Effect of Pactamycin on Synthesis of Poliovirus Proteins: a Method for Genetic Mapping

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We have studied the effect of the drug pactamycin on protein synthesis in polio-virus-infected HeLa cells. At a concentration which primarily inhibits initiation of protein synthesis, the spectrum of poliovirus proteins synthesized is markedly changed. The amount of NCVP 1, the capsid precursor, is greatly reduced relative to NCVP 2 and the amount of NCVP X is slightly reduced. Since it is believed that there is only one major site for the initiation of protein synthesis on the poliovirus genome, we interpret this differential effect on the poliovirus proteins to be an indication of their relative distance from the initiation site. On this basis, we propose a gene order for the poliovirus genome (5' → 3') of NCVP 1, NCVP X, NCVP 2.

The poliovirus genome is a single strand of ribonucleic acid (RNA) which encodes the amino acid sequence for a number of proteins. All of these proteins are apparently formed by prote-olytic cleavage of a single large polypeptide, sug-gesting that the genome has only one major site for initiation of protein synthesis (1, 13, 14; D. Baltimore et al., In Strategy of the Viral Genome, Ciba Found. Symp., in press).

This situation affords the opportunity for se-quencing the genome by treating infected cells with a drug which specifically inhibits initiation of protein synthesis. After initiation is inhibited, synthesis of the protein encoded by the 5'-terminal end of the genome should be most rapidly de-pressed followed in order by the proteins encoded by regions increasingly nearer to the 3'-end.

Pactamycin is known to selectively inhibit ini-tiation of protein synthesis in bacteria (4, 5) and in intact or cell-free extracts of rabbit reticulo-cytes (17; H. F. Lodish et al., Biochemistry, in press). In this paper, we will show that, in both uninfected and poliovirus-infected HeLa cells, there is a transient period of protein synthesis after addition of pactamycin followed by com-plete inhibition, suggesting that in these systems, too, the drug is a selective inhibitor of the initia-tion of protein synthesis. During the transient period of protein synthesis after addition of pactamycin there is a differential inhibition of synthesis of the various poliovirus-specific poly-peptides. This allows a tentative ordering of the regions of the genome which encode the various polypeptides.

MATERIALS AND METHODS

Infection of cells. The growth of HeLa cells in spinner-modified Eagle's medium (9) plus 7% horse serum and their infection with poliovirus in the pres-ence of 5 μg of actinomycin D per ml at 4×10^6 cells per ml has been described previously (2).

Incorporation studies. Cells at 3 hr after infection were removed from growth medium and resuspended at 4×10^6 cells per ml in Earle's saline (9) for labeling. Radioactive amino acids were added, and samples were diluted with cold Earle's saline at various times thereafter. The samples were washed with cold Earle's saline, resuspended in water, made 0.1 N in NaOH, incubated for 10 min at 37 C, and precipitated with cold 5% trichloroacetic acid. The precipitates were collected on membrane filters (Millipore Corp.) and counted in a liquid scintillation spectrometer in 10 ml of a mixture of 5 g of 2,5-diphenyloxazole and 100 g of naphthalene in 1 liter of dioxane.

Gel electrophoresis. Cytoplasmic extracts were pre-pared by swelling cells for 5 min in distilled water at 0 C and homogenizing with a Dounce homogenizer. Nuclei were removed by centrifugation. The cyto-plasmic extract was adjusted to 1% mercaptoethanol, 1% sodium dodecyl sulfate, 15% glycerol and boiled for 2 min. It was then subjected to the Maizel and Laemmli modification of sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (16; J. V. Maizel, personal communication) for approximately 3.5 hr at 100 v. For densitometric analysis of the gel patterns, they were sliced longitudinally into four slices. One of the center slices was placed on a blotter paper and lyophilized overnight. The dried gel was taped to Kodak RB 2 X-ray film and exposed for 2 days. The autoradiogram was scanned at 500 nm with a Gilford spectrophotometer modified for gel scanning.

Sucrose gradient analysis. Preparation of cyto-

plasmic extracts and analysis on sucrose gradients have been previously described (2, 12, 18).

Materials. [³⁵S]methionine (~6 Ci/mmole) was prepared as previously described (19) and was provided by Harvey Lodish.

