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Poliovirus Replication: Interactions with Cytoplasmic Membranes and with Compounds Which Affect Virus Biosynthesis

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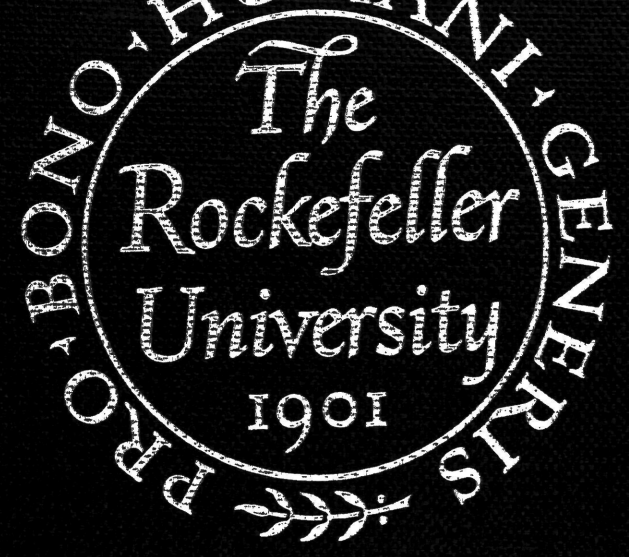
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POLIOVIRUS REPLICATION: INTERACTIONS WITH CYTOPLASMIC MEMBRANES AND WITH COMPOUNDS WHICH AFFECT VIRUS BIOSYNTHESIS

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by

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Preface

The studies described in this dissertation explore the replication of poliovirus RNA from two approaches. We have investigated the interactions between viral RNA synthesis and the production and distribution of cytoplasmic membranes. The quantitative aspects of poliovirus multiplication under the combined effects of a specific virus inhibitor, guanidine hydrochloride, and compounds which block that inhibition, have also been studied.

I am grateful for the privilege of studying at The Rockefeller University. I have enjoyed its atmosphere of intellectual excitement and the many beneficial associations with its faculty and students.

For the guidance, encouragement and enthusiasm of Dr. Lawrence Caliguiri and Dr. Igor Tamm, I am deeply indebted. Thanks are also due to the other members of the virology laboratory for fruitful discussions, and to its technical and clerical staff for much appreciated help. I gratefully acknowledge the help of Dr. Andreas Scheid, who performed the phospholipid analyses reported here. I would also like to thank Dr. Wilhelm Stoffel who suggested the use of glycerol-2-^{3}H for pulse-chase studies of lipid synthesis.

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Summary

Picornavirus multiplication is intimately associated with intracellular membranes and affects their production and distribution within the cell. We have shown that the density distribution of cytoplasmic membranes separated by isopycnic centrifugation in discontinuous sucrose gradients is progressively altered after infection of HeLa cells with poliovirus. The most striking change is a very large increase in the smooth microsomal membranes with which viral RNA synthesis is associated. These membranes begin to increase between 2.5 and 3.5 hours after infection, and continue to proliferate late in the virus growth cycle, at a time when cellular protein and nucleic acid syntheses are much reduced.

These smooth membranes differ from the membranes in the corresponding fraction from uninfected cells in several ways. They have a higher phospholipid/protein ratio and the phospholipid composition is altered. We have confirmed that the viral RNA polymerase activity is firmly associated with the membranes in this fraction and that it may be localized in a complex with a sedimentation constant of 130 S after lysis of the membranes with deoxycholate.

We have utilized ^{3}H-choline as a specific precursor of choline-containing phospholipids, to study the synthesis of cytoplasmic membranes. We have confirmed that the incorporation of ^{3}H-choline into cytoplasmic membranes is greatly stimulated by poliovirus infection. When uninfected or infected cells are pulsed for 3 minutes with ^{3}H-choline, and then fractionated on discontinuous sucrose gradients, the highest activity is found in the rough microsomal membranes which form a thick pellicle in the lower third of the centrifuge tube. These membranes probably constitute the intracellular location for the synthesis and incorporation of phospholipids into membranes. Uninfected cells still show this distribution of ^{3}H-choline after 30 minutes of

labeling, but in infected cells, a bimodal distribution is observed. The radioactivity is preferentially incorporated into the accumulating smooth microsomal membranes as well as into the pellicle of rough microsomal membranes.

When infected and uninfected cells are pulse-labeled for 3 minutes with tritiated glycerol, the infected cells incorporate 4-5 times as much radioactivity as uninfected cells. About 80% of the label is incorporated into the rough endoplasmic reticulum. After a 60-minute chase period, the distribution of radioactivity does not change significantly in uninfected cells. By contrast, in infected cells, much of the label shifts from the rough microsomes into the smooth membranes, particularly those with which viral RNA synthesis is associated. Thus, it appears that there is preferential synthesis in the rough endoplasmic reticulum of lipid destined to become part of the smooth membranes which accumulate throughout infection.

Treatment of infected cells with 1.0 to 2.0 mM guanidine-HCl inhibits viral RNA synthesis, but does not measurably affect cellular metabolism. We have confirmed the observation that early guanidine treatment of cells infected with at least 50 PFU per cell of poliovirus does not block the virus-induced stimulation of choline incorporation, although this stimulation is diminished. However, such guanidine-treated, infected cells do not produce the large quantities of smooth surfaced membranes which normally accumulate in infected cells. Indeed, the density distribution of membranes in guanidine-treated, infected cells resembles closely that of uninfected cells. This suggests that poliovirus products may affect the control of cellular membrane synthesis in two separable ways: incorporation of precursors, and the conversion of rough to smooth-surfaced membranes.

We have also studied the choline-mediated blocking of the inhibitory action of guanidine on poliovirus biosynthesis in three cell types. Poliovirus biosynthesis shows increasing sensitivity to

inhibition by guanidine in the different cells in the order: HeLa cells, LLC-MK_2 cells, and primary rhesus monkey kidney cells. When viral replication is more strongly inhibited in a given cell type, a higher concentration of choline is required to block the inhibition. Kinetic studies in all three cell types show that 10 mM choline rapidly reverses the inhibitory effects of 0.4 mM guanidine. After addition of choline, viral multiplication proceeds at a rate indistinguishable from that in control cells that have received neither guanidine nor choline. Choline does not appear to enhance the rate of exit of ^{14}C-guanidine from cells incubated in guanidine-free medium.

A variety of amino acids and compounds structurally related to choline are also known to have the ability to block guanidine inhibition of poliovirus replication. Although choline and methionine act synergistically in blocking the virus-inhibitory action of guanidine, choline and dimethylethanolamine are incapable of synergism. This suggests that guanidine antagonists may be grouped into several classes.

Choline, dimethylethanolamine and methionine all suppress the growth-supporting activity of 0.4 mM guanidine on a guanidine-dependent strain of poliovirus type 1.

Possible mechanisms by which choline and other guanidine antagonists might block the action of guanidine on poliovirus multiplication are considered, and the implications of the results of our studies for these mechanisms are discussed.

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Abbreviations

Most of the abbreviations used in this dissertation are those for which the Journal of Biological Chemistry requires no definition [J. Biol. Chem. 246, 1-8 (1971)]. The following have also been used:

MW - molecular weight
PE - phosphatidylethanolamine
PFU - plaque forming units
PI - phosphatidylinositol

1. GENERAL INTRODUCTION

Chapter 1

General Introduction

Poliovirus is one of the picornaviruses, a group of animal viruses consisting of a single-stranded RNA genome surrounded by a protein coat. Other picornaviruses which are quite similar to poliovirus in the biochemistry of the viral replication cycle are foot-and-mouth disease virus, mengovirus, coxsackieviruses and some of the echoviruses. Occasionally results of studies with these viruses will be cited in discussion of poliovirus replication.

The poliovirus genome has a molecular weight which has been reported as 2×10^6 daltons (Schaffer, 1962) or, more recently, as 2.6×10^6 daltons (Granboulan and Girard, 1969). Its coat contains 4 proteins (Maizel, 1963; Summers et al., 1965). The molecular weights of the virus capsid proteins, which vary somewhat with different types of poliovirus, are as follows: VP1, 32-35,000; VP2, 25-28,000; VP3, 23-24,000; VP4, 4-6,000 (Jacobsen et al., 1970; Holland and Kiehn, 1968; Maizel and Summers, 1968). The molecular weight of the infectious virus particle is about 7×10^6 daltons.

The Virus Cycle

Early Events

Poliovirus adsorbs to specific protein receptors on the surface of susceptible cells (Holland and McLaren, 1961). Adsorption of the virus to plasma membrane receptors results in labilization of the capsid to proteolytic enzymes (Holland, 1962a; Holland and Hoyer, 1962). It is followed by penetration of the virion into the cell and proteolytic digestion of the capsid protein. Mandel (1967) suggests that penetration is achieved by a process of cellular phagocytosis, and that the capsid protein is digested within the phagocytic vesicle. The viral RNA is then released into the cytoplasm, and this is where the events of virus replication occur.

Viral Protein Synthesis

The viral genome acts as messenger RNA, utilizing cellular ribosomes and transfer RNA molecules in association with cellular membranes in the production of viral protein. Early in the infective cycle viral polyribosomes have an average sedimentation constant of 380 to 400 S; each polyribosome contains about 30 to 50 ribosomes (Penman et al., 1963; Rich et al., 1963; Scharff et al., 1963; Summers et al., 1965, 1967). The viral polyribosomes are significantly larger than the typical polyribosomes of uninfected HeLa cells, which have an average sedimentation constant of 200 to 250 S (Penman et al., 1963; Scharff et al., 1963). Late in the infective cycle the size and activity of viral polyribosomes declines (Summers et al., 1967).

Viral protein is most likely made in one long piece which is later cleaved several times in specific places to form the various viral capsid and noncapsid proteins (Summers et al., 1965; Summers and Maizel, 1968; Jacobsen and Baltimore, 1968b; Jacobsen et al., 1970). Jacobsen et al. (1970) have shown that these cleavages may be blocked by treating infected cells with the amino acid analogs canavanine, p-fluorophenylalanine and azetidine-2-carboxylic acid. When this is done, a polypeptide (NCVP 00) with a molecular weight greater than 200,000 daltons is produced. This could represent the translation product of the entire poliovirus genome.

Viral RNA Synthesis

Three classes of virus-specific RNA have been isolated from and identified in poliovirus-infected cells. Single-stranded RNA, which is the size of mature poliovirus RNA, has a sedimentation constant of 33 S (Granboulan and Girard, 1969). There is an 18 S ribonuclease-resistant, double-stranded RNA, called replicative form (Bishop and Koch, 1967). Finally, there is a structure which has been called the replicative intermediate (Erickson et al., 1964; Baltimore and Girard, 1966); it is a partially ribonuclease-resistant multistranded complex. As isolated from infected cells, it has characteristics of both double- and single-

stranded RNA, but its double-strandedness may be an artifact of the isolation procedure.

Viral RNA is replicated in a complex which includes the RNA-dependent viral RNA polymerase and replicative intermediate RNA. This "replication complex", which has been discussed by Girard et al. (1967), operates in association with membranes in the infected cell (Caliguiri and Tamm, 1970b). RNA strands complementary to the virus RNA, which have been termed "minus strands" are formed first; these are then used for the synthesis of more RNA identical to the virus RNA, which is also called the "plus strand". It is not known whether these two processes, production of minus and plus strands, are performed by a single enzyme or more than one. The plus strands may be utilized in any one of three ways: in the synthesis of more viral RNA; in viral protein synthesis; and in the formation of the mature virion. Ribosomes (Caliguiri and Tamm, 1970b) and viral proteins (Caliguiri and Mosser, submitted for publication) probably bind to the nascent viral RNA, still attached to the membrane-associated replication complex. This provides an efficient mechanism for the initiation of viral protein synthesis or morphogenesis of virions.

Assembly and Release of Mature Virions

The process of morphogenesis involves a final cleavage of a viral precursor protein, called NCVP6 or VP0 (MW: 40,000), into two of the viral capsid proteins, VP2 (MW: 25-28,000) and VP4 (MW: 4-6,000) (Jacobsen and Baltimore, 1968a). Infected cells contain many particles with the morphological appearance of empty poliovirus capsids. These empty capsids contain VP0 and two viral capsid proteins, VP1 and VP3, but no viral RNA (Schwerdt, 1957; Maizel et al., 1967). This structure may associate with the viral RNA after its constituent proteins have assembled, or the whole structure may assemble in association with the nascent viral RNA strands.

Mature virus accumulates intracellularly and is eventually released into the surrounding medium either by lysis of the whole cell (Lwoff et

al., 1955; Amako and Dales, 1967a) or by pinching off of cytoplasmic blebs which then lyse to release their contents (Levinthal et al., 1969).

Effects of Poliovirus Infection on its Host Cell

During the course of picornavirus infection, cellular metabolism is altered radically. This has been extensively documented in the literature. I will confine my discussion primarily to studies in which poliovirus was utilized. Although there are some quantitative differences observed when picornaviruses other than poliovirus are used, qualitatively the results are in good agreement.

Inhibition of Cellular Biosynthetic Processes

Between one and two hours after infection cellular protein synthesis declines sharply (Zimmerman et al., 1963; Holland and Peterson, 1964; Penman and Summers, 1965). Willems and Penman (1966) have presented evidence that the initial manifestation of this inhibition is a disaggregation of cellular polyribosomes. Inhibition of cellular DNA synthesis appears to be secondary to inhibition of cellular protein synthesis (Ensminger and Tamm, 1970). Cellular RNA synthesis is also inhibited soon after infection, although not always as much as protein synthesis (Baltimore and Franklin, 1962; Zimmerman et al., 1963; Holland and Peterson, 1964; Holland, 1964). This inhibition seems to be the result of the inactivation of the cellular DNA-dependent RNA polymerase, rather than a loss of template activity of host DNA (Baltimore and Franklin, 1962; Holland, 1962b). The kinetics with which the cellular synthetic processes are inhibited have been shown to depend on the multiplicity of infecting virus (Penman and Summers, 1965) and on the cell type used (Bablanian et al., 1965a).

Stimulation of Phospholipid Synthesis

Concomitant with these inhibitory effects, poliovirus infection stimulates the production of cellular phospholipids. Miroff and coworkers (1957) reported that by 4 hours after infection with poliovirus the incorporation of $^{32}PO_4$ into phospholipids of HeLa cells was greatly

stimulated. These results were confirmed by Cornatzer and co-workers (1961), who showed that the specific activities of ^{32}P in various phospholipids were increased after poliovirus infection. At 4 hours after infection, the greatest increases in specific activity were found in phosphatidylethanolamine and phosphatidylcholine. Penman (1965) studied the time course of incorporation of ^{3}H-choline into trichloroacetic acid-precipitable structures, and found that enhanced incorporation could be demonstrated between 2.5 and 3 hours after infection. This enhancement was abolished by treatment of the cultures with puromycin at 2 hours after infection, but only slightly reduced if puromycin was not added until 3 hours after infection. Virus which had been irradiated with ultraviolet light prior to inoculation was unable to stimulate the incorporation of choline into acid-precipitable structures. Thus, an active viral genome and some protein synthesis are necessary for the stimulation of phospholipid synthesis.

