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# Chloroquine Induces Empty Capsid Formation during Poliovirus Eclipse

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**The poliovirus capsid (160S) is modified during eclipse in HeLa cells, which results in at least three types of particles having sedimentation coefficients of 135, 110, and 80S. The lysosomotropic agent chloroquine redirected the production of eclipse products from 135 and 110S particles (containing RNA) to 80S particles (without RNA). The effect started at 5 μM and was fully developed with 20 μM chloroquine. Viral protein synthesis and virion production remained unaffected. The results show that chloroquine can redirect the processing of input virions without interfering with productive uncoating.**

Poliovirus is endocytosed (12) after binding to an immunoglobulinlike receptor (9, 11). During eclipse, at least three species of cell-associated, modified viral particles can be detected: the 160S intact virions are quickly modified, without loss of RNA, to more slowly sedimenting 135S particles, which are eventually converted to 110S (1). A small—and variable—amount of 80S particles devoid of RNA is also formed, but its relation to the other eclipse products is unclear. Neither the intracellular location of the particles nor their role in uncoating has been established.

The cellular uptake of virions into endosomes and/or lysosomes should submit the virus particles to a gradient of descending pHs generated by cellular proton pumps. It was therefore anticipated that the acid pH inside these organelles somehow induced viral uncoating. Substances which perturb the intracellular pH have indeed been shown to exert an effect on viral multiplication: weak amines like $NH_4Cl$ or chloroquine were shown to affect an early stage in poliovirus uncoating, and an analogous effect was observed for the ionophore monensin (6, 12). Recently, however, it was suggested (2) that the effect of monensin could be attributed to inhibition or retarding of endocytosis and that chloroquine, when present in a high concentration, acted directly on the viral capsid, stabilizing it against thermodenaturation.

The effect of chloroquine on formation of the various eclipse products, however, has not been fully documented. To investigate this effect, we performed the following experiment: $^{35}S$-labelled poliovirus type 1 (input multiplicity of approximately 6,000 virions, or 30 PFU per cell) was allowed to adsorb to suspended HeLa cells ($10^6$ cells per ml in Eagle's minimal essential medium) for 2 h at 26°C. This temperature was chosen to maximize cell-associated radioactivity while not allowing the capsid modifications that occur at higher temperatures: the virions taken up at 26°C still sediment at 160S. We have evidence (5) that under these conditions, intact virus is bound to the plasma membrane but is also internalized into vesicular structures, probably endosomes. After removing the unbound virions by two washings, the cell suspension was divided into six parts and finally resuspended at 37°C in prewarmed culture medium supplemented with 0, 2.5, 5, 10, 20, or 40 μM chloroquine. (The 5 mM chloroquine-phosphate [Sigma] solution was freshly made in water; the addition to Eagle's minimal essential medium did not cause a measurable pH change at the concentrations used.) Cells were collected after 30 min, washed twice, lysed with 1% Nonidet P-40 in phosphate-buffered saline (PBS), pH 8, and analyzed on 15 to 30% sucrose gradients, also made in PBS, pH 8. Centrifugation

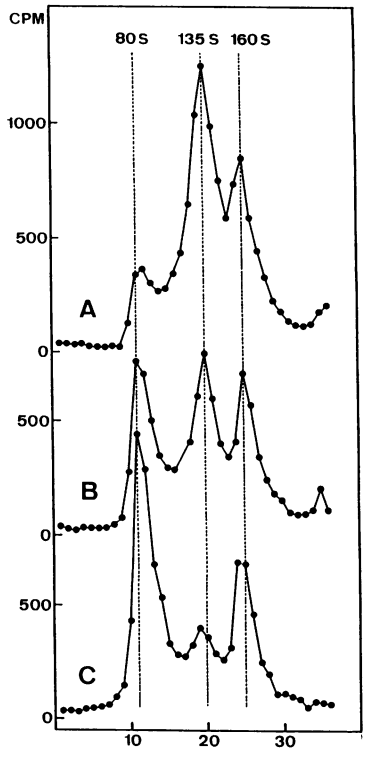


FIG. 1. Effect of chloroquine concentration on eclipse product formation. HeLa cells preadsorbed for 2 h at 26°C with $^{35}S$-labelled poliovirus were further incubated for 30 min at 37°C without (A) or with 10 μM (B) or 20 μM (C) chloroquine. Detergent lysates were analyzed by sucrose gradient centrifugation.