Pactamycin was provided by Gunther S. Fonken of the Upjohn Co. Actinomycin D was provided by Merck, Sharpe and Dohme. [¹⁴C]agal hydrolysate was obtained from New England Nuclear Corp., [³H]methionine was obtained from Amersham/Searle Corp. (5.2 Ci/mmole), and DL-p-fluorphenylalanine was obtained from the Sigma Co.

RESULTS

Concentration of pactamycin for selective inhibition. At low concentrations (~5 × 10⁻⁷ M), pactamycin selectively inhibits initiation of protein synthesis in a reticulocyte cell-free system, but, at higher concentrations, inhibition of translational steps is evident (17; H. F. Lodish et al., Biochemistry, in press). Lacking a specific assay for initiation in intact poliovirus-infected HeLa cells, we determined the minimal concentration of pactamycin which would eventually stop protein synthesis completely and have used that concentration in further experiments.

In poliovirus-infected HeLa cells, 10⁻⁷ M pactamycin was the lowest concentration of the drug which ultimately produced complete inhibition of protein synthesis (Fig. 1); 10⁻⁸ M produced a partial inhibition, whereas 10⁻⁶ M resulted in more rapid inhibition. As in a reticulocyte cell-free system, complete inhibition was not instantaneous but rather an accelerating rate of inhibition occurred over a few minutes.

Effect of pactamycin on the spectrum of proteins synthesized. To investigate whether pactamycin selectively decreased the labeling of any of the poliovirus polypeptides, cells infected for 3 hr were exposed to 10⁻⁷ M pactamycin and 3 min later [³⁵S]methionine was added. After 7 min of exposure to the [³⁵S]methionine, the cells were harvested. A second sample of cells was labeled with [³⁵S]methionine in the absence of inhibitor for 5 min followed by a chase of 5 min. Electrophoresis on polyacrylamide gels of the labeled protein from the two samples followed by autoradiography and densitometry of the gels showed that the protein formed during the exposure to pactamycin contained much less of the coat protein precursor polypeptide, NCVP 1 (14, 21), than that made in the untreated cells (Fig. 2). The gels resolved more poliovirus-specific polypeptides than have previously been noted (21), and the nomenclature has been extended to include them. Specifically, NCVP 3 has been split into two components, NCVP 3a and 3b; NCVP 3a is also lacking in the treated sample. Resolution of smaller proteins including NCVP X (13,

14) and the capsid proteins (VP) was not achieved in this analysis so the effect of pactamycin on their synthesis is not clear.