Morphological Alterations

The infected cell also shows gross cytopathological effects which are visible with the light microscope. Early in the viral cycle, inclusions are detectable in the nucleus. The nuclear chromatin condenses in clumps around the nuclear envelope. Later the nucleus becomes highly irregular in outline and shrunken; it is displaced to one side of the cell by 8 hours after infection (Reissig et al., 1956; Dunnebacke, 1956). Barski et al. (1955) noted a large pale-staining area in the center of the cytoplasm which appeared more granular and compact than the remainder of the cytoplasm. Other authors have commented on a juxtanuclear area which stained differently from the remainder of the cytoplasm also (Dunnebacke, 1956; Reissig et al., 1956). By 6 hours after infection, a large percentage of the cells have become vacuolated and have retracted their cytoplasmic processes from the glass or plastic surface on which they were growing. They round up, develop breaks in the plasma membrane, and eventually lyse (Reissig et al., 1956; Bablanian et al., 1965a).

In electron microscopic studies these changes have been confirmed and extended. The nuclear margination of the chromatin is among the

first changes to be noted. The Golgi vesicles, which normally are seen as stacks of flattened cisternae, are no longer morphologically identifiable (Jezequel et al., 1966) and are replaced by an extensive accumulation of smooth surfaced cisternae. The cisternae first become noticeable in the centrosphere region of the cell at 3 hours after infection, and proliferate rapidly until they completely fill the central portion of the cell, displacing and squashing the nucleus to one side (Dales et al., 1965). This phenomenon partially explains two of the morphological alterations noted in the light microscopic studies, the changes in nuclear size and shape and the large pale staining area noted by Barski et al. (1955), which is probably identical with the area occupied by these cisternae.

Amako and Dales (1967b) performed autoradiography in cells which had been labeled with ^{3}H-choline before and after infection. In both cases many grains were located over the area of proliferating cisternae. Differential centrifugation to separate various cellular organelles revealed the greatest amount of ^{3}H label per milligram of phospholipid was in the microsomal fractions of both infected and uninfected cells. All fractions of infected cells incorporated more radioactivity than the comparable fractions of uninfected cells, but the increase in radioactivity was greatest in the microsomal fraction.

Also prominent in electron micrographs of cells infected with poliovirus for 3 hours or more are regions of dense randomly oriented filamentous or granular material. These have been termed viroplasm by Dales et al. (1965), who assumed that they are centers of virus assembly. Virus progeny can be detected readily by electron microscopy by 5 hours after infection. It is difficult to identify virus particles at earlier times in infection when they are not in characteristic arrays or in great numbers, for the size of poliovirus particles is similar to that of ribosomes. Paracrystalline arrays of virus are commonly seen in the cytoplasm of infected cells by 5 hours after infection (Dales et al., 1965).

Release of Lysosomal Enzymes

Many of the cytopathological changes produced by picornavirus infection have been attributed to the release of hydrolytic enzymes from cellular lysosomes (Salzman et al., 1959; Wolff and Bubel, 1964; Flanagan, 1966; Amako and Dales, 1967b; Blackman and Bubel, 1969). Release of hydrolases has been studied in several laboratories using poliovirus (Flanagan, 1966; Bartsch et al., 1969; Wolff and Bubel, 1964) and mengovirus (Hotham-Inglewski and Ludwig, 1966) and release of lysosomal enzymes into a nonparticulate form was first observed at times ranging from 3 hours to 9 hours after infection. This variation correlates fairly well with differences in the viral growth kinetics observed in these studies, and is probably due to differences in virus and cell strains used and in experimental techniques. In all cases it is possible that some or even all of the release of the enzymes occurred during the cell fractionation rather than in cells *in situ*. Release of lysosomal enzymes probably reflects increased membrane fragility in picornavirus-infected cells.

Evidence for the Importance of Cellular Membranes in Poliovirus Replication

Investigations in many laboratories have shown that the biosynthetic processes of poliovirus replication are closely associated with cellular membranes. Poliovirus RNA polymerase activity is found in the microsomal fraction of infected cells (Baltimore et al., 1963; Tershak, 1966; Girard, 1969). All polymerase preparations so far studied are crude enzyme-template complexes which do not respond to added RNA primers of viral or cellular origin. Under the best conditions, the *in vitro* product is largely single-stranded viral RNA (Baltimore, 1964; Girard, 1969), but solubilization of the enzyme complex from the membranes results in the loss of activity or the synthesis of a product in which the proportion of single-stranded to double-stranded product is somewhat altered (Arlinghaus and Polatnick, 1967, 1969; Ehrenfeld et al., 1970; Girard et al., 1967; Polatnick and Arlinghaus, 1967; Plagemann and Swim, 1968).

Differential centrifugation of infected cells yields a cytoplasmic membrane-associated structure which has been shown to contain poliovirus-specific polyribosomes, viral RNA polymerase activity and much of the newly synthesized viral RNA, protein and mature virus (Becker et al., 1963; Penman et al., 1964). Because the electron micrographs of infected cells ruptured by several cycles of freezing and thawing showed virus particles enclosed in membranous cisternae (Horne and Nagington, 1959), Penman and co-workers (1964) proposed the term "virus synthesizing body" for the cytoplasmic structures they had isolated, and assumed that all of the events in virus replication were performed here. More recently, however, Caliguiri and Tamm (1969, 1970a,b) were able to separate from each other the cellular membranes with which viral RNA and protein synthesis are associated. This was done by isopycnic centrifugation of cytoplasmic extracts in discontinuous sucrose gradients. Most of the viral RNA synthesis is associated with a band, containing smooth microsomes, which comes to equilibrium in the upper part of the gradient. The bulk of viral protein synthesis is found in a rough microsomal fraction, which sediments as a thick, coherent pellicle.

We have used the same fractionation method to separate the membranes of infected cells for the investigations described in Chapters 3 and 4 of this dissertation. Since it provides a means of distinguishing the cellular membranes used for two different viral functions, RNA and protein synthesis, it provides an excellent technique for the study of the interrelationships between virus multiplication and cellular membranes.

Guanidine Hydrochloride - A Specific Virus Inhibitor

Action of Guanidine

Guanidine hydrochloride prevents the multiplication of poliovirus and many other picornaviruses when used at a concentration that has no measurable effect on cellular metabolism or multiplication (Rightsel et al., 1961; Crowther and Melnick, 1961; Brown et al., 1966). Guanidine exerts its effect by inhibition of viral RNA synthesis (Eggers et al., 1963; Holland, 1963; Caliguiri et al., 1965) while allowing viral protein

synthesis to continue relatively undiminished (Caliguiri and Tamm, 1968b; Jacobsen and Baltimore, 1968a). Cultures treated with guanidine shortly after infection synthesize relatively little viral protein, probably as a secondary effect of the inhibition of production of viral RNA, which acts as messenger. Results of studies by Caliguiri and Tamm (1968a,b) lead to the conclusion that the primary process inhibited by guanidine is the initiation of synthesis of viral RNA strands.

Guanidine has been used as a tool to study viral functions by many investigators. We can interpret these studies in the light of recent understanding that its mode of action is the inhibition of ongoing viral RNA synthesis without concomitant inhibition of viral protein synthesis.

It has been shown that treatment of infected cells with guanidine does not prevent the virus-mediated suppression of cellular protein and nucleic acid synthesis (Bablanian et al., 1965a; Penman and Summers, 1965). This seems to be a multiplicity dependent phenomenon. Cells infected at a low multiplicity of 10 PFU/cell may continue to synthesize cellular protein and nucleic acid at nearly normal rates in the presence of guanidine (Holland, 1964; Penman and Summers, 1965). However, these biosynthetic processes are rapidly inhibited if the cells are infected with 1,000 to 10,000 PFU/cell regardless of the presence or absence of guanidine (Holland, 1964). Similarly, Penman (1965) showed that in cells treated with 1.0 mM guanidine, the virus-controlled stimulation of choline incorporation is multiplicity dependent. At input multiplicities of 100 PFU/cell or greater, the stimulation of cellular membrane synthesis caused by poliovirus infection is only slightly reduced by guanidine treatment. In both of these cases the metabolic changes due to poliovirus infection may be controlled by proteins synthesized using the parental viral RNA as messenger. As mentioned earlier, studies, utilizing ultraviolet inactivation of the input virus or complete inhibition of protein synthesis, have shown that some translation of the viral genome is required; the capsids of the input virus seem incapable of performing the function of altering the cell's metabolic processes.

In contrast to the lack of effect of guanidine on virus control of cellular metabolism at high multiplicities of infection, cells treated with guanidine at any time up to 2 hours after infection do not show the cytopathological changes caused by virus infection in untreated cells within the time span of a single cycle of virus multiplication (Bablanian et al., 1965b). These changes are delayed for many hours until a time when they might well reflect the continuing inhibition of cellular protein and nucleic acid synthesis (Holland, 1963; Bablanian et al., 1965a).

If guanidine is added at 4 hours after infection, virus-induced cell damage is not prevented even though virus yield is greatly reduced (Bablanian et al., 1965b). Most likely the morphological changes are produced in response to the synthesis of virus proteins, but these proteins do not accumulate in concentrations capable of damaging the cells under conditions of limited messenger RNA production in cultures treated with guanidine early in the viral cycle.

Blocking of Guanidine Inhibition

The action of guanidine may be completely reversed by the removal of guanidine from the culture medium (Crowther and Melnick, 1961; Eggers et al., 1965). In 1964, it was discovered that the action of guanidine can also be reversed by the addition of certain compounds that are components of many culture media (Lwoff and Lwoff, 1964; Dinter and Bengtsson, 1964). Guanidine blockers include amino acids, methylated and ethylated amino alcohols such as choline, and methylated amines (Loddo et al., 1966; Philipson et al., 1966).

There is evidence that the amino acids which block the inhibitory effect of guanidine act synergistically with compounds such as choline and dimethylethanolamine (Philipson et al., 1966). Apparently, different amino acids do not act synergistically with each other (Dinter and Bengtsson, 1964). We have tested the activity of combinations of choline and methionine, and choline and dimethylethanolamine against guanidine inhibition. Our results are reported in Chapter 5.

It has been reported that the degree of blocking of guanidine action by a variety of compounds is strongly dependent on the host cell (Philipson et al., 1966). We initiated our studies of the blocking of guanidine inhibition in the hope that exploitation of such cellular differences would enable us to probe the mechanism of action of these compounds. Our results, discussed in Chapter 5, indicate that the major difference between various cell types is in the sensitivity of the virus biosynthetic processes to guanidine in each.

Guanidine-dependent Mutants of Poliovirus

Almost immediately after the inhibitory action of guanidine was recognized, it was discovered that poliovirus mutants that are resistant to guanidine may be isolated. In infected cultures that have been treated with moderate concentrations of guanidine (e.g., 0.3 mM), some virus is produced. Most of this virus is genetically resistant to 0.3 mM guanidine and multiplies in its presence (Melnick et al., 1961). Continual passage of such mutant stocks in gradually increasing concentrations of guanidine produces stocks of virus resistant to higher concentrations of guanidine. If these stocks are cloned, it is discovered that some clones are guanidine-dependent. They will not multiply in the absence of guanidine.

The property of drug dependence has proved to be entirely congruent to drug sensitivity. Thus, the processes involved in drug dependance and drug sensitivity begin early in the viral cycle and extend through the exponential increase phase (Eggers et al., 1963, 1965). Viral RNA synthesis is blocked in guanidine-deprived cultures infected with guanidine-dependent poliovirus or in guanidine-treated cultures infected with guanidine-sensitive poliovirus (Eggers et al., 1963; Caliguiri et al., 1965).

There is some disagreement in the literature as to the action of compounds such as choline and methionine on the multiplication of the guanidine-dependent strain of poliovirus. Both Lwoff and Lwoff (1965) and Loddo and co-workers (1966) found that the poliovirus-supporting

action of 0.2 mM to 0.4 mM guanidine could be blocked by guanidine antagonists. Philipson et al. (1966), using 1.0 mM guanidine and a series of guanidine antagonists, could not detect any effect on the multiplication of guanidine-dependent poliovirus type 1. However, even high concentrations of guanidine blockers would probably not inhibit the action of 1.0 mM guanidine measurably. We investigated the effect of several guanidine antagonists on the replication of guanidine-dependent poliovirus type 1 in HeLa cells in the presence of 0.4 mM guanidine, as described in Chapter 5.

We have carried out a detailed quantitative study of the kinetics of virus multiplication after choline-mediated blocking of the inhibitory action of guanidine. The use of antiserum against the residual virus in the inoculum in such a kinetic study allowed us to determine accurately the speed and effectiveness of choline action.

2. MATERIALS AND METHODS

Chapter 2

Materials and Methods

Materials

Viruses

Poliovirus type 2 (P712-ch-2ab) was used for most of the experiments described in this dissertation. It was plaque purified (Dulbecco and Vogt, 1954) three times before use in the experiments described and was subsequently passaged twice in HeLa cell monolayers and no more than three times in S_3 HeLa cells. Virus was purified by isopycnic centrifugation in CsCl twice before use. This virus strain is sensitive to guanidine.

Guanidine-dependent poliovirus type 1 (Brunhilde), received from Dr. N. Ledinko, was passaged in HeLa cell monolayers in the presence of 1.0 mM guanidine.

Cells

S_3 HeLa cells were obtained from Dr. J. V. Maizel and were maintained in suspension in Eagle's spinner medium (Eagle, 1959), with 7% calf serum. They were used for the experiments described in Chapters 3 and 4. HeLa cell monolayers obtained from Dr. B. Mandel were maintained in 32 oz prescription bottles in reinforced Eagle's medium (Bablanian et al., 1965a) supplemented with 10% calf serum. LLC-MK_2 cells, a continuous rhesus monkey kidney cell line, were obtained from the American Type Culture Collection Cell Repository and maintained in the same way as HeLa cell monolayers. Primary cultures of rhesus monkey kidney cells (MKC) were prepared as described by Eggers and Tamm (1961), and were used on the fifth day after seeding.

All continuous cultures were periodically treated with 50 μg/ml of tylosin tartrate (Ralston Purina Company, St. Louis, Mo.) to control contamination by mycoplasma (Friend et al., 1966).

Chemicals

All chemicals used in the preparation of media and buffers were commercially available, analytical grade products.

Lipostabilized calf serum was obtained from Grand Island Biologicals, Grand Island, N. Y. For plaque assays and growth of virus stocks, calf serum which was certified as non-inhibitory to poliovirus (Special calf serum, Microbiological Associates, Inc., Bethesda, Md.) was used. All calf serum was heated to 56° C for 30 minutes before use in culture media, to destroy complement.

Ribonuclease-free sucrose, used for the sucrose gradients, was obtained from Schwarz Bioresearch, Inc., Orangeburg, N. Y. EDTA was purchased from Fisher Scientific Co., Fair Lawn, N. J.

Recrystallized guanidine·HCl and actinomycin D were obtained through the courtesy of Dr. A. F. Wagner of Merck, Sharp and Dohme Research Laboratories, Rahway, N. J. Choline and methionine were purchased from Mann Research Laboratories, New York, N. Y. Dimethylethanolamine was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Phosphoenolpyruvate and phosphoenolpyruvate kinase were obtained from Calbiochem, Los Angeles, Calif. ATP, GTP and CTP were purchased from P. L. Biochemicals, Inc., Milwaukee, Wis.

