Effects of Temperature and Lipophilic Agents on Poliovirus Formation and RNA Synthesis in a Cell-Free System

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The translation and primary processing events of poliovirus polyproteins in HeLa cell extracts were more efficient at 34°C than at 30 or 36°C. The cleavage products of P2 such as 2APPr, 2BC, and 2C appeared early in the reaction before the appearance of the cleavage products of P1 and of 3CDPr, an observation suggesting that P2 was cleaved in cis by 3CDPr. Proteolytic processing of the capsid precursor P1 into VP0, VP1, and VP3 was also more efficient at 34°C than at either 30 or 32°C. Surprisingly, processing of 3CDPr to 3Cpre and 3Dpol was almost completely inhibited at 36°C. The synthesis of virus in the cell extract was greatly enhanced at 34°C over that at 30 or 32°C, whereas incubation at 36°C yielded very little virus. Cerulenin, an inhibitor of lipid synthesis, did not appear to affect virus-specific translation or protein processing, but it almost completely inhibited viral synthesis in vitro. Oleic acid drastically inhibited in vitro translation at 100 μM and in vitro poliovirus synthesis at 25 μM. Addition of HeLa cell smooth membranes partially restored translation but not virus formation. Our observations suggest that in vitro translation, proteolytic processing, and virus formation require intact membranes. Analysis of the in vitro translation products revealed that viral RNA polymerase activity increased linearly during incubation of the translation mixture. RNA polymerase in the crude mixture was inhibited by oleic acid but not by cerulenin. Surprisingly, oleic acid had no direct effect on oligo(U)-primed, poly(A)-dependent poly(U) synthesis catalyzed by purified 3Dpol.

Cell-free synthesis of nucleic acids and polypeptides allows for a detailed biochemical analysis of the participating components and mechanisms involved in the process of viral replication. The cell-free, de novo synthesis of poliovirus (18, 19) results from the successive events of virus-specific polypeptide synthesis, proteolytic processing, RNA replication, and encapsidation in which viral gene products might interact with cellular components at every step. The cell extract used for poliovirus synthesis (18, 19) contains membranous material, but the nature of the membranes is unknown. We have made use of this novel in vitro system and examined the effect of two lipophilic compounds, cerulenin and oleic acid, on virus formation. The choice of these compounds was guided by observations made several years ago that implicated the involvement of cellular membranes in poliovirus genome replication (13, 28).

Membranous poliovirus replication complexes have been isolated from infected HeLa cells that are able to carry out both the initiation and elongation steps of viral RNA synthesis (29, 30). These crude complexes appear to be vesicles that sequester all virus-specific proteins and template RNAs. Only endogenous (but not exogenously added) viral RNAs serve as templates. Moreover, it was found that the initiation of RNA synthesis ceased when nonionic detergents were added to the complex, an observation suggesting that this process requires intact membranes (30, 31). The mechanism by which the replication vesicles form is obscure, but genetic and biochemical evidence has implicated viral polypeptides 2BC, 2C, and 3AB (2, 3, 7, 15, 27). As poliovirus infection progresses, numerous vesicular structures are formed, concomitantly with a significant increase in phosphatidylcholine synthesis (4). Cerulenin, an inhibitor of lipid synthesis, has been reported to inhibit poliovirus RNA synthesis in vivo (8). It was suggested that de novo lipid synthesis is required for the synthesis of the membranous replication factories and is essential for viral RNA synthesis (8).

Exogenously added fatty acids exert various effects on cultured animal cells, such as increased lipid synthesis and membrane fluidity (16, 23). Oleic acid has been shown to alter membrane fluidity without affecting lipid synthesis. Unexpectedly, treatment of poliovirus-infected HeLa cells with oleic acid resulted in the inhibition of viral RNA replication (9). This effect was seen at concentrations at which host cell metabolism appeared normal. It was suggested that oleic acid may block viral RNA synthesis by altering an essential membranous component of the replication complex.