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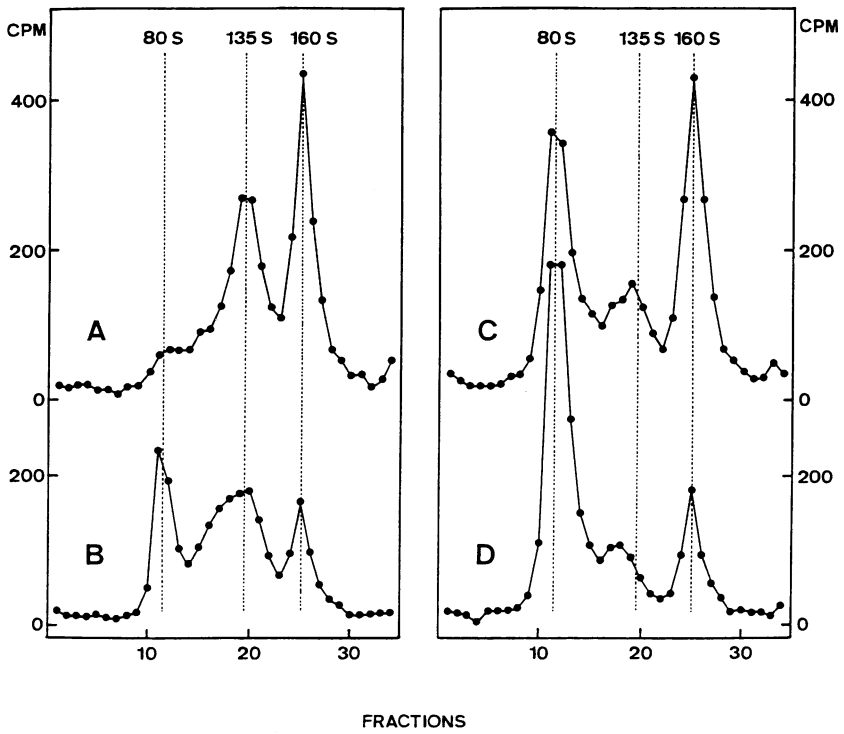


FIG. 2. Kinetics of chloroquine action. HeLa cells preadsorbed for 2 h at 26°C with $^{35}$S-labelled poliovirus were incubated at 37°C without chloroquine for 15 (A) or 60 (B) min or with 50 μM chloroquine for 15 (C) or 60 (D) min. Detergent lysates were analyzed by sucrose gradient centrifugation.

was for 5.5 h at 110,000 × $g_{av}$ in an MSE 30.6 swing-out rotor.

The results for 0, 10, and 20 μM chloroquine are shown in Fig. 1. Without chloroquine, the profile showed the expected conversion of native 160S virions to particles sedimenting at 135S and a smaller peak sedimenting at 80S. Addition of chloroquine drastically redirected virion modification to 80S and away from 135S. With 10 μM chloroquine, roughly one-half of the radioactivity was shifted from 135S to 80S, and from 20 μM and up this transfer was essentially complete. The amount of residual native virions (160S) was unaffected by chloroquine.

The chloroquine concentration selected for further experiments was 50 μM, sufficient to saturate the redirection effect, including a 2.5-fold safety margin.

