that the viral effect on cellular protein synthesis is more likely to affect phospholipids. It is difficult to imagine that inhibition of host RNA alone could be involved. In addition, the experiments of Pastan and Friedman on the effect of actinomycin D on phospholipid synthesis led them to conclude that the drug was effective too rapidly to be acting via inhibition of RNA synthesis. They suggested that it might act directly on the enzymes involved in phospholipid synthesis. In direct contrast to our results with infected cells, they also observed that, in the presence of actinomycin D, the synthesis of the various phospholipids was inhibited to different extents, ranging from 33% for phosphatidylethanolamine to 78% for sphingomyelin (9). The inhibition caused by the virus was much more uniform (Fig. 1). Thus, whereas viral inhibition of host RNA synthesis could be involved in the decline of phospholipid synthesis in CEF, the experiments with actinomycin D do not support it. Since, in CEF, the inhibition of choline incorporation caused by the virus is greater than that caused by actinomycin D or cycloheximide alone, both RNA and protein synthesis may be implicated. A direct viral effect on the enzymes involved in phospholipid synthesis cannot be excluded.

In BHK cells, phospholipid synthesis is much less sensitive to actinomycin D, cycloheximide, or Sindbis infection than in CEF. This may be due to the presence of longer-lived proteins involved in phospholipid synthesis, or to some other metabolic difference between avian and mammalian cells, or between confluent monolayers of primary cultures and of cell lines.

The contrast between the effects of Sindbis virus and picornavirus infection on phospholipid synthesis is still marked, although the difference was reduced when more comparable host cells were studied. Both Sindbis and the picornaviruses (6) inhibit cellular RNA and protein synthesis. In BHK cells where Sindbis virus grows well, it did not inhibit phospholipid synthesis, but it also failed to cause the stimulation seen in picornavirus infections of other mammalian cells (2, 5, 10). Since Sindbis virus is an avian virus, the state which obtains in CEF may well be that which normally occurs, although cessation of phospholipid synthesis is clearly not required for viral growth.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of William J. Gilbert.

This investigation was supported by Public Health Service research grant AI 08238 from the National Institute of Allergy and Infectious Diseases. M. R. F. W. was supported by Public Health Service training grant 5TI GM174 and predoctoral fellowship 1 F01 GM45629-01 from the National Institute of General Medical Sciences.

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