The cell-free system for the synthesis of poliovirus occurs in cytoplasmic extracts of uninfected HeLa cells containing a delicate balance of concentrations of viral mRNA and cations (18, 19). The yield of in vitro-synthesized virus can vary widely from preparation to preparation, a result that we cannot explain at present but is probably due to the turnover of essential cellular components during extract preparation. In addition to studies with lipophilic compounds, we report here the effect of increased temperatures on polypeptide synthesis and virus formation in vitro.

MATERIALS AND METHODS

Preparation of RNA. Suspension cultures of HeLa S3 cells (5 × 10⁶ cells per ml) were infected with poliovirus type 1 Mahoney [PV1(M)] at a multiplicity of 20 PFU per cell. Cells were harvested at 7 h postinfection and lysed by three rounds of freezing and thawing in RSB containing Mg²⁺ (0.01 M NaCl, 0.01 M Tris hydrochloride [pH 7.4], 1.5 mM MgCl₂). The cell lysate was centrifuged at 10,000 rpm for 10 min (SS34 rotor) to remove cell debris and nuclei. The supernatant containing poliovirus was made 2 mM EDTA.

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and 0.1% sodium dodecyl sulfate (SDS), and the virus was pelleted by centrifugation at 30,000 rpm for 3 h (Beckman 50.2 Ti rotor). Pelleted virus was resuspended in 20 mM phosphate buffer (pH 7.4) and purified by CsCl. The virus band was collected, resuspended in phosphate buffer, and centrifuged at 30,000 rpm for 3 h (Beckman 50.2 Ti rotor). Pelleted virus was taken up in phosphate buffer containing 0.1% SDS and extracted with phenol-chloroform, and the poliovirus RNA was precipitated with ethanol.

Preparation of smooth membranes. The method used for the fractionation of HeLa cells was that of Caligiuri and Tamm (5). Briefly, suspension cultures of HeLa S3 cells were broken in a glass Dounce homogenizer with a tight-fitting pestle. The nuclei and cell debris were removed by centrifugation. The supernatant containing cytoplasmic extract was fractionated on a sucrose density gradient. Fractions 2 and 3 containing the smooth membranes were diluted with RSB and repelleted, and the pellets were resuspended in RSB containing Mg++. In vitro translation and product analysis. HeLa cell extract was prepared, treated with micrococcal nuclease, and used for translation as described previously (18, 19). A 150-μl volume of micrococcal nuclease-treated extract was mixed with 50 μl of 10X translation mixture (10X translation mixture: 76 μl of 1 M K-HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.4], 40 μl of 100 mM ATP, 6 μl of 40 mM GTP, 40 μl of 1 M creatine phosphate, 10 μl of 10-mg/ml creatine phosphokinase, 8 μl of 1 M dithiothreitol, 10 μl of 10-mg/ml calf liver RNA, 50 μl of 1 mM each amino acid [all 20 except methionine], 10 μl of 100 mM spermidine, and 250 μl of water), 22 μl of 2 M potassium acetate, 3 μl of 50 mM magnesium acetate, 16 μl of 20 mM MgCl₂, and 25 μl of [35S]methionine (1 mCi/ml; NEN Research). An 8-μl volume of the above mixture was mixed with 100 ng of poliovirus RNA in a total volume of 12.5 μl. The samples were incubated at 30, 32, 34, and 36°C for 15 h. At each time point the samples were removed; mixed with RNase A (20 μg/ml), RNase T₂ (100 U/ml), and cycloheximide (5 μg/ml); incubated for 5 min at 30°C; and frozen immediately. A 3- to 6-μl portion was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (13.5% polyacrylamide). Gels were fixed, treated with EnHANCE, dried, and exposed to X-ray film for 15 to 24 h at ~70°C. The extent of cleavage was quantitated by scanning X-ray films with an LKB laser densitometer.