Rather than attempt to differentiate protein NCVP X from capsid proteins VP 0, VP 1, and VP 3, all of which have similar migration rates during electrophoresis, we chose to inhibit all capsid protein formation with p-fluorophenylalanine (FPA). As shown previously (14), 1.1 mM FPA prevents cleavage of NCVP 1 to form VP 0, VP 1, and VP 3 but does not prevent the cleavage of larger precursors. Therefore, in 1.1 mM FPA, only three major polypeptides are formed (NCVP 1, NCVP 2, and NCVP X) plus a number of less prominent ones. Using FPA-treated, infected cells, a comparison was made between the polypeptides made in the presence and absence of pactamycin. Differentially labeled extracts were analyzed on a single gel with the data normalized such that the peaks of NCVP 2 were the same height (Fig. 3). In the presence of FPA, pactamycin again preferentially inhibited synthesis of NCVP 1. Furthermore, a small decrease of NCVP
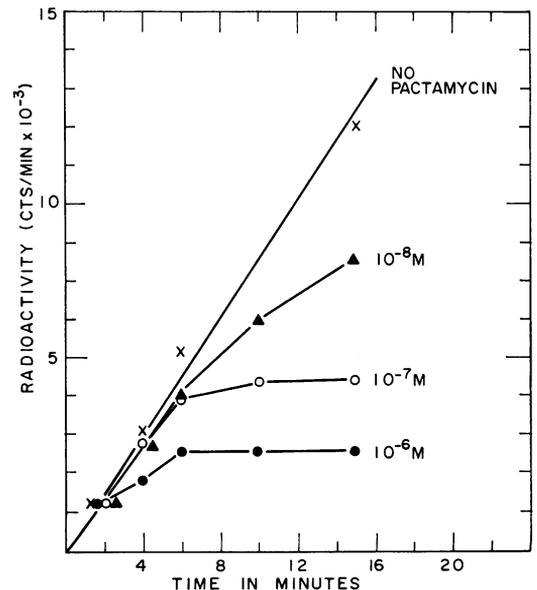
FIG. 1. *Time course of incorporation of amino acids in poliovirus-infected HeLa cells at different concentrations of pactamycin. At 3 hr after infection, cells were harvested and resuspended in Earle's saline at 4 × 10⁶ cells/ml. The cells were separated into four equal portions and pactamycin at a final concentration of 10⁻⁶ M (●), 10⁻⁷ M (○), or 10⁻⁸ M (▲), or no drug (×) was added to each. Radioactive [¹⁴C]agal hydrolysate (0.5 μCi/ml) was added to each sample simultaneously with the pactamycin. Samples were taken at the indicated times and treated as described in Materials and Methods.*
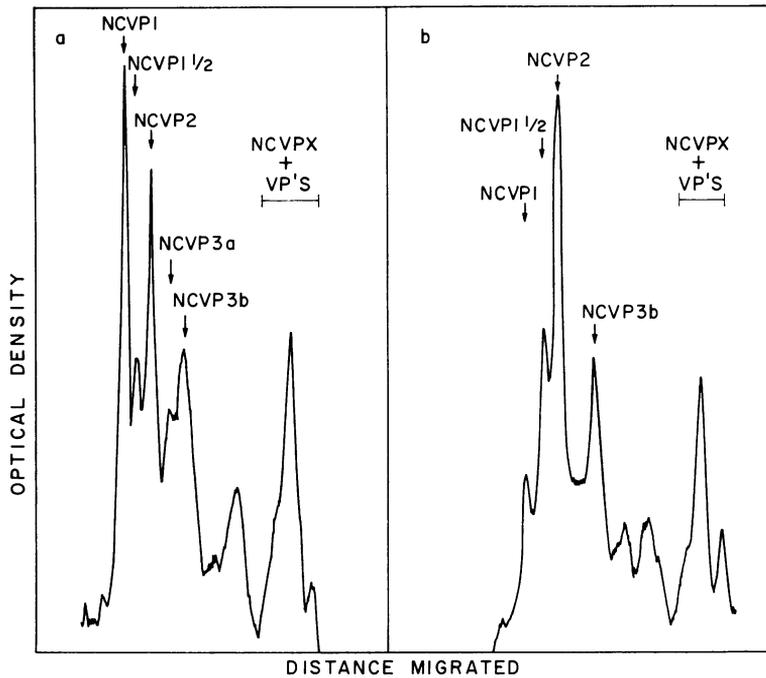
FIG. 2. *Polyacrylamide gel electrophoresis of cytoplasmic extracts of poliovirus-infected cells in the absence (a) or presence (b) of pactamycin. Tracings of an autoradiogram of the acrylamide gels are shown. At 3 hr after infection, cultures of 2×10^7 cells were suspended in Earle's saline. After 10 min, (a) was labeled with [^{35}S] methionine (5 μCi/ml) for 5 min and chased for 5 min with a 10^5 excess of cold methionine, and (b) was made 10^{-7} M in pactamycin and labeled 3 min later with [^{35}S] methionine and incubation was stopped at 10 min.*
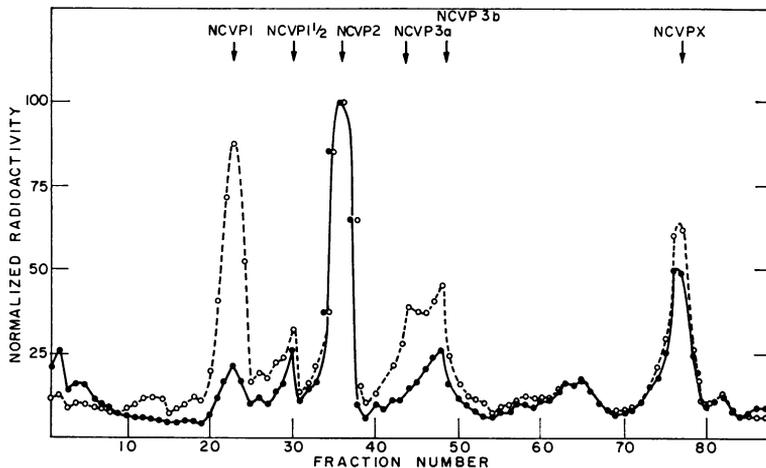