Trypsin, 1 x crystallized, and bovine pancreatic deoxyribonuclease I, electrophoretically purified, were purchased from Worthington Biochemicals, Freehold, N. J.

^{3}H-Choline (methyl labeled, specific activity 100 mCi/mmole or greater) was obtained from Amersham/Searle, Des Plains, Ill., or from New England Nuclear, Boston, Mass. Glycerol-2-^{3}H (380 mCi/mmole) and uridine-5-^{3}H (20 Ci/mmole were obtained from Amersham/Searle, Des Plains, Ill. ^{14}C-Guanidine-HCl (40 mCi/mmole) was obtained from International Chemical and Nuclear Corporation, City of Industry, Calif. (5-^{3}H)-Uridine-5'-triphosphate (17.8 Ci/mmole) was purchased from Schwarz Bioresearch, Orangeburg, N. Y.

Methods

Media and Solutions

All nutrient media contained 500,000 units of penicillin G, 100 mg streptomycin, and 25,000 units of mycostatin per liter. They were sterilized by filtration.

Eagle's spinner medium (Eagle, 1959) was used with 7% calf serum for maintenance of S_3 HeLa cells and was used unsupplemented for all experiments with these cells.

Reinforced Eagle's medium was prepared according to Bablanian et al. (1965a). Eagle's minimum essential medium (MEM) (Egale, 1959) was used for all experiments with cells in monolayer culture, except as otherwise stated.

RSB is reticulocyte standard buffer, and was prepared according to Caliguiri and Tamm (1970a).

Phosphate-buffered saline (PBS) was prepared as described by Dulbecco and Vogt (1954). PBS-deficient is identical except that it lacks $CaCl_2$ and $MgCl_2$.

Trypsin-versene solution, used in cell passages, is 0.25% trypsin in 0.05% versene (EDTA) solution. It was sterilized by filtration.

Polymerase buffer consists of 0.1 M Tris, pH 7.4; 1.0 mM $MgCl_2$; and 5 μg/ml DNase.

Immune Sera

Poliovirus types 1 and 2 antisera were prepared in this laboratory by hyperimmunization of rabbits.

Maintenance of Cell Stocks

Monolayers of cells in continuous culture were subcultured every 2 or 3 days. Confluent monolayers in 32 oz prescription bottles were washed twice with 20 to 50 ml warm PBS-deficient. Warm trypsin-versene solution (4.5 ml) was pipetted onto the cells and they were incubated

at 37° C for 5 minutes. The bottles were then shaken to detach the cells and the cell suspension was pipetted into a conical centrifuge tube. The suspension was centrifuged at 900 rpm for 5 to 10 minutes in an IEC centrifuge and the supernatent decanted. The pellet of cells was dispersed in fresh medium and dispensed into bottles or tissue culture dishes. Bottles were gassed with 5% CO_2 in air, tightly capped, and incubated at 36 to 37° C. Tissue culture dishes were swirled to spread the cells and incubated in a warm, humidified atmosphere of 5% CO_2 in air.

S_3 HeLa cells were maintained at a concentration of 1 to 4 x 10^5 cells/ml by addition of medium to suspension cultures. At least once a week they were sedimented by centrifugation and resuspended in fresh medium.

Growth and Purification of Virus

S_3 HeLa cells in suspension were infected with approximately 10 PFU/cell of virus at a cell density of approximately 5 x 10^6 to 1 x 10^7 cells/ml. After one hour the cells were diluted to approximately 2 x 10^6 cells/ml with warm Eagle's spinner medium containing 2% special calf serum. Cell-associated virus was harvested at 6.5 hours after infection by centrifugation at 1500 rpm x 15 minutes in an IEC refrigerated centrifuge. The pellet was resuspended in a small volume of PBS and cells were disrupted by 3 cycles of freezing and thawing. Cell debris was then removed by centrifugation at 10,000 x g for 20 minutes. Virus was purified from the supernatant by two isopycnic centrifugations in 45% w/v CsCl (Harshaw Chemical Co., Hastings-on-Hudson, N. Y.), and CsCl was removed from the final band by passage through a 0.9 x 30 cm column of G-25 Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.).

Plaque Assays

Plaque assays were performed as described by Eggers and Tamm (1962) except that the final concentration of calf serum was 5%, and cultures were generally incubated only two days before being read. Plaques could be read easily without staining with neutral red. All plaque assays were performed using HeLa cell monolayers.

Infection of S_3 HeLa Cells for Experiments

HeLa cells were sedimented by centrifugation and washed once in 200 ml cold (4° C) Eagle's spinner medium. They were then resuspended in cold Eagle's spinner medium and infected with 25-150 PFU/cell of poliovirus in PBS. Control cultures received an equal volume of PBS. Cultures were stirred at 37° C for one hour and then diluted to their final volume. For details see individual experiments.

Cell Fractionation

Cells were harvested by pouring over crushed frozen PBS and centifuging immediately at 1500 rpm for 7.5 minutes at 4° C. They were washed once with 30 ml cold PBS and recentrifuged. The pellets were resuspended in 6.5 ml cold RSB and maintained at the temperature of melting ice for 20 minutes. They were then disrupted with approximately 7 strokes with a tight-fitting dounce homogenizer or until inspection under the phase microscope showed that almost all of the cells had been broken. Nuclei, unbroken cells and large cell debris were sedimented by centrifugation at 900 x g for 10 minutes at 4° C in an IEC refrigerated centrifuge. Five ml of the supernatant (cell extract) were mixed with 5 ml 60% w/w sucrose-RSB and layered into a discontinuous sucrose gradient (Caliguiri and Tamm, 1970a) which was modified from that of Bosman et al. (1968). This gradient is illustrated in Figure 1, p. 26. Gradients were centrifuged at 86,000 x g (Rav) for about 17 hours in a Spinco SW27 rotor. In our estimation, equilibrium was probably reached before this time.

After centrifugation fractions were collected into 5/8" diameter x 3" cellulose nitrate tubes by means of Pasteur pipettes. The volume was brought up to 11.0 ml with cold RSB and the tubes were centrifuged at 95,000 x g for 2 hours in a Spinco type 40 rotor. The supernatants were decanted and pellets were resuspended in RSB using a Teflon pestle. Typically, 1.0 ml RSB was used for resuspension and the preparation was analyzed as follows: 0.2 ml was analyzed for protein; 0.2 ml was precipitated with trichloroacetic acid and radioactivity determined; and

lipids were extracted from 0.5 ml and used for phosphorus and radioactivity determinations. Approximately 40% of the total protein of the cytoplasmic extract and 95% or greater of the lipid phosphorus or lipid-associated radioactivity were recovered in the pelleted fractions.

Experiments on Blocking of Guanidine Action

All experiments were performed in confluent monolayers in 60 mm plastic tissue culture dishes. Reinforced Eagle's medium was removed and cultures were washed once with warm PBS and inoculated with 0.5 ml of poliovirus diluted in PBS to give a multiplicity of 30-180 PFU/cell. After an adsorption period of one half hour at 37° C the cultures were washed once with warm PBS and treated with 0.5 ml of rabbit antiserum against the poliovirus type used. The antiserum was diluted so that it would reduce the background of input virus to 0.01% of the inoculum, or about 0.01 PFU/cell. The cultures were then incubated for an additional half hour, washed twice with warm PBS and 1.4 to 1.8 ml of MEM was added to give a final volume of 2.0 ml in each plate after the additions. Additions of guanidine and guanidine antagonists in PBS or MEM were made in 0.2 ml volumes. All cultures were incubated at 37° C in an atmosphere of 5% CO_2 in air until harvested. All experiments on the kinetics of virus multiplication were carried out in a 37° C constant temperature room.

Samples were harvested by scraping cells from the plates and collecting culture fluid and cells. Duplicate cultures were pooled and cells frozen immediately in an alcohol-dry ice bath. Subsequently, they were thawed and frozen twice more and stored at -20° C until assayed. Before assaying, the suspensions were thawed and centrifuged at 2000 rpm for 20 minutes in an IEC refrigerated centrifuge to remove cell debris.

Lipid Extraction

Lipid extraction was performed as described by Folch et al. (1957). The surface of the non-aqueous phase was washed with chloroform:methanol: 0.03 M $MgCl_2$ in water, in the proportions 3:48:47 by volume. Samples were dried by heating to 56° C in a water bath overnight.

Phosphorus Determination

Lipid phosphorus was determined by the method of Rouser et al. (1966) except that samples were digested in an oven at 150-160° C for 3 hours rather than in a Kjeldahl rack, and all volumes were reduced by 50%. Diphenylphosphate (Sigma Chemical Corporation, St. Louis, Mo.) was digested in the same manner as the samples and was used as a standard. Phospholipid content was occasionally calculated from the phosphorus content by assuming 770 as the average molecular weight of the phospholipids.

Phospholipid Analysis

Aqueous samples were sonicated in a Branson sonifier and protein contents were determined. The chilled sonicates were then precipitated by the addition of one half volume of 30% cold trichloroacetic acid. After 30 minutes in an ice bath, the precipitates were collected by centrifugation at 1600 rpm for 10 minutes in an IEC refrigerated centrifuge.

The precipitates were extracted once with 1 ml of cold methanol and three times with 2 ml of chloroform:methanol, 2:1 by volume, at 20° C, 37° C and 60° C, respectively. The combined extracts were washed once with one sixth volume of water and twice with one third volume of chloroform:methanol: 0.1% NaCl in water (0.1:1:1) (Hakomori and Murakami, 1968).

Washed extracts were evaporated to dryness under N_2 and resuspended in a small volume of chloroform:methanol (2:1). Aliquots were taken for phosphorus determination. Two dimensional chromatography was performed on triplicate aliquots on silica gel-precoated glass plates (E. Merck, A. G., Darmstadt, Germany). The plates were developed with chloroform: methanol:water (65:25:4), air dried for 10 minutes, and then developed in a direction 90° to the first with butanol:acetic acid:water (60:20:20) (Rouser et al., 1965).

Dry plates were stained with iodine vapor and the phospholipid-containing silica gel was scraped from them for phosphorus determination.

Protein Determination

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (Armour Pharmaceutical Company, Chicago, Ill.) in the appropriate buffer as a standard.

Determination of Radioactivity

Aliquots of cell fractions or whole cells were precipitated with an equal volume of cold 10% trichloroacetic acid. After 20 minutes in an ice bath, samples were filtered onto Whatman GF/C glass fiber filters and washed twice with 5% trichloroacetic acid. Filters were placed in glass scintillation vials and dried in a 70° C oven. Ten ml of scintillation fluid was added, and the radioactivity was determined in a Packard Tri-carb Scintillation Spectrometer. The scintillation fluid was made by adding 160 ml Liquifluor (New England Nuclear, Boston, Mass.) to one gallon of toluene (Merck and Company, Rahway, N. J.).

Chloroform-methanol extracts were dried directly in vials and counted in the same scintillation fluid.

RNA Polymerase Assay

The reaction mixture, modified from that used by Girard (1969), contained 0.05 mmoles Tris, pH 8.5; 4.15 μmoles magnesium acetate, 4.0 μmoles phosphoenolpyruvate, 10 μg pyruvate kinase, 0.1 μmoles each of ATP, CTP and GTP, 25 μCi of ^{3}H-UTP (17.8 Ci/mmole) and 0.20 ml of the enzyme preparation in a final volume of 0.5 ml. Samples were incubated in a 36.7° C water bath. The reaction was terminated by the rapid addition of 0.1 ml of 0.1% bovine serum albumin and 0.6 ml cold 10% trichloroacetic acid containing 0.02 M sodium pyrophosphate. The tube was agitated on a vortex mixer and plunged into an ice bath. After 20 minutes on ice, the tubes were centrifuged at 2000 rpm for 15 minutes in an IEC refrigerated centrifuge and the pellets washed twice by resuspension in 0.3 ml ice-cold 0.2 N NaOH and reprecipitation with 10% trichloroacetic acid with 0.02 M sodium pyrophosphate (Maitra and Hurwitz, 1965). Each time the precipitate was kept on ice for 20 minutes before centrifugation.

The final precipitate was collected by filtration onto Whatman GF/C glass fiber filters and was washed twice with cold 5% trichloroacetic acid containing 0.025 M sodium pyrophosphate. Filters were dried and counted as described earlier.

It should be noted that this cell-free polymerase system is a crude system which utilizes endogenous template and is not responsive to added RNA. The presence of various degradative enzymes, notably ribonuclease, complicate the interpretation of results.

3. CYTOPLASMIC MEMBRANES IN POLIOVIRUS-INFECTED CELLS

Chapter 3

Cytoplasmic Membranes in Poliovirus-infected Cells

Introduction

Poliovirus replication is closely associated with cellular membranes. The studies of Caliguiri and Tamm (1969, 1970a,b) showed that the membranes of infected cells could be fractionated on a discontinuous sucrose gradient. When this is done, viral RNA and viral protein synthesis are separated from each other; the two processes are associated with different membranes in the infected cell. Because of the close association between the biosynthesis of poliovirus and cellular membranes demonstrated by these experiments, and because the phospholipid and protein contents of these fractions differed in uninfected and infected cells (Caliguiri and Tamm, 1970a), we decided to study the chemical composition of these membranes throughout the course of infection.

We also investigated the RNA-synthesizing capacity of the membranes from the various fractions and demonstrated this activity in a complex with a sedimentation constant of 130 S, which may be obtained by lysis of the membranes in Fraction 2.

The gradient used for all of our experiments is shown in Figure 1. A cytoplasmic extract, prepared as described in Materials and Methods, is mixed gently with an equal volume of 60% sucrose in RSB, and layered into the gradient. Gradients are centrifuged at 86,000 x g (Rav) until the contents are at equilibrium. After centrifugation, six opaque bands, centered on the previous interfaces in the tube, can be seen. Fraction 1 is a fatty, scumlike layer at the air-buffer interface. Fractions 2 through 6 are the bands pictured in Figure 1, and Fraction 7 consists of the pellet plus a small amount of 60% sucrose lying immediately above it. These fractions are collected, diluted with RSB and centrifuged at 95,000 x g in a Spinco 40 rotor for 2 hours. Electron micrographs of the fractions after pelleting are shown in Figures 2 and 3. These micrographs were prepared by Dr. Richard W. Compans.