Time course of virus formation. Virion RNA was translated in the presence of cold methionine, 200 μM each CTP, GTP, and UTP, and 1 mM ATP in a total volume of 25 μl at the temperatures indicated above. At each time point, the samples were removed, treated with RNase A and RNase T₂ at the concentrations indicated above, and incubated at room temperature for 30 min. The samples were diluted with phosphate-buffered saline and added to the HeLa cell monolayers. Plaques were scored after 48 h of incubation as described previously (24).

Fractionation of translation reaction by sucrose density gradient centrifugation. In vitro translation reaction (0.5 ml) was treated with RNase A and RNase T₂, as described above. The sample was made 0.5% Nonidet P-40 and 5 mM EDTA and then loaded onto a 15 to 30% sucrose gradient (10.4 ml). A parallel gradient was run with purified PV3 (M) (about 10⁵ PFU). The samples were centrifuged at 28,000 rpm for 3 h in an SW41 rotor at 4°C and pumped into equivalent fractions. Portions (1 to 50 μl) of each of the fractions were applied to separate monolayers of HeLa cells, and plaques were scored after 48 h of incubation.

Effect of lipophilic agents on translation and virus formation. Virion RNA was translated in the presence or absence of different concentrations of either cerulenin or oleic acid in the presence of either [35S]methionine or cold methionine for 15 h at 34°C. The samples were then analyzed by both SDS-PAGE and plaque assay.

Polymerase activity. Virion RNA was translated in the presence of cold methionine with or without each drug at 34°C. At each time point, samples were removed and kept on ice. Reactions (25 μl) containing 50 mM HEPES (pH 7.8), 15 mM MgCl₂, 10 mM DTT, 1 μM common TP, 1 μCi of [32P]UTP (3,000 mCi/mmol; Amersham), 25 ng of primer p(dT)₁₅, 500 ng of template poly(A), and 2 to 3 μl of in vitro translation reaction with or without drugs were incubated at 30°C for 1 h. The mixtures were applied to DEAE 81 filters (Whatman), immersed in 100 ml of 5% Na₂HPO₄, agitated for 5 min, and washed twice with the same solution, once with water, and once with ethanol. The filters were dried under a heat lamp and counted by scintillation. Polymerase activity was also measured by using purified 3Dpol (a generous gift of Stephen Plotch) in the presence or absence of cerulenin or oleic acid. The reaction mixtures (25 μl) were the same as above except that they contained 1 μCi of [3H]UTP, 3.8 ng of 3D, and 125 ng of poly(dT)₁₅.

RESULTS

Optimal synthesis and processing of viral polyepitides. As reported previously, the efficiency of translation of viral polyepitides in a HeLa cell extract is highly dependent on the concentration of poliovirus mRNA (18, 19). Synthesis of polypeptide is maximal around 8 μg of RNA per ml but is almost completely inhibited at 100 μg of RNA per ml of reaction. Moreover, translation requires Mg²⁺ and K⁺ ions in a very narrow range of optimal concentrations, and it requires chloride and acetate ions (17). It should be noted that translation of picornavirus RNAs other than poliovirus requires different concentrations of RNA and salt (17), as reported previously (11, 12). The reason for the strict concentration dependence of these components is unknown but may relate to the stability of higher-order structures of the RNA and to the binding parameters of these RNAs to trans-acting factors.