FIG. 3. *Polyacrylamide gel electrophoresis of cytoplasmic extracts of p-fluorophenylalanine (FPA)-treated cells. Poliovirus-infected cells 3 hr after infection at 4×10^6 cells/ml were made 1.1 mM FPA in Earle's saline and incubated for 10 min. To one culture, 100 μCi of [^{3}H]methionine per ml was then added and cells were labeled for 25 min (○). To the other culture, 10^{-7}M pactamycin was added, cells were incubated for 2.5 min, 60 μCi of [^{35}S]methionine per ml was added, and incubation was continued for an additional 22.5 min (●). Cytoplasmic extracts were prepared separately, and portions of each were mixed and analyzed by electrophoresis. Radioactivity in gel slices was normalized to 100 for both (^{3}H) and (^{35}S) in fraction 36. This represents 4,840 counts/ min in the [^{3}H]methionine sample (○) and 2,149 counts/min in the [^{35}S]methionine sample (●).*

X relative to NCVP 2 was evident in the treated sample along with the elimination of NCVP 3a. Effects on other minor peaks were evident but cannot be interpreted until the relationship of these proteins to the major ones is clarified.

Effect of pactamycin on protein synthesis and polyribosomes. The results in Fig. 2 and 3 were reproducible in other experiments, but attempts to change the ratio of NCVP 2 to NCVP X were unsuccessful even when the time between addition of pactamycin and addition of labeled amino acid was lengthened. This led us to look further at the effects of the drug on uninfected and infected HeLa cells.

In the reticulocyte system, even at 25 C, only 1 to 2 min was required to give a complete cessation of synthesis (H. F. Lodish et al., Biochemistry, *in press*), whereas poliovirus-infected HeLa cells required more than 6 min (Fig. 1). This could reflect the time necessary for entry of the drug into the cells or the longer messenger RNA for poliovirus proteins than for hemoglobin, or both. If the length of the messenger RNA is a factor, uninfected HeLa cells should cease synthesis sooner than infected cells. To test this possibility, infected and uninfected cells were simultaneously exposed to 10^{-7} M pactamycin and [^{35}S]methionine. In uninfected cells, the accumulation of labeled protein in the treated sample deviated from that in an untreated sample within 2 min after treatment and inhibition was 90% complete

by about 4 min (Fig. 4b). In contrast, in the presence of pactamycin, the poliovirus-infected cells maintained their rate of synthesis near that of the untreated, infected cells for about 4 min and 90% inhibition occurred at about 7 min (Fig. 4a). Similar results have been obtained by measuring rates of synthesis in uninfected and infected cells by using 1-min pulses of [^{3}H]leucine.

In uninfected HeLa cells, pactamycin markedly reduced the average size of the polyribosomes by 2 min after addition and by 5 min few polyribosomes were evident (Fig. 5). In poliovirus-infected cells, the drug required 5 min to decrease the size of the major polyribosome peak. A slow disappearance of polyribosomes then ensued which was complete by 25 min (Fig. 6). Most of the [^{35}S]methionine incorporated during pactamycin treatment was initially localized in polyribosomes and was released from the polyribosomes by 25 min after the addition of the drug (Fig. 7). The results with uninfected cells are consistent with a preferential blockage of initiation in pactamycin-treated cells. The results with infected cells are also consistent with this idea but are less clear and will be discussed below.

DISCUSSION

Our hope in performing these experiments was to provide a method for sequencing the gene order on the poliovirus genome. Because it appears that the genome is translated into a single polypeptide
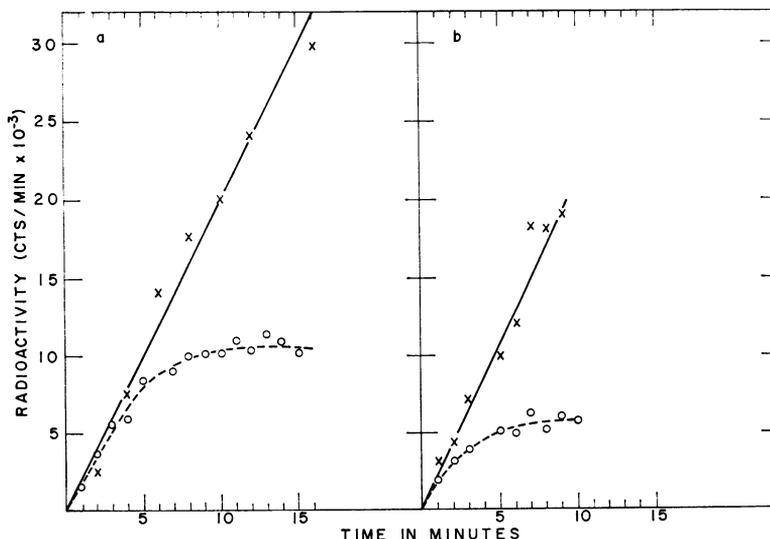