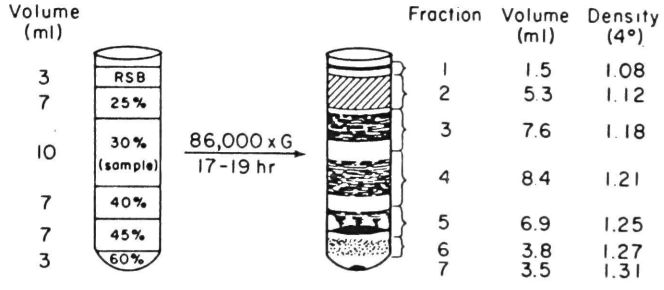

Figure 1. Schematic representation of discontinuous sucrose gradient used to fractionate cell membranes. The bands that are visible after centrifugation to equilibrium are indicated in the tube on the right. The brackets indicate the fractions. The volumes and densities of each fraction in a representative experiment are listed in the two columns on the right. (From Caliguiri and Tamm, 1970a)

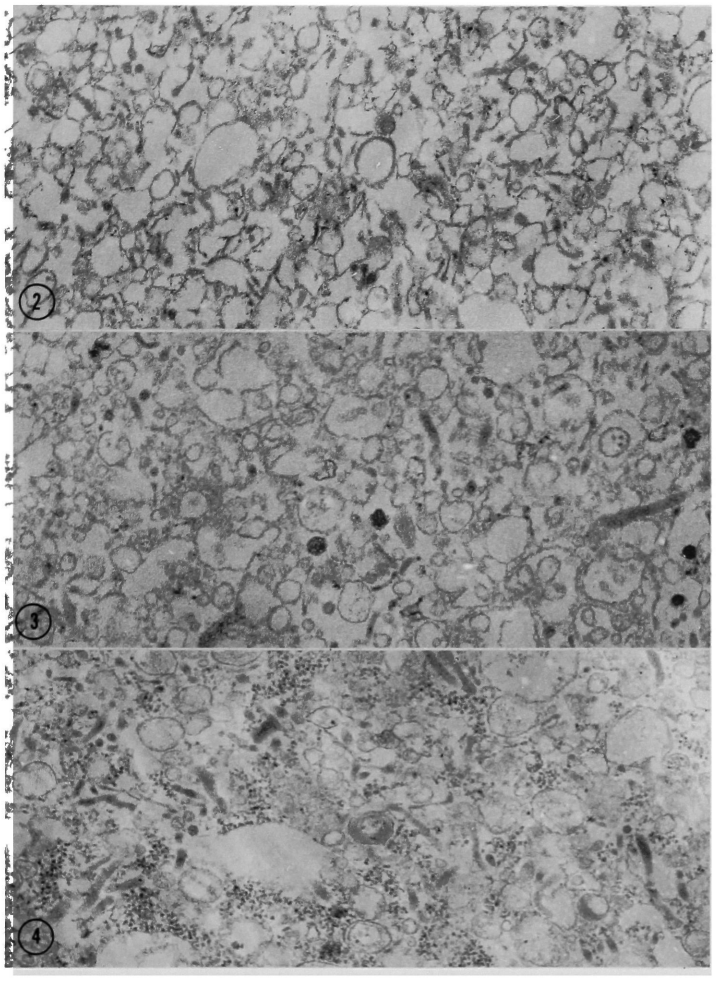

Figure 2. Electron micrographs of the pellets of Fractions 2 through 4 from poliovirus-infected cells. HeLa cells (5×10^8) were infected at a multiplicity of 50 PFU/cell in medium containing 5 μg/ml actinomycin D. The culture was harvested 3.25 hours after infection and the cells were fractionated on a discontinuous sucrose gradient as described in Materials and Methods. Fractions were pelleted and prepared for electron microscopy. The fraction number appears in the lower left-hand corner of each micrograph. Magnification x 40,000. (From Caliguiri and Tamm, 1970a)

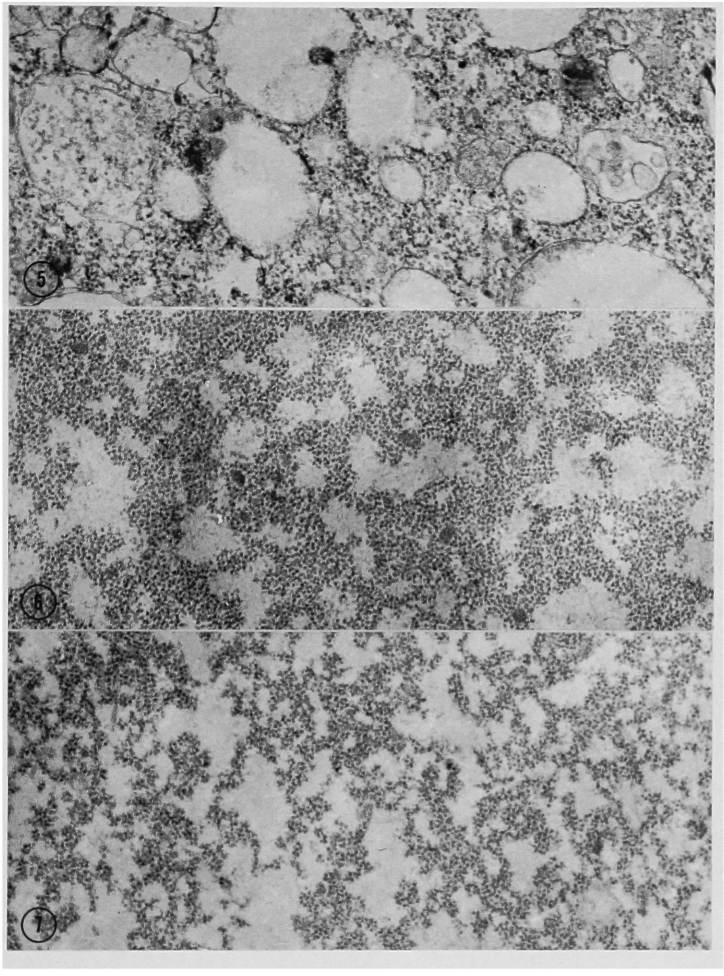

Figure 3. Electron micrographs of the pellets of Fractions 5 through 7 from poliovirus-infected HeLa cells. The preparation was made as in Figure 2. Magnification x 40,000. (From Caliguiri and Tamm, 1970a)

The work of Caliguiri and Tamm (1969, 1970a,b) leads to the conclusion that viral RNA synthesis is associated with the fraction labeled 2 in Figures 1 and 2 by the following criteria. This fraction contains most of the labeled RNA after a very short pulse of ^{3}H-uridine is given to infected cells just prior to harvesting. Analysis of RNA extracted from this fraction by sedimentation velocity centrifugation or polyacrylamide-agarose gel electrophoresis reveals viral replicative intermediate and replicative form RNA, but no completed single-stranded viral RNA. And finally, the bulk of the viral RNA polymerase activity determined in a cell-free assay is found here. This fraction consists of smooth microsomes as shown by electron microscopy and by the absence of whole ribosomes as determined by sedimentation velocity analysis of deoxycholate-treated material from this fraction. When cells are labeled with ^{3}H-uridine for 36 hours prior to infection, and harvested 3.25 hours after infection, over 95% of the radioactivity is in ribosomal RNA. Only 0.3% of the labeled ribosomal RNA sediments in Fraction 2, and the sedimentation constant of labeled material after deoxycholate treatment is less than 74 S. This material probably represents ribosomal subunits. Enzymatic studies have shown that some 5'-nucleotidase activity (plasma membrane marker) also sediments here. However, most of the 5'-nucleotidase activity is sedimented in the low speed centrifugation (see Materials and Methods), and thus most plasma membrane fragments are never layered into the gradient (Caliguiri, unpublished observations).

The fraction labeled 5 in Figures 1 and 3 is visualized in the centrifuge tube as a thick, coherent pellicle, and electron microscopy shows that it contains rough microsomal membranes. When cells are labeled with ^{3}H-uridine for 36 hours prior to infection and harvested 3.25 hours after infection, over 50% of the labeled ribosomal RNA is found in this fraction. The pellicle fraction contains structures with the sedimentation characteristics of viral polyribosomes and 3-minute pulses with ^{14}C-labeled amino acid mixture label this fraction preferentially. Because of these facts, it is concluded that viral protein synthesis is associated with cellular membranes which sediment in the pellicle.

This fraction also contains the highest specific activities of cytochrome oxidase and β-glucuronidase, and therefore mitochondria and lysosomes probably come to equilibrium here (Caliguiri, unpublished observations).

The findings summarized briefly above are discussed more completely by Caliguiri and Tamm (1970a,b).

Results

Protein and Phospholipid Analyses of Fractions from Infected Cells

HeLa cells (7.5×10^8) in Eagle's spinner medium were infected with 28 PFU/cell of poliovirus type 2 in a total volume of 100 ml. After one hour the volume was brought to 480 ml with additional warm medium. Note that actinomycin D was not used in this experiment. At selected times, 80 ml aliquots were harvested by pouring over crushed frozen PBS and centrifuging to sediment the cells. Cell extracts were prepared as described in Materials and Methods and layered into discontinuous sucrose gradients. They were centrifuged for 17 hours. The appearance of the gradients after centrifugation is pictured schematically in Figure 4. The appearance changed progressively with time after infection, and the fractions which changed most were precisely those which seem most intimately involved in the virus biosynthetic processes. The gradient from cells harvested 2.5 hours after infection looked like one produced from uninfected cells. As infection continued, Fraction 2 became progressively more opaque, and the width of the visible band increased. The thick pellicle of rough microsomes, indicated in the drawing as a heavy black line, came to equilibrium at progressively higher densities. At the same time, it became noticeably smaller and less coherent.

The protein and lipid phosphorus contents of the pelleted fractions are listed in Table I. The values for Fraction 1 have been omitted. This fraction consisted of a scumlike layer of fatty material which had significant quantities of lipid phosphorus, but could not be pelleted or handled quantitatively. Its content of lipid phosphorus did not change significantly over the course of infection. Its protein content was negligible.

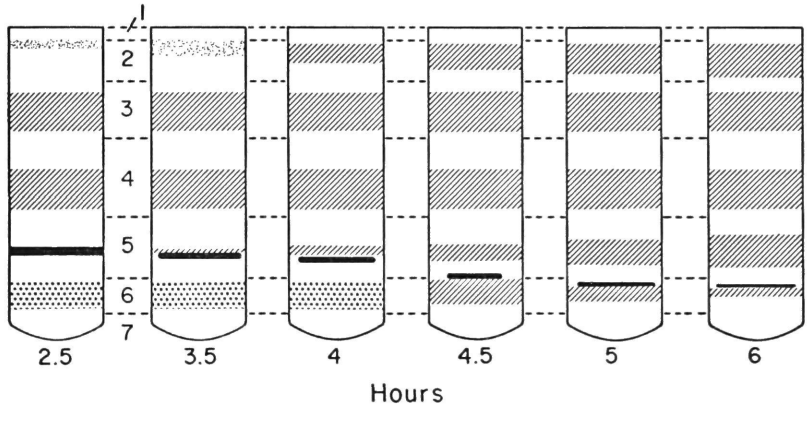

Figure 4. Schematic representation of discontinuous sucrose gradients prepared from HeLa cells at various times after infection with poliovirus. Preparation of gradients is described in text. Times indicated below each tube are hours after infection.

Table I

Protein and Lipid Content of Fractions from Poliovirus-infected Cells

Sample		Protein		Lipid P		
Hours after infection	Fraction	mg	% total	mmoles	% total	mg PL/ mg protein
2.5	2	.029	0.54	.046	1.70	1.24
	3	.600	11.18	.494	17.92	.634
	4	.690	12.86	.174	6.32	.194
	5	3.480	64.8	1.992	72.35	.441
	6	.380	7.08	.026	0.94	.053
	7	.200	3.72	.020	0.77	.080
	total	5.379		2.752		.394
3.5	2	.062	1.04	.146	5.36	1.81
	3	.555	9.32	.418	15.41	.580
	4	.930	15.62	.242	8.90	.200
	5	3.960	66.50	1.472	67.80	.357
	6	.310	5.20	.038	1.44	.097
	7	.140	2.35	.028	1.05	.157
	total	5.957		2.344		.303
4.0	2	.098	1.74	.254	9.63	2.00
	3	.630	11.16	.480	18.19	.588
	4	1.020	18.07	.284	10.72	.214
	5	3.240	57.40	1.540	58.23	.366
	6	.320	5.67	.054	2.06	.131
	7	.340	6.02	.032	1.18	.071
	total	5.648		2.644		.361

Table I (Cont.)

Protein and Lipid Content of Fractions from Poliovirus-infected Cells

Sample		Protein		Lipid P		
Hours after infection	Fraction	mg	% total	mmoles	% total	mg PL/ mg protein
4.5	2	.146	2.70	.398	14.10	2.096
	3	.600	11.11	.402	14.30	.533
	4	1.035	19.16	.338	11.98	.251
	5	.660	12.22	.148	5.25	.173
	6	2.730	50.52	1.488	52.50	.420
	7	.230	4.26	.042	1.48	.139
	total	5.401		2.816		.402
5.0	2	.174	3.59	.496	18.87	2.193
	3	.525	10.85	.400	15.22	.589
	4	1.170	24.17	.506	19.27	.333
	5	1.170	24.17	.484	18.38	.318
	6	1.590	32.88	.666	25.30	.322
	7	.210	4.34	.078	2.97	.286
	total	4.839		2.630		.418
6.0	2	.230	5.27	.616	22.18	2.097
	3	.630	13.83	.494	17.64	.603
	4	1.155	25.36	.576	20.60	.384
	5	1.300	28.54	.500	17.83	.295
	6	1.140	25.03	.576	20.60	.390
	7	.100	2.19	.028	1.02	.220
	total	4.555		2.790		.472

Changes in Composition of Fraction 2. The most dramatic changes were those found in Fraction 2. The protein and phospholipid contents of Fraction 2 from the 6 gradients are plotted in Figure 5. These are expressed as percent of the total for Fractions 2 through 7 in each gradient to minimize sampling error and variation due to the loss of damaged cells during processing at late times after infection. Also plotted is the phospholipid/protein ratio. In Fraction 2 the protein content increased approximately 8-fold and the lipid phosphorus content increased 13.4-fold between 2.5 and 6 hours after infection. This resulted in almost a doubling of the phospholipid/protein ratio in this 3.5-hour period, an indication that the membranes sedimenting in this fraction were substantially different at the two times.

Further evidence for the changing character of these membranes may be found in the phospholipid analysis of membranes from Fraction 2 of uninfected and infected cells. HeLa cells were sedimented and washed free of serum. Half of the cells were infected with approximately 50 to 100 PFU/cell. Uninfected cells were mock-infected with PBS and were treated identically to the infected cells throughout. At 5 hours after infection, both cultures were harvested and fractionated as described in Materials and Methods. Lipids were extracted from each fraction and phospholipids were separated by thin layer chromatography. The phosphorus content of each spot was determined and the averages of results of two experiments, expressed as percents of total phospholipids, are shown in Table II. These analyses were performed by Dr. Andreas Scheid.

The most striking alteration in the phospholipids of Fraction 2 which occurred after infection was the sharp decrease in sphingomyelin content. This change was regularly observed. The decrease in sphingomyelin content is accompanied by an increase in the proportion of phosphatidylcholine present. The somewhat smaller change in lysophosphatidylethanolamine plus phosphatidylinositol content has not proven to be reproducible. There are no significant changes in the phospholipid composition of whole cells or of Fraction 3 as a result of infection.