We have observed previously that, at 30°C, nearly 1 h of translation in the HeLa cell extracts is required before newly synthesized capsid precursor P1 could be detected. Most virus-specific proteins were observed after 3 h of translation, although nearly 12 h was necessary until the processing pattern of virus-specific polyepitides was identical to that of infected-cell extracts (references 18 and 19 and unpublished data). We have now found that the time required for P1 synthesis shortened with increasing temperature of translation (Fig. 1). The extent of cleavage was determined by densitometric scanning of the autoradiograms. The relative amount of P1 translation products was 1.33-fold higher after 2 h at 34°C than was the relative amount obtained at 32°C and 2.12-fold higher after 4 h at 34°C than was the relative amount obtained at 32°C. The relative amount of the processing product of VP3 of P1 was almost the same at both temperatures after 2 h of translation. However, after 4 h the yield of VP3 increased 1.43-fold over that obtained at 32°C. Inspection of the translation and processing patterns shown in Fig. 1 revealed several interesting features. First, it appears that, overall, translation and processing are more efficient at 34°C than at lower or higher temperatures. Second, certain cleavage-specific products of the polypro-
proteins such as 2APro, 2BC, and 2C were always visible as early as 3CDPro, long before the band corresponding to 3CPro appeared (compare lanes 4 and 8 in Fig. 1B). This observation suggests that P2 (containing the sequences for 2A, 2B, and 2C) is cleaved predominantly, if not exclusively, by 3CDPro in cis as soon as the P2-P3 polypeptide has been synthesized. Third, certain processing pathways are inhibited at 36°C. This is most pronounced for the cleavage of 3CDPro to 3DP and 3CPro since neither of these products can be detected in the gel (Fig. 1C). Moreover, at 36°C 3BCD and P3 are prominent after 12 h of incubation. This suggests that processing of these large precursors is impaired at this temperature and explains why little 3AB is found at 36°C even after extended incubation (Fig. 1C, lane 14).

Effect of temperature on in vitro synthesis of poliovirus. The best yield of poliovirus obtained previously by translation at 30°C was 40,000 plaques per ml of reaction (18, 19). To test the possibility that translation and processing at higher temperatures affect the kinetics of formation as well as the final virus yield, we performed plaque assays at various times of incubation at different temperatures. As can be seen in Table 1, both the time of maximal virus synthesis and the yield of infectious particles changed dramatically. Remarkably, virus yield was highest at 34°C, whereas very little virus was formed after incubation at 36°C. This sharp drop in virus synthesis correlated well with the aberrant processing pattern seen at 36°C (Fig. 1C).

Sedimentation analysis of in vitro-synthesized poliovirus. Polioviruses have a characteristic sedimentation coefficient of 160S in sucrose gradients, whereas all intermediates of the maturation pathway (provirions, empty capsids, etc.) sediment at a lower velocity (25). We have subjected 0.5 ml of the complete translation mixture, incubated at 34°C for 12 h in the presence of cold methionine, to sedimentation velocity

TABLE 1. Effect of temperature on viral synthesis in vitro

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Viral synthesis (PFU/ml) after incubation time (h) of:</th>
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* Conditions of translation reactions were same as those for Fig. 1.
experiments in sucrose gradients. As determined by plaque assays, the newly formed virus sedimented with a velocity similar to that of wild-type virus analyzed in a parallel experiment (Fig. 2). This result confirms our previous conclusion that mature virus was synthesized in vitro.

**Effect of cerulenin on translation and poliovirus synthesis.**

Cerulenin is an antibiotic whose mode of action is thought to be the inhibition of fatty acid and lipid synthesis in host cells. Guinea and Carrasco (8) reported that this drug inhibited poliovirus RNA replication, an effect that was correlated with the inhibition of lipid synthesis. We have tested whether cerulenin inhibits in vitro translation of poliovirus RNA and virus formation and found no effect on protein synthesis and processing at concentrations of the drug up to 200 \( \mu \text{M} \) (Fig. 3). However, at 200 \( \mu \text{M} \) cerulenin, no virus was formed with two independently prepared cell extracts, translated at 30 or 34°C (Table 2). Even at 12.5 \( \mu \text{M} \) cerulenin, the virus yield was significantly reduced.

We considered it possible that the inhibition of virus formation is the result of an inhibition of lipid synthesis in the translation mixture. This hypothesis was tested by measuring the incorporation of \(^{3}\text{H}\)glycerol into components soluble in chloroform (8). Although a significant fraction of labeled glycerol in the extract was converted to chloroform-soluble material, 200 \( \mu \text{M} \) cerulenin had no effect on the reaction (data not shown).