The kinetics of redirection were studied by using a similar experimental design: following a preadsorbtion of 2 h at 26°C, cells were resuspended in Eagle's minimal essential medium prewarmed at 37°C with or without 50 μM chloroquine. Samples were collected at 15 and 60 min after the temperature shift-up and analyzed by sucrose gradient centrifugation (Fig. 2). In the untreated cells, 135S particles were formed within 15 min (tracing A) and subsequently converted to 110S particles (discernible as a left shoulder to the 135S peak, tracing B). In the chloroquine-treated cells, 135S production had already been shifted towards 80S after 15 min (tracing C), and after 1 h, virtually all cell-associated radioactivity had become 80S material (tracing D).

To test whether the 80S particles formed (either with or without chloroquine) contained RNA, cells were infected with a mixture of $^{3}$H-uridine-labelled and $^{35}$S-methionine-labelled virus, keeping the input multiplicity at 6,000 virions per cell. After 2 h of preadsorption at 26°C, the culture was divided into two parts. One part received medium prewarmed at 37°C, and the other part received prewarmed medium containing 50 μM chloroquine. After 30 min at 37°C, the cell suspension was washed twice and the detergent lysates were analyzed by sucrose gradient centrifugation. From Fig. 3 it can be seen that the 80S material in both chloroquine-treated and untreated cells remained free of $^{3}$H-uridine label, thus confirming the absence of RNA (1). An excess of RNA instead appeared in the 110 to 135S region, indicating that the RNA that was released from virions to yield 80S particles was probably not free (free RNA sediments at about 35S [3a]).

The formation of 80S empty capsids might reflect productive uncoating: the step that delivers the viral RNA to the cellular translation system. If so, it was of interest to find out whether chloroquine actually influenced viral protein synthesis, one of the earliest markers of successful infection.

Cells were infected by preadsorbing $^{35}$S-labelled virus for 2 h at 26°C. The culture was then divided into two parts, and medium prewarmed at 37°C (lacking methionine) was added, with or without 50 μM chloroquine. Samples were collected every 0.5 h up to 4 h and pulsed with $^{35}$S-methionine, and trichloroacetic acid-precipitable radioactivity was measured after 20 min of incorporation time; after 25 min, the remainder was prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To check chloroquine's action on the eclipse products, additional samples were taken 1 h after the temperature shift-up, lysed with Nonidet P-40, and analyzed by sucrose gradient centrifugation.

The incorporation studies (Fig. 4A) showed that there was little difference in the incorporation rates of chloroquine-treated versus untreated cells, except for a slightly faster