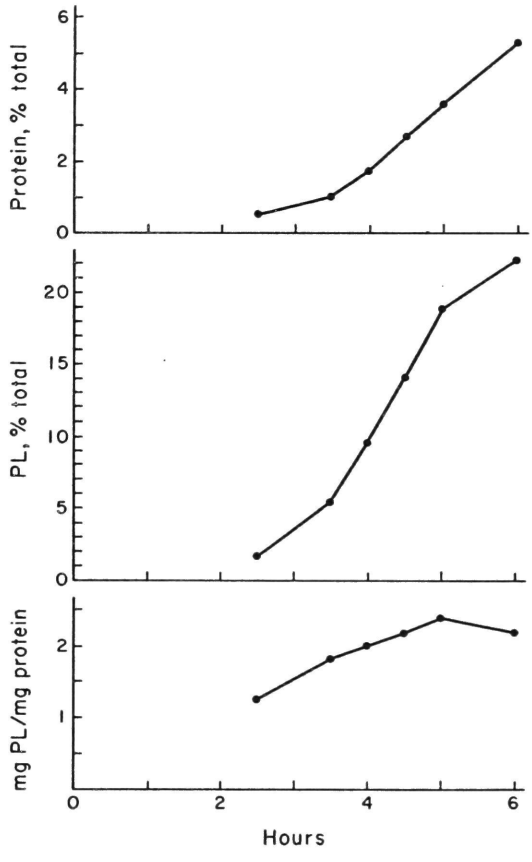

Figure 5. Protein and phospholipid content of Fraction 2 from poliovirus-infected HeLa cells. HeLa cells were infected and fractionated on discontinuous sucrose gradients at various times after infection. Protein and lipid phosphorus were determined. Values for lipid phosphorus were converted to phospholipid as described in Materials and Methods. Both protein and phospholipid are plotted as % of the total for each gradient.

Table II

Percentage Phospholipid Distribution in Fractions 2 and 3 of Poliovirus-infected HeLa Cells and Uninfected Cells

	Controls			Infected		
	Whole cells	Fraction 2	Fraction 3	Whole cells	Fraction 2	Fraction 3
Lyso-phosphatidyl-choline	10.1	10.2	7.9	9.6	10.0	8.2
Sphingomyelin	6.1	11.7	16.0	5.9	3.8	14.9
Phosphatidyl-choline	52.7	46.5	38.3	54.3	59.6	39.9
Phosphatidyl-serine	8.0	14.9	18.5	8.3	14.7	18.3
Lyso-PE/PI	7.0	8.5	10.1	6.5	4.4	10.1
Phosphatidyl-ethanolamine	16.1	8.0	9.3	15.3	7.5	8.5
Total	100.0	99.8	100.1	99.9	100.0	99.9
Lipid-P/protein (mμmoles/mg)	118.5	1539.	503.5	140.5	2830.	533.0

Changes in Composition of Other Fractions. In contrast to the marked changes in the mass and composition of membranes in Fraction 2, changes in the other fractions were small (Table I). The protein and phospholipid content of Fraction 3 remained quite uniform throughout the course of infection as seen in Figure 6. Fraction 4 increased in mass and its phospholipid/protein ratio doubled in this experiment; in other experiments these changes were not as great. It should be emphasized that the changes in Fraction 2 described above were highly reproducible.

The changes in Fractions 5 and 6 were related to the changing size and position of the pellicle. It was collected in Fraction 5 from the first 3 gradients in the series, and in Fraction 6 from the last 3. The totals of the protein and phospholipid contents of both fractions are plotted in Figure 6. Here it is apparent that the total mass of the pellicle did decrease, but the phospholipid/protein ratio remained fairly stable. The apparent increase in density might be due to variation in RNA content of the pellicle.

The protein and phospholipid contents of Fraction 7 were highly variable and have proven to be so over the course of many experiments. The composition of this fraction, which appears in electron micrographs to consist of ribosomes without recognizable membranes, may be influenced greatly by small differences in cell physiology or in the processing procedures.

Poliovirus RNA Polymerase in Membranes from Fraction 2

A major difference between the membranes which sediment in Fraction 2 in gradients prepared from infected and uninfected cells is the association of the viral RNA polymerase with membranes from infected cells. This association is firm enough to withstand pelleting or recentrifugation on continuous sucrose or tartrate gradients, and is broken only by treatment with deoxycholate and lysis of the membrane.

Figure 7 shows the RNA polymerase activity associated with the microsomal fraction of infected cells. HeLa cells were infected with 140 PFU/cell of poliovirus type 2, and harvested 4 hours later. Cytoplasmic

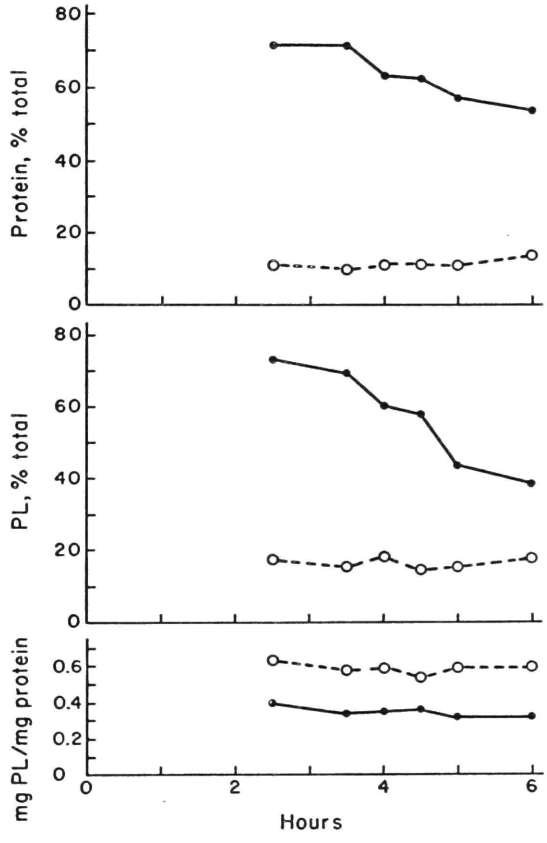

Figure 6. Protein and phospholipid content of Fraction 3 and Fractions 5 plus 6 from poliovirus-infected HeLa cells. The preparation was made as described for Figure 5. o---o, Fraction 3; •——•, Fractions 5+6.

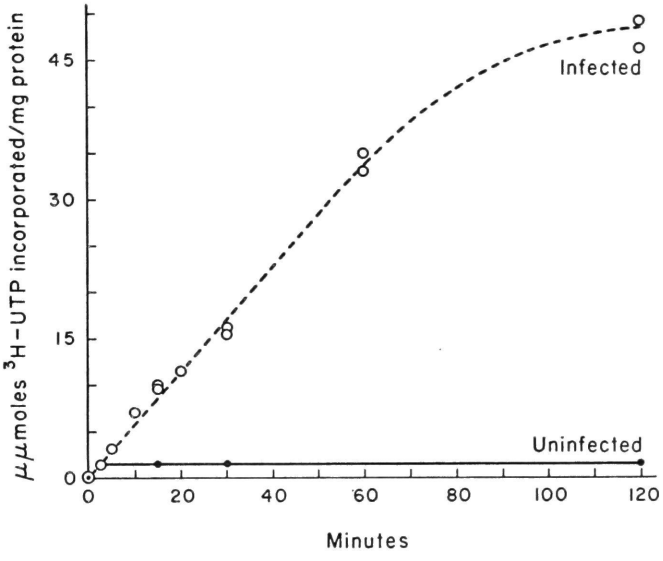

Figure 7. Kinetics of in vitro RNA polymerase activity in poliovirus-infected and uninfected HeLa cells. HeLa cells (3.2×10^8 cells) were divided into 2 cultures. One was infected with 140 PFU/cell of poliovirus type 2. Both were harvested 4 hours later. Cytoplasmic extracts were prepared and centrifuged at 95,000 x g for 1 hour. Pellets were resuspended in 4 ml of polymerase buffer and centrifuged at 2000 rpm for 10 minutes. The supernatants were assayed for RNA polymerase activity as described in Materials and Methods. ·——·, uninfected cells; o---o, infected cells.

extracts were prepared and centrifuged at 95,000 x g for one hour. The pellet was resuspended in polymerase buffer and used in a cell-free assay for RNA polymerase activity. The preparation from infected cells demonstrated linear incorporation of ^{3}H-UTP for one hour. An identical preparation from uninfected cells contained no demonstrable RNA polymerase activity.

When cells were fractionated in a discontinuous sucrose gradient, the highest specific activity was found in Fraction 2, as shown in Table III. This confirms the previous results of Caliguiri and Tamm (1969).

If membranes from Fraction 2 of infected cells are mixed with deoxycholate and centrifuged in a gradient of 15-30% sucrose in RSB at 86,000 x g (Rav) for 90 minutes in a Spinco SW27 rotor, a complex with an estimated sedimentation constant of 130 S may be detected which contains pulse labeled viral RNA (Caliguiri and Tamm, 1970b). We have demonstrated that this complex also contains viral RNA polymerase activity.

HeLa cells (3.2×10^{8} cells) were infected with 150 PFU/cell of poliovirus type 2 and divided into two portions. One culture received 5 μg/ml actinomycin D at one hour after infection and 1.0 mCi of ^{3}H-uridine at 3 hours 55 minutes after infection. The other culture received neither. At 4 hours after infection, both cultures were harvested by pouring over crushed frozen PBS, and centrifuged to sediment cells. Cytoplasmic extracts were prepared and centrifuged in discontinuous sucrose gradient as described in Materials and Methods. Fraction 2 was collected from each gradient and pelleted by centrifugation at 95,000 x g for 2 hours. Some material from the unlabeled gradient was refrigerated for use in the RNA polymerase assay. The pellets were resuspended in 1.0 ml of 2.0% deoxycholate in RSB and layered onto 15-30% linear sucrose gradients. These were centrifuged for 90 minutes at 86,000 x g (Rav) in a Spinco SW27 rotor and 1.0 ml fractions were collected by pumping 45% sucrose into the bottom of the

Table III

In Vitro RNA Polymerase Activity in Fractions from Poliovirus-infected HeLa Cells

	cpm incorporated[a]	mg protein	$\frac{\mu\mu mole\ ^3H\text{-}UTP}{mg\ protein}$
Cell extract[b]	34,749	.896	3.88
Fraction 2[c]	43,528	.093	46.85
Fraction 3	8,050	.076	10.71
Fraction 4	7,220	.100	7.22
Fraction 5	8,029	.195	4.12
Fraction 6	2,384	.058	4.11

[a] Incubated for one hour.

[b] Infected culture harvested at 4 hours after infection.

[c] 100,000 x g pellets of fractions collected after centrifugation in discontinuous sucrose gradient as described in Materials and Methods.

tubes. The replication complex, with a sedimentation constant of approximately 130 S, was located by assaying each fraction from the labeled gradient for acid-precipitable radioactivity. The results are shown in Figure 8. Fractions from the unlabeled gradient corresponding to the peak of radioactivity in the labeled gradient (bracketed in Figure 8) were pooled and assayed for RNA polymerase activity. Our experiments have shown that the ^{3}H-uridine label and RNA polymerase activity are distributed identically in such a gradient. The activity of the pooled fractions was compared to the activity of the membrane-bound complex in Fraction 2 and to Fraction 2 material prepared identically from uninfected cells. These results are shown in Table IV. The specific activity of the released 130 S complex and the kinetics of incorporation of ^{3}H-UTP into this complex are very similar to the specific activity and kinetics for the membrane-bound replication complex from infected cells. Note that in neither case is the incorporation linear for an hour; since we have shown that the product of this system is primarily ribonuclease-resistant double-stranded RNA, it is possible that nuclease contamination is responsible for non-linearity of precursor incorporation. This experiment has demonstrated viral RNA polymerase activity in the 130 S complex released from membranes in Fraction 2 from infected cells.

Discussion

The distribution of cytoplasmic membranes into fractions with different densities changes with time after infection of HeLa cells with poliovirus. These changes are most evident as a marked increase of membranes with low density and as a decrease in quantity and increase in density of the rough microsomal membranes which form a pellicle in the discontinuous sucrose gradient used for fractionation.

Viral RNA synthesis is associated with the low density membranes found in Fraction 2 (Caliguiri and Tamm, 1970a,b). We consider that these membranes are identical to the proliferating smooth membrane-bound cisternae seen in electron micrographs of picornavirus-infected cells (Dales et al., 1965; Amako and Dales, 1967b). Electron microscopy has

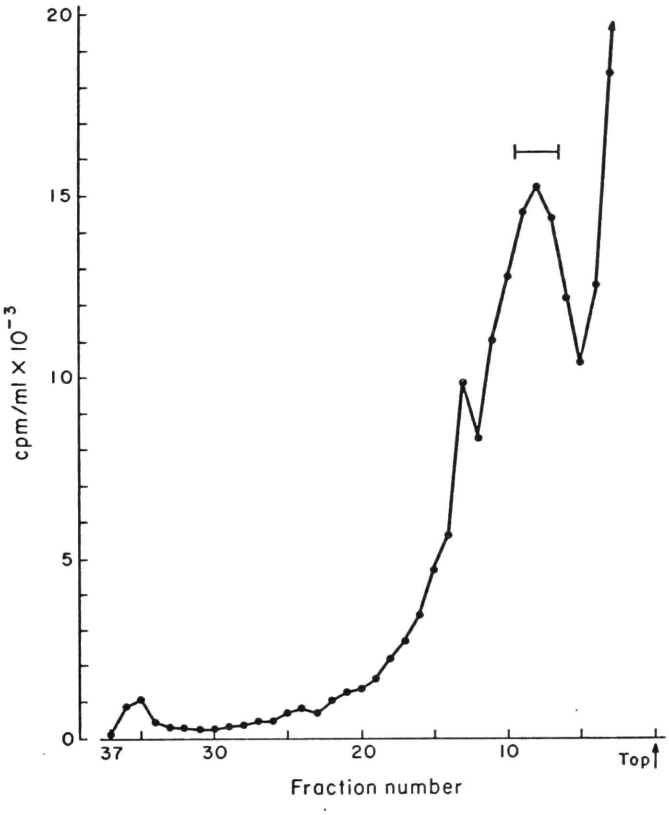

Figure 8. Localization and isolation of the 130 S replication complex for viral RNA polymerase assay. HeLa cells were infected and fractionated as described in the text. Deoxycholate-treated pellets from Fraction 2 of an unlabeled culture and a culture labeled with ^{3}H-uridine for 5 minutes were centrifuged in 15-30% sucrose-RSB gradients at 86,000 x g for 90 minutes. The peak of acid-precipitable radioactivity of the labeled gradient shows the location of the replication complex. Fractions of the unlabeled gradient corresponding to those bracketed in the labeled gradient were pooled and used for an *in vitro* polymerase assay as the 130 S complex.

Table IV

In Vitro RNA Polymerase Activity in the Replication Complex Before and After Lysis of Membranes

	minutes incubated	cpm incorporated	mg protein	$\frac{\text{mole } ^3\text{H-UTP}}{\text{mg protein}}$
Fraction 2				
control cells	60	1,550	.0174	10.1
Fraction 2[a]	15	25,002	.0064	442.0
infected cells	60	55,097	.0064	973.0
130 S complex	15	3,973	.0008	560.5
from Fraction 2	60	8,061	.0008	1,138.5

[a] Infected cells harvested at 4 hours after infection.

shown that they are smooth cytoplasmic membranes (Caliguiri and Tamm, 1970a; see Figure 2). The time course of the increase in protein and phospholipid content of this fraction, plotted in Figure 5, corresponds well with the development of smooth-walled cisternae in thin-sectioned poliovirus-infected cells. Dales and co-workers (1965) found few of these bodies at 3 hours after infection, and increasing amounts at 5 and 7 hours after infection.