FIG. 4. *Time course of incorporation of* [^{35}S]*methionine in uninfected and poliovirus-infected HeLa cells.* (a) *Poliovirus-infected cells were treated as in Fig. 1 and labeled with* [^{35}S]*methionine (2 μCi/ml) either in the presence* (○) *or the absence* (×) *of* 10^{-7} M *pactamycin.* (b) *Uninfected cells were treated as in Fig. 1 and labeled with* [^{35}S]*methionine (2 μCi/ml) either in the presence* (○) *or the absence* (×) *of* 10^{-7} M *pactamycin.*
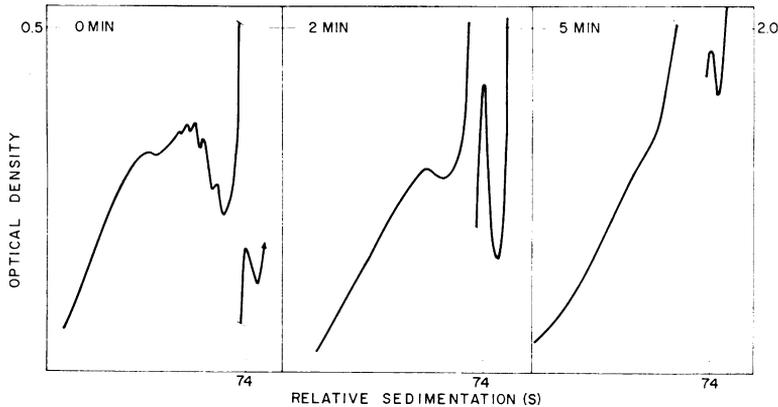
FIG. 5. *Sucrose gradient pattern of polyribosomes from pactamycin-treated uninfected HeLa cells. Cells suspended in Earle's saline at $4 \times 10^6/ml$ were adjusted to 10^{-7} M pactamycin. Samples of 2×10^7 cells were placed into cold Earle's saline at 0, 2, and 5 min after pactamycin addition. Cytoplasmic extracts were prepared and layered onto 15 to 30% sucrose gradients made up in 0.01 M tris (hydroxymethyl)aminomethane, pH 7.4, 0.01 M NaCl, and 0.0015 M $MgCl_2$. Centrifugation was for 100 min at 25,000 rev/min in the Spinco SW27 rotor at 4 C.*

chain, this would be equivalent to sequencing the order of proteins in this polypeptide.

The experiments suggest that NCVP 1 is encoded by information at the 5' end of the genome. The reasons for this conclusion are: (i) within 2.5 to 3 min after pactamycin treatment only NCVP 1 synthesis is markedly inhibited and (ii) the evidence for translation of the viral genome into a single polypeptide implies that only one initiation site exists on the viral genome (12–14) and this should be at the 5' end (3). This conclusion agrees with the genetic mapping data of Cooper which placed the capsid proteins at an end of the genome (7).

Only a tentative conclusion can be made about the location of the other two major viral genes, those for NCVP 2 and NCVP X, because their specific activities (Fig. 3) are not very different. The differences are reproducible, however, and so the gene for NCVP 2 would appear to be at the 3' end of the genome because its synthesis is least affected by pactamycin. NCVP X would then be between NCVP 1 and NCVP 2 because its synthesis is more severely inhibited by pactamycin than the synthesis of NCVP 2.

Polypeptides smaller than NCVP X have not been investigated. The intermediate peaks, which are not made in molar amounts relative to NCVP 1, NCVP 2, and NCVP X, are hard to interpret until their relation to the other proteins has been determined by, for instance, peptide composition. The coincidental behavior of NCVP 1 and NCVP 3a suggests a relation between these two species.