**Effect of oleic acid on translation and poliovirus synthesis.**

In view of the proposed role of membranes in poliovirus replication in vivo, and a report of an inhibitory effect of oleic acid (9-octadecenoic acid) on poliovirus RNA replication (9), we have tested translation and virus synthesis in vitro in the presence of this fatty acid. At concentrations of up to 25 \( \mu \text{M} \), oleic acid had little, if any, effect on poliovirus translation in vitro (Fig. 4A, lanes 2 to 5), whereas at 100 \( \mu \text{M} \) oleic acid the overall translation was severely inhibited (lane 7). However, at 50 \( \mu \text{M} \) oleic acid, the only protein that is affected by oleic acid is 3AB (Fig. 4A, lane 6, and Fig. 4B, lane 9); this suggests that 3AB may have to be properly associated with the membrane in order to be processed correctly. Apart from an overall reduction of incorporation of \(^{35}\text{S}\)methionine into viral protein at 100 \( \mu \text{M} \) oleic acid, it is also apparent that only low levels of the polypeptides 3CP\(^{0.00}\) and 3AB were produced. This result was due to the inhibition of processing of the P3 precursor (Fig. 4A, lane 7). Addition of smooth membranes to the translation mixture containing 100 \( \mu \text{M} \) oleic acid partially restored translation, although the processing of P3 and P2 precursors remained inhibited (Fig. 4A, lanes 8 to 10).

Oleic acid had a much more drastic effect on virus synthesis than on translation. At oleic acid concentrations as low as 12.5 \( \mu \text{M} \), the virus yield was significantly reduced to

![In vitro synthesized virus](image)

**FIG. 2.** Sucrose gradient profile of PV1(M) (●) and in vitro-synthesized virus (■). Virion RNA was translated for 12 h at 34°C in the presence of cold methionine and all nucleoside triphosphates as indicated in the text. The translation mixture containing in vitro-synthesized virus and PV1(M) stock virus was fractionated on a sucrose gradient as described in the text. Portions from each gradient fraction were analyzed by plaque assay.

![Effect of cerulenin on in vitro translation of virion RNA in HeLa cell extract. Virion RNA was translated for 15 h at 30°C in the presence or absence of cerulenin at the concentrations indicated above the lanes. The products were analyzed by SDS-PAGE (13.5% polyacrylamide).](image)

**FIG. 3.** Effect of cerulenin on in vitro translation of virion RNA in HeLa cell extract. Virion RNA was translated for 15 h at 30°C in the presence or absence of cerulenin at the concentrations indicated above the lanes. The products were analyzed by SDS-PAGE (13.5% polyacrylamide).

**TABLE 2.** Effect of cerulenin and oleic acid on in vitro synthesis of poliovirus*

| Conc (\( \mu \text{M} \)) | Cerulenin [Viral synthesis (PFU/ml) in presence of:]| Oleic acid [Viral synthesis (PFU/ml) in presence of:]| |
|-----------------|-----------------------------------|-----------------------------------||
|                 | 30°C | 34°C | 30°C | 34°C | 30°C | 34°C |
| 0               | 9,600 | 6.8 \( \times 10^6 \) | 9,900 | 6.8 \( \times 10^6 \) | 6.25 | ND \( ^{b} \) | ND |
| 6.25            | ND \( ^{b} \) | ND | 3,100 | 2.1 \( \times 10^6 \) | ND | ND | ND |
| 12.5            | 3,700 | 4.2 \( \times 10^6 \) | 900 | 1.4 \( \times 10^4 \) | 106 | 50.0 | ND |
| 25.0            | ND | 1.9 \( \times 10^6 \) | 0 | 0 | 0 | 0 | ND |
| 50.0            | 2,700 | 1.6 \( \times 10^6 \) | 0 | 0 | 0 | 0 | ND |
| 100.0           | 150 | 1.2 \( \times 10^5 \) | 0 | 0 | 0 | 0 | ND |
| 200.0           | 0 | 0 | ND | ND | ND | ND | ND |

* With different preparation of extract.