It is interesting that these membranes continue to accumulate late into infection, well beyond the peak of viral RNA synthesis, which occurs between 3.5 and 4 hours after infection as measured in a cell-free assay system (Baltimore et al., 1963) or by short ^{3}H-uridine pulses in intact, infected cells (Caliguiri and Tamm, 1968a). This indicates that continuing rapid viral RNA synthesis is not necessary for the appearance of membranes in this fraction.

The phospholipid analysis of Fraction 2 (Table II) demonstrates that these membranes are qualitatively as well as quantitatively different in infected and uninfected cells. The very low sphingomyelin content which is the most striking change is indicative, incidentally, of a lack of contamination of this fraction by plasma membrane fragments, most of which are sedimented by the low speed centrifugation prior to layering the samples into the gradients. Plasma membranes are relatively rich in sphingomyelin (Fleischer and Rouser, 1965; Klenk and Choppin, 1970). The membranes of Fraction 2 from infected cells demonstrate an extremely low content of this phospholipid, considerably lower than most analyses of microsomal membranes have indicated (Dallner et al., 1965b; Fleischer and Rouser, 1965).

The lysophosphatidylcholine contents listed in Table II are quite high. Analysis of cell extracts that were frozen immediately after preparation has confirmed that these values are not the result of degradation of phosphatides during storage. We consider that these values are not artifacts of the method used for phospholipid analysis, since previous experience has shown the method to be reliable.

The pellicle of rough microsomes with which viral protein synthesis is associated decreases in size and increases in density as infection progresses. These changes might be related to late events in the viral growth cycle or to the action of lysosomal enzymes that may be released in poliovirus-infected cells (see Chapter 1).

4. SYNTHESIS OF CELLULAR MEMBRANE PHOSPHOLIPIDS - INCORPORATION OF ^{3}H-CHOLINE AND ^{3}H-GLYCEROL

Chapter 4

Synthesis of Cellular Membrane Phospholipids - Incorporation of ^{3}H-Choline and ^{3}H-Glycerol

Introduction

We have demonstrated that poliovirus infection produces striking changes in the quantity and composition of certain membranes in its host cell, and that these membranes may be separated on the basis of their densities. It was of interest to investigate the synthesis of the phospholipids of these membranes not only because it represents a case of viral control of cellular synthesis, but especially because of the rapid accumulation of the membranes which sediment in Fraction 2, with which viral RNA synthesis is associated.

As mentioned in Chapter 1, poliovirus infection leads to a marked, rapid decline in cellular protein synthesis. Slightly later in the viral cycle, at about 3 hours after infection, the incorporation of ^{3}H-choline into macromolecular structures is stimulated in infected cells. This stimulation requires some protein synthesis. However, treatment of infected cells with actinomycin D does not abolish the stimulation of choline incorporation (Penman, 1965), and thus the synthesis of cellular messenger RNA is not required. In fact, Amako and Dales (1967b) found that they had to treat both infected and uninfected cells with actinomycin D to "unmask" the stimulated synthesis in mengovirus-infected L cells. Drug treatment lowered the incorporation of choline in both groups of cells, but the effect was greater in uninfected cells. We did not use actinomycin D in the experiments discussed in this chapter.

The usefulness of choline as a label for membrane phospholipids has been demonstrated in intact animals (Nagley and Hallinan, 1968) and in cell culture (Amako and Dales, 1967b; Plagemann, 1968). In all of these studies 90% or more of ^{14}C- or ^{3}H-labeled choline that was incorporated could be detected in membrane lipids. Thin layer chromatography of lipid extracts have shown that in our experiments, after 2 hours of labeling poliovirus-infected and uninfected cells with ^{3}H-choline,

about 94% of the label is in phosphatidylcholine, 5% is in lysophosphatidylcholine, and 1% or less in sphingomyelin.

It is not possible to perform pulse-chase experiments using very short pulses of labeled choline in most animal cells (Amako and Dales, 1967b; Plagemann, 1968). Apparently, choline enters an expandable intracellular pool which is not exchanged with the extracellular medium. Plagemann (1968) has presented evidence that in Novikoff rat hepatoma cells, the nonexchangeable pool consists primarily of phosphorylcholine. To overcome this difficulty, we have used glycerol-2-^{3}H to demonstrate the precursor-product relationships between membrane components in the rough and smooth membranes of infected cells.

Results

Labeling of Cellular Membrane Fractions with ^{3}H-choline in Infected and Uninfected Cells.

HeLa cells (3.6×10^{8} cells) from suspension culture, were sedimented, washed and resuspended in cold Eagle's spinner medium. Half of the cells were infected with 60 PFU/cell of poliovirus type 2. Both cultures were incubated at 37° C. One hour later both cultures were diluted with warm spinner medium. At 3.5 hours after infection, 0.5 mCi of ^{3}H-choline was added to each culture and 30 minutes later they were harvested by pouring over crushed frozen PBS. The cells were sedimented and washed with cold PBS to remove ^{3}H-choline from the medium, and discontinuous sucrose-RSB gradients were prepared as described in Materials and Methods. After centrifugation for 17 hours, fractions were collected from the top using a specially designed fraction collector. The fatty material from the top of the gradient was removed in a volume of 1 ml and the rest of the gradient was collected in 3 ml fractions. The fractions were diluted with RSB and membranes were pelleted by centrifugation at 95,000 x g for 2 hours in a Spinco S40 rotor. Pellets were resuspended in 1.0 ml RSB and analyzed for protein, lipid phosphorus and lipid-associated radioactivity. We collected fractions of uniform volume in this experiment to eliminate technical problems that could be introduced by manual collection of visualized bands.

There was good agreement between results obtained using the two collection methods.

The results of the protein and lipid phosphorus analyses are plotted in Figures 9 and 10, respectively. Note that the lipid phosphorus values have been converted to mg of phospholipid, based on an average molecular weight of 770 for the "typical" phospholipid. There was an increased amount of both protein and phospholipid in the upper fractions of the infected gradient, where the smooth microsomes of Fraction 2 would sediment.

Figure 11 shows the total radioactivity which was extracted into chloroform-methanol from the pelleted fractions. Again, there was a definite increase in radioactivity in the upper fractions from the sucrose gradient prepared from the cytoplasmic extract of infected cells. It should be noted that the total radioactivity in the entire gradient from infected cells exceeded the total in the gradient from uninfected cells by 67%, although this is not immediately obvious in Figure 11.

Division of the lipid-associated radioactivity in each fraction by the amount of phospholipid per fraction yields the results plotted in Figure 12. In uninfected cells, the highest activities were in the fractions corresponding to the pellicle of rough membranes. In infected cells the distribution of radioactivity was bimodal, indicating stimulation of synthesis of membranes which sediment in the upper part of the gradient.

This method of presenting the data gives a figure which is analogous to a specific activity, although a greater amount of radioactivity per mg phospholipid could be achieved either by a generalized stimulation of phospholipid synthesis or by a specific stimulated production of those phospholipid molecules that contain the choline label. Our studies and those of Plagemann (1968) and Nagely and Hallinan (1968) have shown that ^{3}H-choline label is found largely in phosphatidylcholine, with minor quantities in lysophosphatidylcholine and sphingomyelin. Since we have shown that the relative proportion

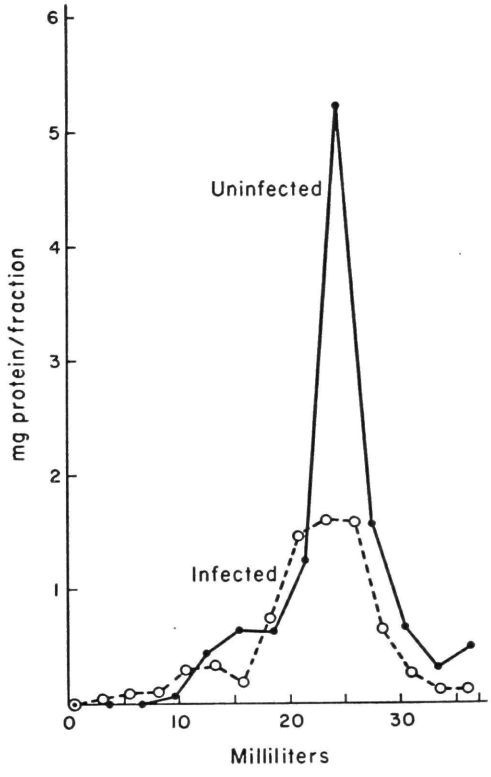

Figure 9. Protein content of fractions from discontinuous sucrose gradients from uninfected and infected HeLa cells harvested 4 hours after infection with poliovirus. HeLa cells were infected, labeled and fractionated as described in the text. The fractions were pelleted by centrifugation at 95,000 x g for 2 hours. Protein was determined for each fraction. Top of the gradients is on the left. .——., uninfected cells; o---o, infected cells.

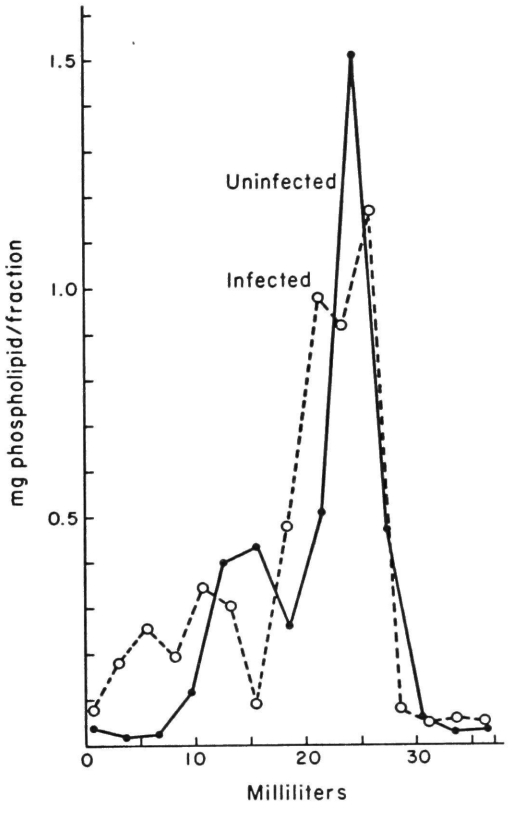

Figure 10. Phospholipid content of fractions from discontinuous sucrose gradients from uninfected and infected HeLa cells harvested 4 hours after infection with poliovirus. Preparation is as described for Figure 9. Lipid phosphorus was determined for each pelleted fraction and the values converted to phospholipid as described in Materials and Methods. Top of the gradients is on the left. •——•, uninfected cells; o---o, infected cells.

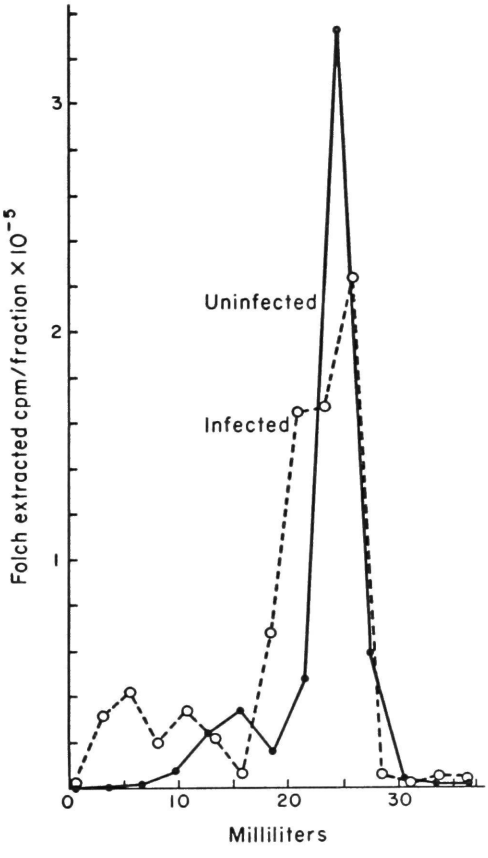

Figure 11. Lipid associated ^{3}H-choline radioactivity of fractions from discontinuous sucrose gradients from uninfected and infected HeLa cells harvested 4 hours after infection with poliovirus. Fractions were prepared as described for Figure 9. Radioactivity extracted by chloroform-methanol was determined. Top of gradients is on the left.
•——•, uninfected cells; o---o, infected cells.

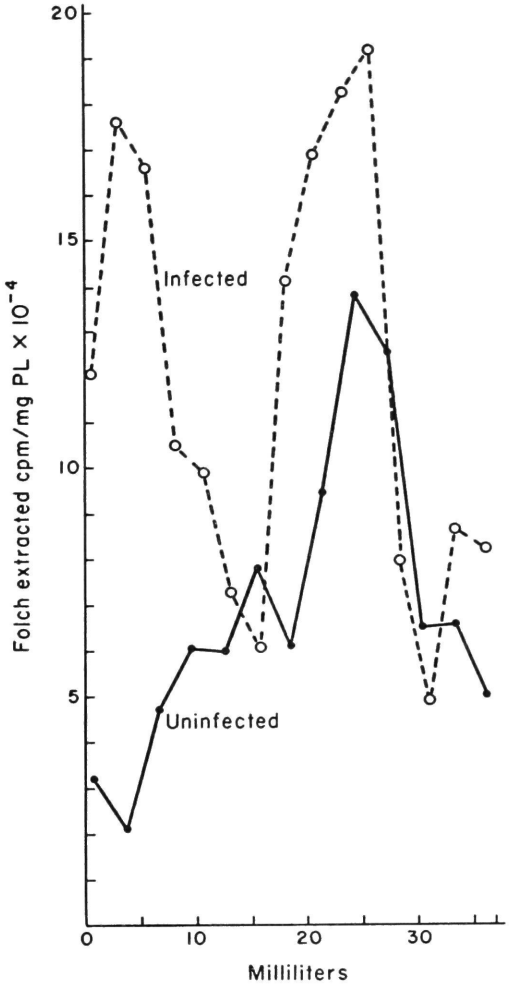

Figure 12. Lipid-associated ^{3}H-choline radioactivity per mg phospholipid of fractions from discontinuous sucrose gradients from uninfected and infected HeLa cells harvested 4 hours after infection. Values were calculated as described in the text. Top of the gradients is on the left. .——., uninfected cells; o---o, infected cells.

of the different phospholipids is significantly altered in Fraction 2 after 5 hours of infection, it is probable that some of the increased radioactivity per mg of phospholipid in this part of the gradient reflects the relative increase in phosphatidylcholine compared to other phospholipids. However, the increase in phosphatidylcholine is not large enough to account for all of the excess radioactivity, and we may assume that the incorporation of phospholipids is increased in general. This has been demonstrated for the entire cell. Cornatzer (1961) found that 4 hours after infection with poliovirus the specific activities of all phospholipids from HeLa cells labeled for 30 minutes with ^{32}P were increased in comparison with those from uninfected cells.

Synthesis of Cellular Membranes Late in the Viral Growth Cycle

It was of interest to determine whether this stimulation of choline incorporation into cellular membranes, and preferential synthesis of certain membranes would continue late into infection. For this purpose 8.3×10^8 cells were centrifuged, washed and resuspended in serum-free medium in two equal portions. One was infected with 100 PFU/cell of poliovirus type 2, and both were incubated at 37° C. After one hour both were diluted to 100 ml with warm Eagle's spinner medium. At 5 hours after infection, 0.3 mCi of ^3H-choline was added to each culture. Three minutes later one half of each culture was harvested by pouring over crushed frozen PBS. At 5.5 hours after infection the remainder of each culture was harvested in the same way. Discontinuous sucrose gradients were prepared and centrifuged, as described in Materials and Methods. The visible bands were collected manually.