A gene order of NCVP 1-NCVP X-NCVP 2 might explain the existence of NCVP 0 in infected cells treated with either amino acid analogues (13, 14) or high temperature (1; D. Baltimore et al.,

In The Strategy of the Viral Genome, Ciba Found. Symp., *in press*). NCVP 0 is always a broad peak and therefore is probably two (or more) species of protein. It could consist of one polypeptide consisting of NCVP 1 plus NCVP X and a second of NCVP X plus NCVP 2.

Inhibition of polypeptide elongation in poliovirus-infected cells. Our experiments were suggested by the observation that in a reticulocyte cell-free system, inhibition of initiation is the major result of adding a critical concentration of pactamycin (H. F. Lodish et al., Biochemistry, *in press*). In uninfected HeLa cells, at 10^{-7} M pactamycin, the time course of breakdown of polyribosomes paralleled the inhibition of protein synthesis, suggesting that initiation of protein synthesis was also selectively affected in this system. In poliovirus-infected cells, however, although the time course of inhibition was lengthened as would be predicted from the existence of a long messenger RNA translated from a single initiation site, there are two anomalies. First, synthesis stops too rapidly. The viral genome is about 2.5×10^6 daltons (11), whereas the average cell messenger RNA is about 0.5×10^6 (15; Baltimore, *unpublished data*). It should therefore require about five times as long for complete inhibition of viral protein synthesis than of cell protein synthesis. The measured difference was no more than twofold. Second, in infected cells, the polyribosomes do not disaggregate in parallel with the inhibition of protein synthesis. A progressive diminution of polyribosome size and number does occur, but, by 10 min, when incorporation is no longer detectable, polyribosomes are still present.
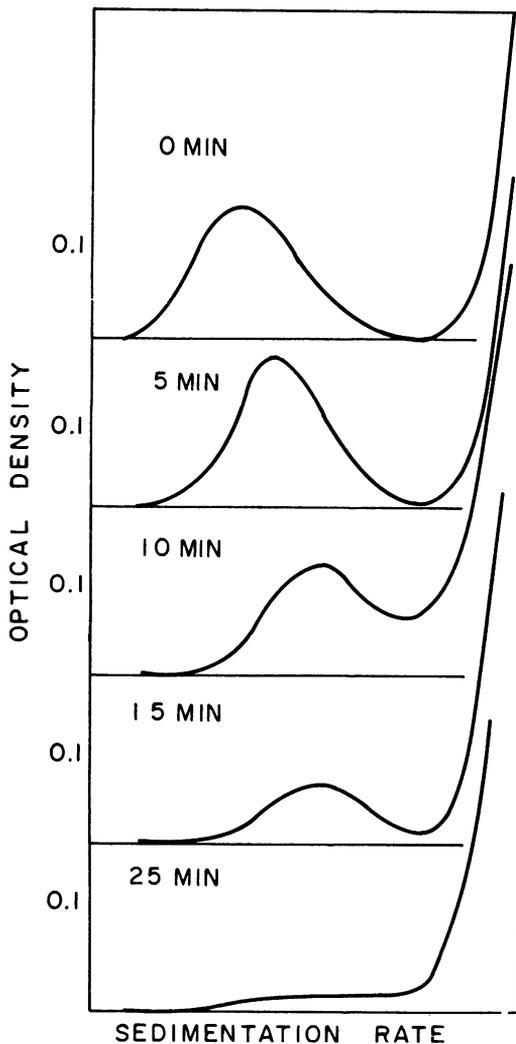
From these data, it appears that in infected

FIG. 6. *Sucrose gradient analysis of polyribosomes from poliovirus-infected HeLa cells. At 3 hr after infection, cells were centrifuged and resuspended at 4×10^6 cells per ml in Earle's saline. Pactamycin was then added to 10^{-7} M. Samples of 2×10^7 cells were taken at 0, 5, 10, 15, and 25 min after the addition of pactamycin. Sucrose gradient analysis of cytoplasmic extracts was carried out as in Fig. 5. Only the portion of the gradients displaying material larger than 80S is shown.*