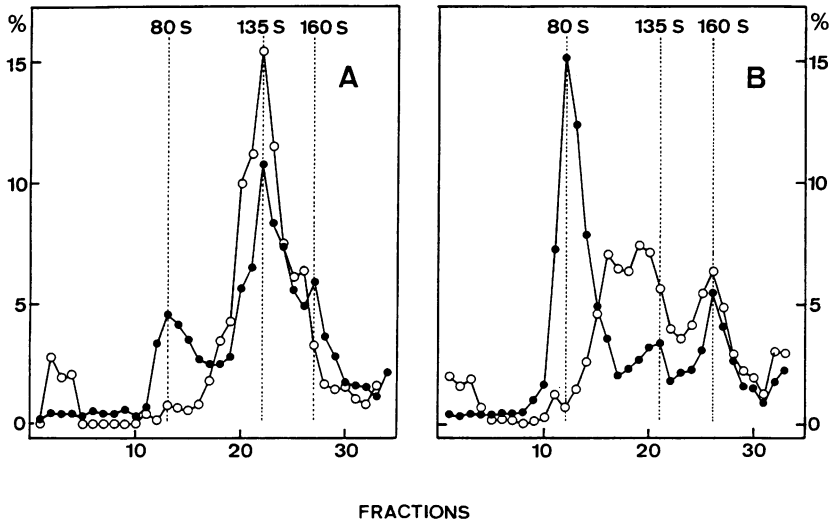


FIG. 3. RNA content of 80S particles. HeLa cells were preadsorbed for 2 h at 26°C with a mixture of $^{3}$H-uridine- and $^{35}$S-methionine-labelled poliovirus and further incubated for 30 min at 37°C without (A) or with (B) 50 μM chloroquine. Detergent lysates of the cells were analyzed by sucrose gradient centrifugation, followed by double-label counting of the fractions. Open circles, $^{3}$H radioactivity; filled circles, $^{35}$S. Results are expressed as percentages of the total counts per minute of a particular isotope in the gradient. The 100% values were 1,875 cpm ($^{3}$H, panel A), 3,681 cpm ($^{35}$S, panel A), 2,534 cpm ($^{3}$H, panel B), and 5,293 cpm ($^{35}$S, panel B).

incorporation in the treated cells. The timing and extent of host shutoff were also unaffected.

The sedimentation profiles from treated and untreated cells (Fig. 4B) confirmed the chloroquine-induced redirection of eclipse product formation, already noted in the previous experiments.

The polypeptide profiles (Fig. 4C and D) furthermore demonstrated that host shutoff and subsequent incorporation

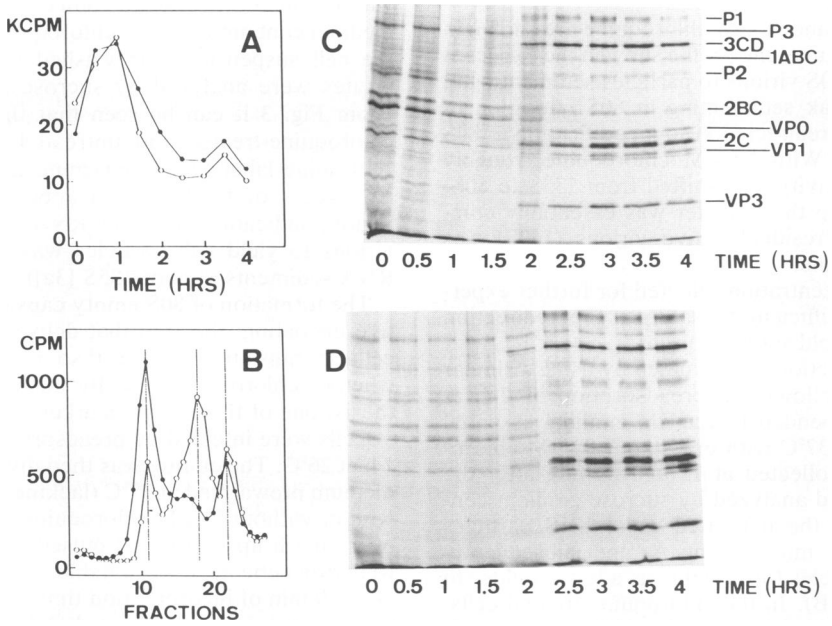


FIG. 4. Effect of chloroquine on viral protein synthesis. HeLa cells preadsorbed with $^{35}$S-labelled poliovirus for 2 h at 26°C were collected and further incubated at 37°C with or without 50 μM chloroquine. Every 30 min, a sample was taken and $^{35}$S-methionine was added to study the incorporation rate (A) and polypeptide synthesis (C and D). In addition, a sample was taken 1 h after the temperature shift-up to study eclipse product formation (B). (A) Incorporation of $^{35}$S-methionine into trichloroacetic acid-precipitable material was measured 20 min after isotope addition. Open circles, untreated cells; filled circles, cells treated with 50 μM chloroquine. (B) Sucrose gradient analysis from untreated (open circles) and chloroquine-treated (filled circles) cells collected 1 h after the temperature shift to 37°C. Centrifugation was in an MSE SW40.6 rotor. Vertical lines indicate the position of 80, 135, and 160S particles as in Fig. 1 to 3. (C and D) SDS-PAGE analysis of untreated (C) and chloroquine-treated (D) cells collected 25 min after addition of $^{35}$S-methionine. For protein nomenclature, see reference 10.

of the methionine label into viral proteins were unaffected by chloroquine: in both treated and untreated cells, the transition from cellular to viral protein synthesis occurred between 2 and 2.5 h after the temperature was raised to 37°C (panels C and D), and the same viral polypeptides were synthesized in the presence or absence of chloroquine. The contribution of the input virus to the final $^{35}S$ labelling was negligible, as shown by the absence of viral capsid proteins before the onset of viral protein synthesis.