The radioactive counts extracted into chloroform-methanol from each pelleted fraction are plotted in Figure 13. After a 3-minute pulse most of the ^3H-choline label was found in the pellicle of rough microsomal membranes: Fraction 5 in uninfected cells, and Fraction 6 in infected cells, indicating that phosphatidylcholine is incorporated into membranes in this fraction. After 30 minutes of incorporation, this was still the case for uninfected cells, but much of the label in infected

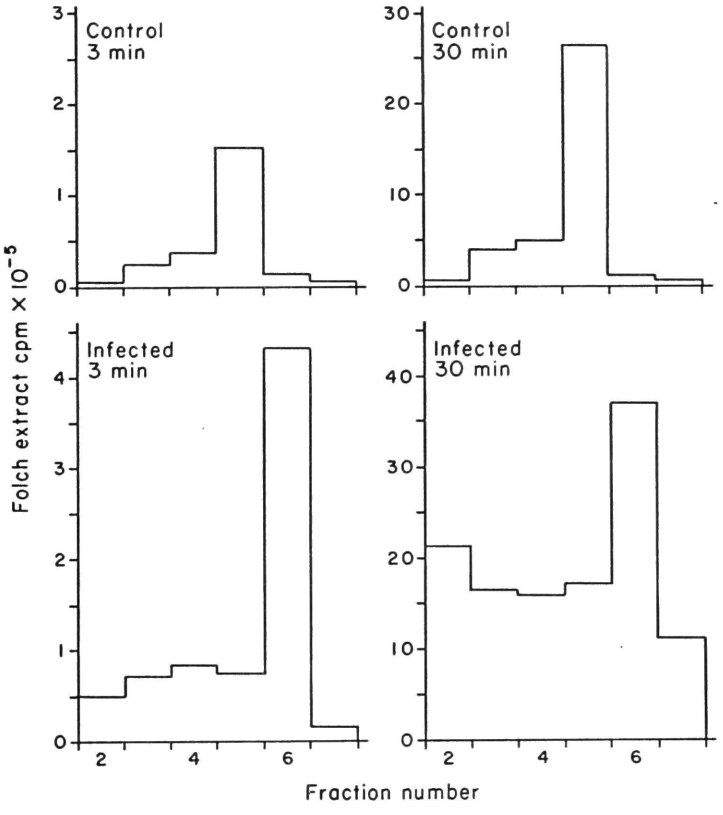

Figure 13. Lipid-associated ^{3}H-choline radioactivity from fractions of discontinuous sucrose gradients from uninfected and infected HeLa cells late in the viral cycle. Cells were infected, labeled and fractionated as described in the text. Pelleted fractions were extracted with chloroform-methanol and the extracts were assayed for radioactivity.

cells was now associated with the smooth membranes which sedimented in Fraction 2. In fact, comparison of the relative amount of radioactivity incorporated into Fraction 2 of infected cells in this experiment with incorporation during half hour pulses at earlier times in the virus cycle indicated that approximately as many of the newly incorporated choline molecules sedimented in Fraction 2 at 5.5 hours after infection as at 3.5 or 4 hours after infection. Over the course of the full half hour, the infected cells incorporated three times as much radioactivity as did uninfected cells.

When the radioactivity of each fraction is divided by the phospholipid content of the fraction, the results are shown in Figure 14. Only the values for infected cells are shown. It is clear from these results that incorporation occurred most rapidly into Fractions 5 and 6, both of which contained material from the original pellicle fraction. After 30 minutes of labeling there was no peak of activity in the upper part of the gradient as there was in the results discussed earlier, but it must be remembered that a large quantity of material has accumulated here by 5.5 hours after infection. This mass of unlabeled phospholipid reduced the calculated activity for Fraction 2 even though newly synthesized phospholipids are found here in quantities as large as at earlier times in the viral cycle.

The continued incorporation of choline and the accumulation of membranes in Fraction 2 occurs at a time when cellular nucleic acid and protein synthesis have been greatly reduced for several hours (Zimmerman et al., 1962; Holland and Peterson, 1964; Penman and Summers, 1965). In fact, pathological rounding of cells has also occurred in a large proportion of the cells. Viral RNA synthesis has declined markedly by 5 to 5.5 hours after infection (Baltimore et al., 1963). The products of viral protein synthesis, however, are still present in large quantities.

Pulse-Chase Labeling of Cellular Membranes with Glycerol-2-^{3}H

Glycerol-2-^{3}H labels lipids containing the glycerol backbone. Under our experimental conditions 95% or more of the radioactivity is

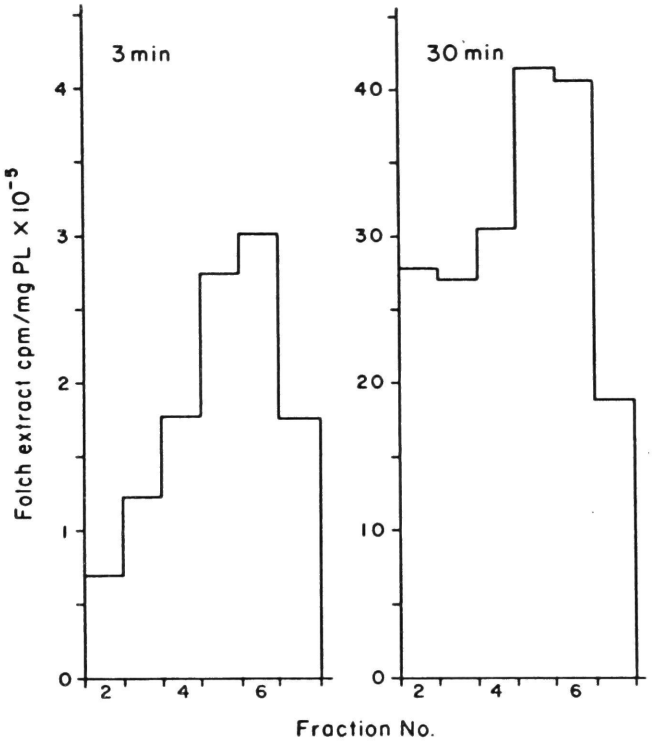

Figure 14. Lipid-associated ^{3}H-choline radioactivity per mg of phospholipid of fractions from discontinuous sucrose gradients from infected HeLa cells late in the viral cycle. Cells were infected, labeled and fractionated as described in the text. Pelleted fractions were analyzed for lipid-associated radioactivity and lipid phosphorus. Values for lipid phosphorus were converted to phospholipid as described in Materials and Methods and divided into the lipid-associated counts for each fraction.

incorporated into substances extracted by chloroform-methanol. Since labeled glycerol molecules can be effectively chased by removal of the labeled medium, we used glycerol as a tracer for studies on the origin of the lipids in the smooth membranes of Fraction 2.

S_3 HeLa cells in suspension (5×10^8 cells) were washed and divided into 2 cultures, of which one was infected with 70 PFU/cell of poliovirus type 2. After one hour at 37° C, both cultures were diluted to 100 ml with warm Eagle's spinner medium. At 3 hours and 27 minutes after infection, 0.3 mCi of glycerol-2-^{3}H (approximately 0.8 μmoles) was added to each culture. Three minutes later, half of each culture was harvested by pouring over crushed frozen PBS. Cell extracts were prepared and fractionated in discontinuous sucrose gradients. The remainder of each culture was poured over 100 ml warm Eagle's spinner medium containing 10 mmoles of unlabeled glycerol. The cells were sedimented by centrifugation, resuspended in 50 ml warm, unlabeled medium, and returned to 37° C. At 4.5 hours after infection these cells were harvested. Cell extracts were prepared and fractionated in discontinuous sucrose gradients. Visible bands were collected manually from the top.

Radioactivity extracted into chloroform-methanol from the pelleted fractions is shown in Figure 15. Note the great increase in incorporation of glycerol in infected cells compared to uninfected cells. As seen when ^{3}H-choline is used as a label, after the 3-minute pulse, most of the radioactivity in both uninfected and infected cells was found in the rough microsomal membranes in Fraction 5. In uninfected cells the distribution of radioactivity did not change significantly during the hour-long chase period. However, in infected cells, a large amount of the label was shifted from the rough microsomal pellicle, in Fraction 6 in this gradient, into the smooth membranes at the top of the gradient, particularly those in Fraction 2. Identical results were obtained in another experiment in which the cells were maintained, during the chase period, in an excess of unlabeled glycerol (1.0 mM), which prevented the reutilization of labeled glycerol.

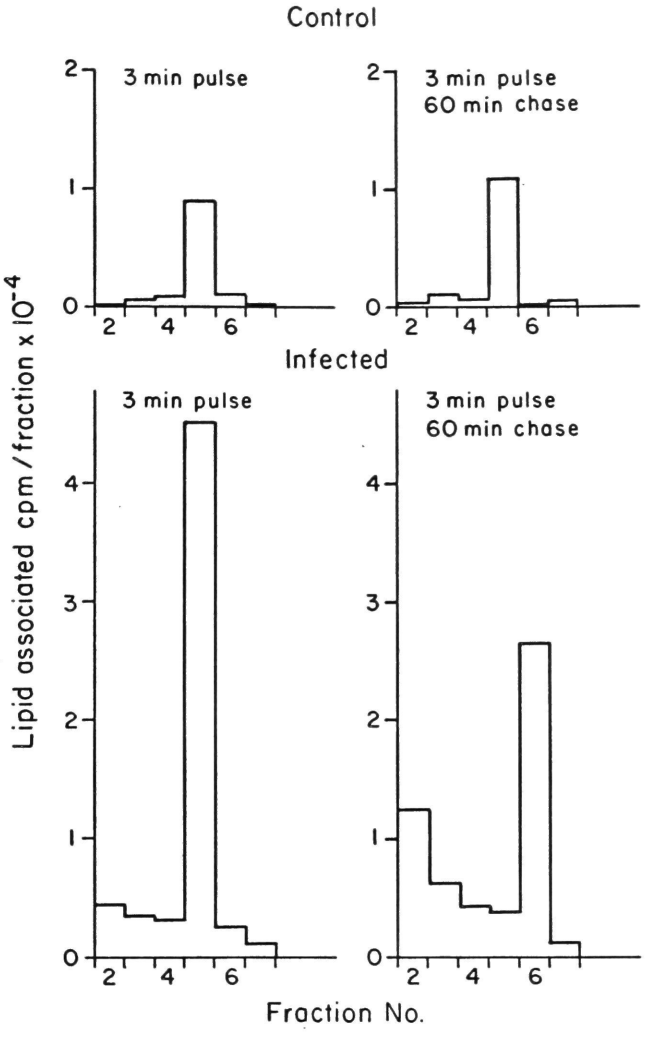

Figure 15. Lipid-associated glycerol-2-^{3}H radioactivity of fractions from discontinuous sucrose gradients from uninfected and infected HeLa cells. Cells were infected, labeled and fractionated as described in the text. Pelleted fractions were extracted with chloroform-methanol and the extracts were assayed for radioactivity.

Thus it appears that in poliovirus-infected cells there is preferential synthesis in the rough endoplasmic reticulum of lipid which is destined to become part of the smooth membranes in Fraction 2.

Inhibition of Viral RNA Synthesis - Effect on Choline Incorporation

Since viral RNA synthesis is associated with the smooth membrane of Fraction 2, it was of interest to study the effect of inhibition of viral RNA synthesis on the incorporation of choline into various fractions of infected cells. We chose guanidine hydrochloride as a specific inhibitor of viral RNA synthesis which would not directly alter the rest of the system in any measurable way. As discussed in Chapter 1, we already knew several pertinent facts about the action of guanidine. Treatment of infected cells with 1.0 to 2.0 mM guanidine completely halts viral RNA synthesis. Viral protein synthesis is unaffected except by limitation of viral messenger RNA as a result of blocked RNA synthesis. Penman (1965) showed that the stimulation of choline uptake into macromolecular structures in infected cells is not abolished by guanidine treatment if the cells have been infected with a large enough multiplicity of virus. He also showed that guanidine treatment has no observable effect on choline incorporation into uninfected cells. We have found that treatment of uninfected cells with 1.0 to 2.0 mM guanidine either reduces slightly the incorporation of choline or has no significant effect.

Other authors have shown that guanidine prevents or delays the development of cytopathic effects in picornavirus-infected cells (Bablanian et al., 1965a) and Dales et al. (1965) observed that the small smooth surfaced cisternae, which we believe correspond to the membranes of Fraction 2, did not develop in cells which had been treated with guanidine. Their experiments were performed using 23 PFU/cell of poliovirus. Since guanidine treatment blocks viral RNA production, this relatively small inoculum may not provide sufficient viral messenger RNA to code for the product active in stimulating phospholipid synthesis.

Penman (1965) found that the stimulation of choline incorporation is greatly depressed by guanidine treatment if the cells are infected with only 10 PFU/cell of poliovirus.

To investigate the effect of guanidine treatment on incorporation of choline and the accumulation of smooth membranes in Fraction 2, cells were infected with a somewhat higher virus multiplicity. HeLa cells (6.6×10^8) were sedimented, washed and divided into 3 equal portions. Two of these were infected with 45 PFU/cell of poliovirus type 2, and a third served as a control. After one hour at 37° C, all were diluted to 100 ml with warm spinner medium. At this time one of the infected cultures was brought to a final concentration of 1.0 mM guanidine. At 3.5 hours after infection, 0.3 mCi of ^{3}H-choline was added to each, and they were harvested at 4 hours after infection by pouring over crushed frozen PBS. Cells were sedimented, and cytoplasmic extracts were prepared and layered into discontinuous sucrose gradients as described in Materials and Methods. After centrifugation for 9.4 hours, the visible bands were collected manually from the top. They were pelleted and the resuspended pellets were analyzed for lipid phosphorus and radioactivity. Results are plotted in Figures 16-18. The uninfected control cultures and the infected cells which were not treated with guanidine behaved as in previous experiments with one exception: the pellicle of rough microsomal membranes in the uninfected cells sedimented in Fraction 6. This was an infrequent occurrence for which we have no explanation. Infected cells which had been treated with guanidine incorporated 82% more ^{3}H-choline than did uninfected control cells. Infected cells not treated with guanidine incorporated 120% more choline than did uninfected control cells. Thus, both infected cultures demonstrated marked stimulation of ^{3}H-choline incorporation. However, in infected cells that had been treated with guanidine, the relative amount of radioactivity incorporated into Fraction 2 is similar to that found in uninfected cells. The lipid phosphorus analysis (Figure 16) shows even more strikingly that there was no extensive accumulation of smooth membranes in Fraction 2 in infected, guanidine-treated cells.