\(^{b}\) ND, not determined.
FIG. 4. Effect of oleic acid and supplementation of HeLa cell smooth membranes on in vitro translation of virion RNA in HeLa cell extract. Virion RNA was translated at 30°C for 15 h in the presence or absence of oleic acid at the concentrations indicated above the lanes with or without purified membranes, and the products were analyzed by SDS-PAGE (13.5% polyacrylamide). (A) Lanes 3 to 7, translation at 6.25, 12.5, 25, 50, and 100 μM oleic acid, respectively; lanes 8 to 10, 100 μM oleic acid with 180, 360, and 720 ng of membrane proteins, respectively. (B) The translation products were diluted and centrifuged at 50,000 rpm for 1 h (TLA-100 rotor, 4°C), and the proteins contained in the supernatant (Sup.) were precipitated with trichloroacetic acid. Both pellets were then analyzed by SDS-PAGE (15% polyacrylamide). Controls in lanes 3, 6, and 9 are the translation products without oleic acid and with 25 μM and 50 μM oleic acid, respectively.

10%, and at 25 μM oleic acid, no virus was formed at either 30 or 34°C (Table 2). The addition of smooth membranes to the translation at 100 μM oleic acid did not restore virus synthesis (data not shown).

Most poliovirus-specific polypeptides synthesized in infected HeLa cells are membrane associated and can be pelleted by low-speed centrifugation (31, 32). We have found the same with poliovirus proteins synthesized in the cell extract (Fig. 4B, compare lanes 4 and 5). It is interesting that the only viral polypeptides found in the supernatant in significant quantities are the capsid proteins VP0, VP1, and VP3 and the proteases 3CDpro and 2Apro. The presence of the capsid proteins and 3CDpro in the supernatant may not be coincidental since the latter is the enzyme which is involved in the processing of the capsid precursor P1 (10, 33). Addition of oleic acid clearly increased the amount of protein found in the supernatant, although the pattern of the released proteins did not change significantly (Fig. 4B). Therefore, the inhibition of virus synthesis by oleic acid cannot be explained simply by the alterations of membrane-bound viral polypeptides.

Effect of cerulenin and oleic acid on 3DPs3 polymerase activity. Barton and Flanagan (1) have recently used the HeLa cell-free system (18, 19) to study poliovirus RNA polymerase (3DPs3) activity that was synthesized during translation. They reported an increase in the amount of 3DPs3 activity concomitant with the increase of translation products. We, in turn, have observed 3DPs3 activity during translation of poliovirus RNA in the HeLa cell extract as early as 1 h, the activity increasing linearly for up to 12 h (Fig. 5) as assayed by primer- and poly(A)-dependent poly(U) synthesis (see Materials and Methods). Surprisingly, this experiment had a high background in extracts incubated without mRNA. Cerulenin at 200 μM had no effect on 3DPs3 activity whereas oleic acid at 25 μM completely inhibited the synthesis of poly(U). The same concentration of oleic acid completely inhibited in vitro synthesis of poliovirus (Table 2).

We then tested the effect of cerulenin and oleic acid on the activity of purified 3DPs3. To our surprise, no inhibition was detectable even at 50 μM oleic acid and up to 200 μM cerulenin (data not shown).

DISCUSSION

In vitro translation of mRNAs is routinely carried out at 30°C (11), a temperature presumed to allow maximal interaction of the numerous participating components prior to the decay of their activities. We were surprised, therefore, to observe that an increase in temperature not only had no deleterious effect on the translation of poliovirus RNA in a HeLa cell extract but also appeared to shorten the time course of the reaction and to increase the overall efficiency. These data have also revealed some interesting aspects of the proteolytic processing of the polyprotein precursors. The capsid precursor P1, mapping to the N terminus of the polyprotein, is, as expected, one of the first products to become visible, because of the cotranslational cleavage between P1 and P2 by 2Apro. However, the cleavage products of P2 also appear early in the reaction, before the appearance of the cleavage products of P1 and of 3CDpro (Fig. 1A and B, lanes 3 and 4). In fact, at these very early
time points, P3, the precursor to 3CDpro, has hardly been processed. We suggest, therefore, that P2 is cleaved in cis by 3Cpro-linked activities residing in the polypeptides such as P3 or 3CDpro. In fact, we consider it highly likely that some of the polypeptide P2 is processed cotranslationally. This is supported by our failure to obtain a viable dicistronic poliovirus by inserting the encephalomyocarditis virus internal ribosome entry site between P2 and P3 (22). This hypothesis also correlates well with the proposed proteolysis of P1 by 3CDpro in trans (10, 33), which we believe to occur after the P2 cleavages in cis. cis cleavage of the nonstructural proteins has also been proposed recently by Semler and colleagues (6, 14).