We showed that low concentrations of the lysosomotropic weak base chloroquine redirected eclipse product formation from the RNA-containing 135 or 110S particles to 80S particles. We presented evidence that 80S particles, whether formed in the presence or absence of chloroquine, were devoid of RNA. We showed that 50 μM chloroquine did not affect viral protein synthesis, either qualitatively or quantitatively.

Similar results were obtained in additional experiments without viral preadsorption. In these experiments we also obtained evidence that production of mature virions (160S particles) was unaffected by 50 μM chloroquine (results not shown).

Our results are compatible with chloroquine's effect on poliovirus replication in HEp-2 cells, measured by plaque titration (12). With these cells, the concentration of chloroquine had to be much higher than 50 μM to inhibit virus reproduction (inhibition started at concentrations greater than 150 μM, and 1.2 mM chloroquine was required for a 5 $\log_{10}$ reduction).

Redirection of eclipse product formation thus occurs at chloroquine concentrations much lower than those required for replication inhibition. It may be of interest to note that in mouse BALB/c 3T3 fibroblasts, 140 μM chloroquine already caused a rise of the endocytic pH above 6.0 (8). Moreover, 50 μM chloroquine inhibited the replication of Semliki Forest virus (3) and other enveloped viruses which are known to use the endocytic pathway.

Two observations suggest that chloroquine induces the release of RNA from 135S particles rather than from the native virions. First, the amount of 80S particles formed equalled the depletion of 135S particles, while the amount of residual 160S virions remained constant (Fig. 1); and second, when the chloroquine concentration was raised up to 5 mM, no more 80S particles were formed than at 20 μM (results not shown). It thus seems as if no more 80S material can be produced than the amount of 160S material that is converted to 135S in the absence of the drug. Moreover, since chloroquine has been shown to exert a stabilizing effect on native virions in vitro (2), the direct formation of 80S from 160S particles seems unlikely.

If chloroquine-induced release of the genome were functionally equivalent to productive uncoating, i.e., the delivery of the viral RNA to the cellular translation system, we would expect a significant enhancement of viral protein synthesis, as more viral RNA was set free. However, since this expectation was not fulfilled, the findings thus rather suggest that the chloroquine-induced uncoating was not productive.

No reduction in viral protein synthesis was observed either, implying that the productive pathway was not affected. Taken together, the findings allow the tentative conclusion that chloroquine merely redirects an abortive pathway (160S → 135S → 110S) to another one (160S → 135S? → 80S + RNA). Different chloroquine sensitivities may reflect different cellular compartmentalization. The low concentrations used in this study may affect only the abortive pathway (e.g., in the lysosomes), whereas the higher concentrations used in other studies (discussed above) may also arrest the productive pathway that leads to viral replication.

Studies with mouse hepatitis virus, a coronavirus, showed that the low chloroquine concentrations we used in this work caused only a delay in viral replication, from which it was concluded that the productive pathway of mouse hepatitis virus did not depend on endocytotic acidification (4). Whether this is also true for poliovirus remains to be seen.

Further work will focus on the following questions: (i) does chloroquine in the 20 to 150 μM concentration range act directly on the virus particles (presumably 135S) or is its action indirect (e.g., interference with intravesicular pH, lysosomal proteases, endolysosomal fusion, or receptor recycling), (ii) how does chloroquine inhibit poliovirus replication at concentrations greater than 150 μM, and (iii) is there a link between our observations (more 80S and thus more freed RNA) and the recently reported chloroquine-induced enhancement of the replication of Semliki Forest and encephalomyocarditis viruses in mice (7)?

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