FIG. 7. *Sucrose gradient analysis of polyribosomes from poliovirus-infected HeLa cells. To cells, treated as in Fig. 6, [35S]methionine (~2 μCi/ml) was added at 3 min after the addition of 10^{-7} M pactamycin. Samples of 2×10^7 cells were taken into cold Earle's saline at 3.5 min (O), 10 min (X), and 25 min (△) after pactamycin addition. Sucrose gradient analysis of cytoplasmic extracts was carried out as in Fig. 5, and acid-precipitable radioactivity was determined. The radioactivity in each gradient was summed (3.5 min, 62,812; 10 min, 381,110; 25 min, 405,156) and normalized to the 25-min total to make the proportion of counts in polyribosomes readily discernible. Single ribosomes (74S) sedimented at fraction 16.*

cells 10^{-7} M pactamycin inhibits both initiation and elongation of polypeptide chains. This could be the result of the long time necessary for complete translation of the long viral messenger RNA which allows a high intracellular concentration of the drug to accumulate, leading to a rapid initial block of polypeptide initiation followed by a slower blockage of elongation. The irreversability of pactamycin inhibition (6, 10; R. Taber and D.
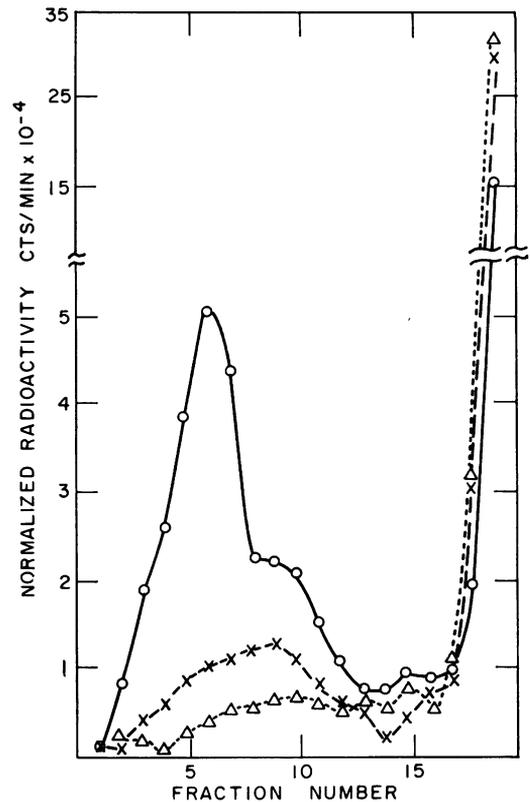
Rekosh, *unpublished data*), in fact, suggests that an increasing intracellular concentration of the drug may accumulate.

Another possible explanation for the slow loss of polyribosomes is that translation by one ribosome in a polyribosome may by chance become inhibited by the drug, and, because of the large size of the viral polyribosome, a larger number of ribosomes would be inhibited, whereas in a smaller polyribosome this effect would be less evident.

Whatever the reason for the apparent inhibition of polypeptide chain elongation by pactamycin in infected cells, it limits the types of experiments

which can be performed by causing an asynchrony of run-off of ribosomes. Probably for this reason, we have been limited to about 3 min between the times of addition of pactamycin and the labeled amino acid; although longer times have been tried, they merely diminish the amount of radioactivity incorporated without changing the patterns on polyacrylamide gels from those in Fig. 2 and 3.

Further utility of pactamycin for genetic mapping. It may be possible to use pactamycin in experiments like those reported here to investigate a number of questions in virology and cell biology. It should be generally true that the synthesis of proteins encoded by separate monocistronic messenger RNA species will be inhibited rapidly and coincidentally by pactamycin. Proteins made from a single messenger RNA and formed by proteolytic cleavage of a precursor polypeptide should be inhibited sequentially. One specific problem amenable to solution is whether the two group A arbovirus (togavirus) proteins are formed by cleavage from a single precursor (20; E. Ginsberg and B. Burge, *unpublished data*).

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ADDENDUM IN PROOF

After submitting this communication, we became aware of similar experiments performed by Summers and Maizel (Proc. Nat. Acad. Sci. U.S.A., *in press*). Their data also indicate that NCVP 1 is coded for by RNA at or near the 5' end of the genome.

Recently, by extending the pactamycin technique, we have sequenced the coat proteins within NCVP 1 and obtained an order (5' → 3') of VP 4, VP 2, VP 3, VP 1 (D. Rekosh, *in preparation*).