Of special interest is the observation that the proteolytic processing pattern of the poliovirus polyprotein changed at 36°C. The fact that there is a deficiency in the cleavage of P3 (resulting in the apparent lack of 3AB, 3Cpro, and 3Dpol [Fig. 1C, lane 14]) correlates with an almost complete inhibition of poliovirus synthesis at this temperature (Table 1). We have previously suggested that only newly synthesized viral RNA, but not endogeneous mRNA, is encapsidated (18, 19), a hypothesis supported by the results reported here. However, the reason for the aberrant cleavage pattern at 36°C in vitro is unknown. This phenomenon is surprising to us since this temperature is almost optimal for viral protein synthesis in vivo.

The yield of infectious virus, even at 34°C, is very low compared with the yield of poliovirus in vivo. Perhaps the concentration of the viral proteins is too low for efficient encapsidation to occur. In addition to a reduced rate of virus formation, particle instability works against the production of high virus titers in vitro. At 34°C, infectious-virus formation reached a peak after an incubation period of 12 h and readily decreased thereafter. A search for conditions that will further increase the virus yield and/or stability is in progress.

Cerulenin failed to exert any influence on translation even at a concentration as high as 200 μM, although virus forma-

This phenomenon was inhibited at this concentration. Our attempts to correlate this effect with lipid synthesis in the extracts were inconclusive. Cerulenin did not inhibit RNA synthesis in a crude membranous replication complex (8), nor did it have an effect on primer-dependent poly(U) synthesis by 3Dpol (Fig. 5; data not shown). The inhibition of viral RNA formation by cerulenin in the translation mixture therefore remains unexplained. It is possible, however, that cerulenin interacts with cellular factors essential for RNA synthesis and for morphogenesis. The drug has recently been implicated in the inhibition of a variety of cellular processes unrelated to fatty acid synthesis (20). It should be added that poly(U) synthesis, catalyzed by purified 3Dpol of encephalomyocarditis virus, has been reported to be strongly inhibited by 100 μM cerulenin (26), a result contrasting with our data obtained by using the purified poliovirus RNA polymerase.

The mechanism by which oleic acid inhibited virus formation in vitro is concentration dependent. At a concentration of 100 μM, the fatty acid interferes with protein synthesis, and that, of course, would abort any viral RNA replication. Since the inhibition of translation, but not the formation of poliovirus, can be reversed by the addition of smooth membranes, it appears that translation and processing of poliovirus polyprotein requires intact membranes. A role of membranes in specific cleavage pathways of the poliovirus polyprotein has also been suggested recently by Lawson and Semler (14) and Giachetti et al. (6). Alternative effects of oleic acid on the virus-specific translational machinery cannot be ruled out, although little change in cellular translation is seen in vivo even at 300 μM oleic acid (9).

Oleic acid inhibited endogenous RNA synthesis in the cell-free translation mixture whereas it failed to do so when poly(A)-dependent poly(U) synthesis was measured with purified 3Dpol (reference 21 and unpublished data). We have now discovered that oleic acid interferes with the stimulatory effect of poliovirus polypeptide 3AB on poly(U) synthesis catalyzed by purified 3Dpol (21). This effect could explain the inhibition of poliovirus RNA synthesis in vivo (9).

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