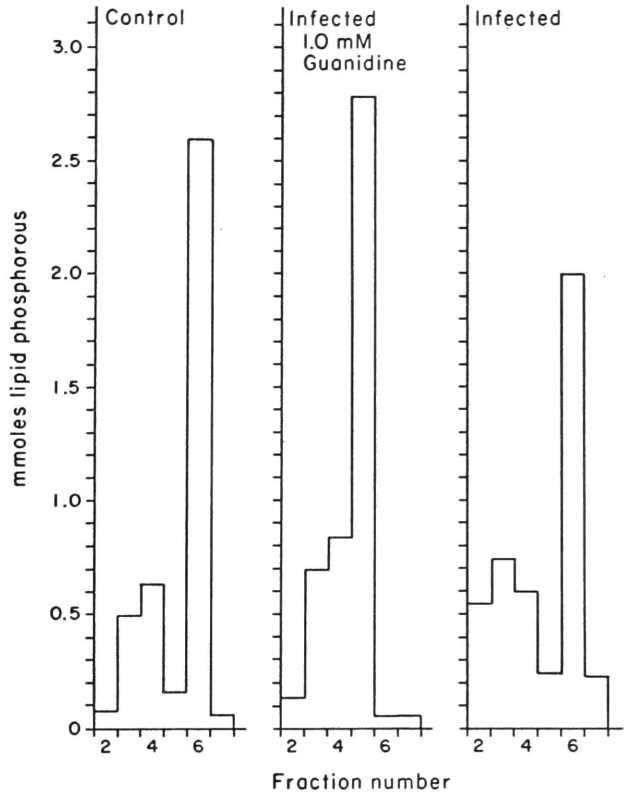

Figure 16. Effect of guanidine treatment on lipid phosphorus content of fractions from discontinuous sucrose gradients from uninfected and infected HeLa cells. Cells were infected, treated and fractionated as described in the text. Pelleted fractions were analyzed for lipid phosphorus.

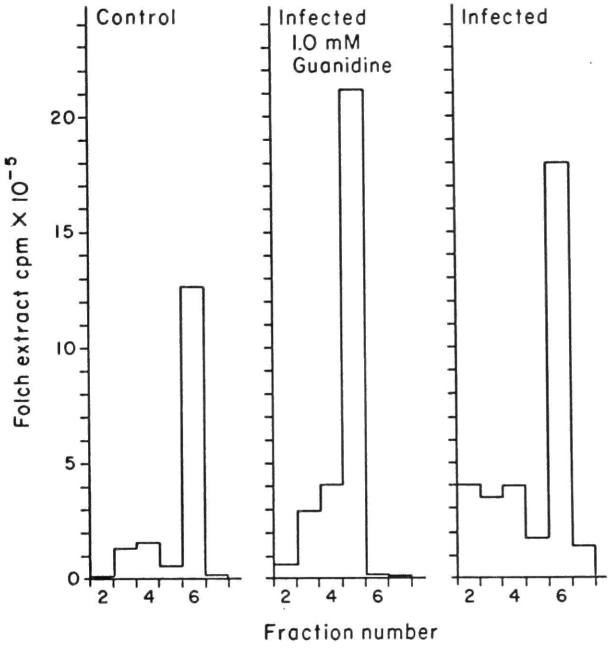

Figure 17. Effect of guanidine treatment on lipid-associated ^{3}H-choline radioactivity of fractions from discontinuous sucrose gradients from uninfected and infected HeLa cells labeled for 30 minutes with ^{3}H-choline. Cells were infected, treated and fractionated as described in the text. Pelleted fractions were analyzed for radioactivity extractable by chloroform-methanol.

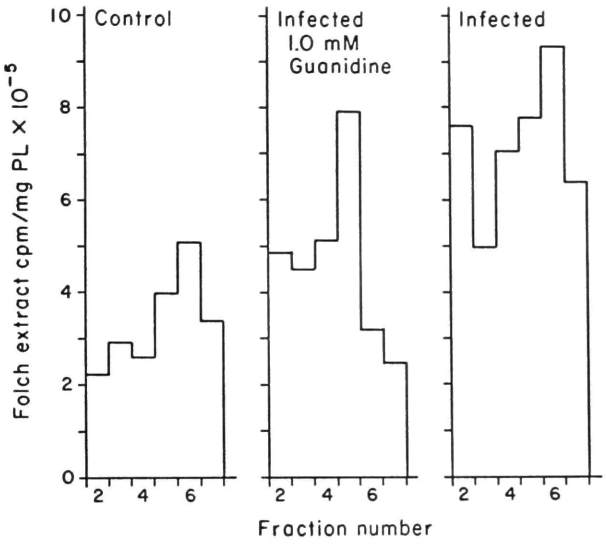

Figure 18. Effect of guanidine treatment on lipid-associated ^{3}H-choline radioactivity per mg of phospholipid from fractions of discontinuous sucrose gradients from uninfected and infected HeLa cells labeled for 30 minutes with ^{3}H-choline. Cells were infected, treated and fractionated as described in the text. Pelleted fractions were analyzed for lipid phosphorus and radioactivity extractable by chloroform-methanol. Lipid phosphorus values were converted to phospholipid and divided into the lipid-associated counts for each fraction.

Distribution of ^{3}H-Choline Label After a Three-Hour Pulse

These results were confirmed in the following experiment, which utilized a longer exposure to ^{3}H-choline. HeLa cells (9.3×10^{8} cells) were sedimented, washed and divided into 3 equal portions. Two of these were infected with approximately 100 PFU/cell of poliovirus type 2. In this experiment, guanidine was added to a final concentration of 1.5 mM in one of the infected cultures at one hour after infection when all three cultures were diluted to 100 ml with warm spinner medium. ^{3}H-Choline (0.3 mCi/culture) was added to all three cultures at 1.5 hours after infection, and duplicate aliquots were withdrawn and precipitated with cold 10% trichloroacetic acid at half hourly intervals thereafter. The counts in each precipitated aliquot, averaged for each pair, are plotted in Figure 19. It may be seen that in this case guanidine diminished the extent to which choline incorporation was stimulated by poliovirus infection, but it was still marked in the guanidine-treated culture by 3.5 to 4.5 hours after infection. It should be noted that control experiments have shown that guanidine treatment of uninfected cells does not lead to increased choline incorporation.

At 4.5 hours after infection the cells were harvested and fractionated on discontinuous sucrose gradients as described earlier. Figure 20 shows the phospholipid content of each fraction from the gradients of the two infected cultures, and Figure 21 shows the lipid-associated radioactivity. Again, the pattern of membrane phospholipids and incorporated counts from the guanidine-treated culture resembled uninfected cells far more than infected cells. Early guanidine treatment seemed to block almost completely the formation of the smooth microsomal membranes which are found in Fraction 2 of gradients from infected cells, even though it diminished only partially the stimulation of choline incorporation into the membranes of infected, guanidine-treated cells.

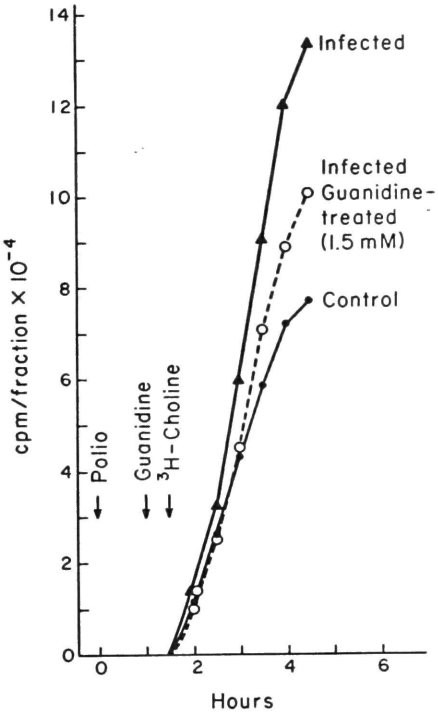

Figure 19. ^{3}H-Choline incorporated into acid-precipitable structures in infected and uninfected HeLa cells. Cells were infected and treated as described in the text. Duplicate aliquots were withdrawn, precipitated with trichloroacetic acid and analyzed for radioactivity. Values for each pair of samples are averaged.

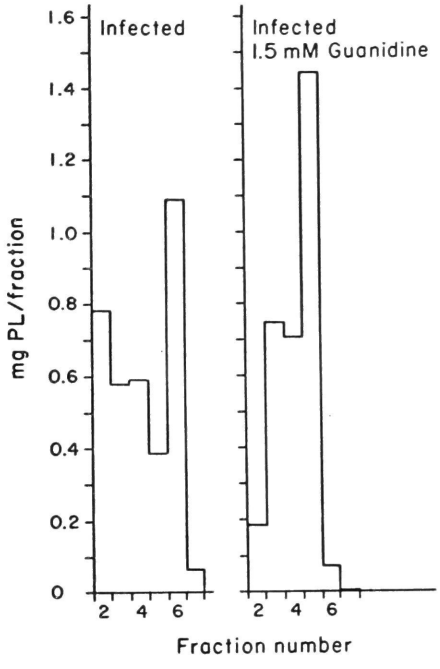

Figure 20. Effect of guanidine treatment on phospholipid content of fractions from discontinuous sucrose gradients of poliovirus-infected HeLa cells. Cells were infected, treated and fractionated as described in the text. Pelleted fractions were analyzed for lipid phosphorus. Values for lipid phosphorus were converted to phospholipid as described in Materials and Methods.

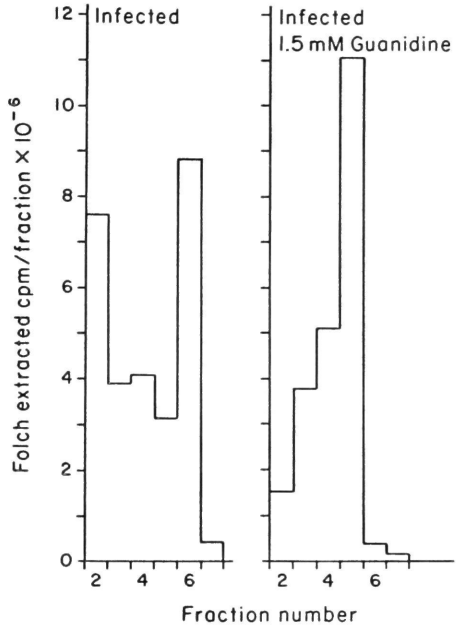

Figure 21. Effect of guanidine treatment on lipid-associated radioactivity of fractions from poliovirus-infected HeLa cells labeled for 3 hours with ^{3}H-choline. Cells were infected, treated and fractionated as described in the text. Pelleted fractions were analyzed for radioactivity extractable by chloroform-methanol.

Discussion

The results presented in this chapter confirm Penman's (1965) findings that poliovirus infection leads to increased incorporation of ^{3}H-choline into cellular membranes. A comparison of the radioactivity per mg phospholipid incorporated into each fraction after a 30-minute labeling period shows that the smooth surfaced cisternae that sediment in Fraction 2 contain a proportionately greater share of the counts, indicating preferential synthesis. The rough membranes which sediment in the pellicle also contain relatively more radioactivity per mg of phospholipid, but since the label is being incorporated into membranes in this fraction at a more rapid rate than in other fractions, the high activity does not necessarily represent preferential synthesis of rough microsomal membranes.

Results of pulse-chase studies with glycerol-2-^{3}H suggest that the smooth membranes with which viral RNA synthesis is associated are produced in the rough endoplasmic reticulum. Incorporation of glycerol-2-^{3}H is greatly increased as a result of poliovirus infection.

Treatment of cells with guanidine at one hour after infection diminishes but does not block the stimulation of ^{3}H-choline incorporation. Yet such cells do not produce great quantities of smooth surfaced membranes which sediment in Fraction 2; in fact, their complement of membranes appears to be quite similar to that of uninfected cells. This suggests that effects of poliovirus infection on cell membrane synthesis may operate on two levels: incorporation of precursors, and the conversion of rough to smooth-surfaced membranes. The production of smooth-surfaced cisternae may depend on the continued production of large amounts of certain viral products, which cannot take place in cells treated with guanidine before quantities of viral messenger RNA can be produced. The increased choline incorporation may be triggered by smaller amounts of the same viral product or by an entirely different mechanism.

5. BLOCKING OF GUANIDINE ACTION ON POLIOVIRUS MULTIPLICATION

Chapter 5

Blocking of Guanidine Action on Poliovirus Multiplication

Introduction

As discussed in Chapter 1, the action of guanidine on poliovirus multiplication may be blocked by treatment of cells with choline, methionine or many other compounds. To characterize the blocking phenomenon in greater detail, we have investigated the kinetics of poliovirus replication after blocking the inhibitory action of guanidine with choline or dimethylethanolamine. Three cell types were used for this study; all were maintained as monolayer cultures. HeLa cells are a continuous line derived from a human cervical carcinoma and are a different subline from the S_3 HeLa cells grown in suspension culture which were used for the experiments described in Chapters 3 and 4. LLC-MK_2 cells are a continuous line derived from rhesus monkey kidney cells. Primary rhesus monkey kidney cells (MKC) were also used.

Preliminary information was needed before kinetic experiments could be performed. Knowledge of the effect of the medium used during experiments on guanidine inhibition was necessary since most nutrient media contain compounds capable of blocking or reducing guanidine inhibition. We also needed to know the effects on virus yield of different concentrations of guanidine and choline in the three cell types used.

Results

Effect of the Medium

The virus-inhibitory activity of guanidine may be influenced by the culture medium if those components of the medium which possess anti-guanidine activity are present in sufficient concentration. MEM, which was used for most of the experiments reported in this paper, contains choline and several amino acids that are guanidine antagonists. Therefore, preliminary experiments were performed to determine the effect of this medium on the inhibitory action of guanidine. MEM was compared with Earle's balanced salt solution plus glucose and glutamine (ESG)

(Eagle and Habel, 1956); the latter medium contains no compounds with known antiguanidine activity. The growth medium on one half of the cultures was changed to ESG at 12 hours prior to infection. After infection with poliovirus at 40 PFU/cell, these cultures were again incubated in ESG, whereas the remaining cultures were incubated in MEM. At 1.5 hours after infection appropriate solutions were added to give final concentrations of guanidine and choline as shown in Table V.

The results summarized in Table V show that, while yields of poliovirus were reduced by approximately 50% in ESG, the effects of guanidine or guanidine plus choline were similar to those in cultures incubated in MEM. It may be concluded that the concentrations of potential guanidine antagonists in MEM are too low to affect measurably the activity of guanidine or of added choline in this system. These results differ from those obtained with foot-and-mouth disease virus in primary calf kidney cultures, where MEM caused some suppression of the inhibitory action of guanidine (Dinter and Bengtsson, 1964). It should be noted that this study did not utilize single cycle conditions of virus infection, and therefore small changes occurring in a single cycle of virus multiplication would be magnified over the course of several viral cycles.

Inhibition of Poliovirus Multiplication by Guanidine in Different Cells

To determine the effect of cell type on the inhibitory activity of guanidine, the following experiment was performed. HeLa cells, MKC and LLC-MK_2 cells were inoculated with 100 PFU/cell of poliovirus. At 1.5 hours after infection, guanidine at varying concentrations was added to pairs of cultures. All were harvested at 8 hours after infection. The results are shown in Figure 22. It is evident that guanidine at all concentrations used produced greater reductions of poliovirus yield in either of the monkey cell cultures than in HeLa cells. Viral replication in primary monkey kidney cells was reproducibly the most sensitive to guanidine. These results show that the degree of inhibition by guanidine at a given concentration is host cell dependent.

Table V

Effect of Culture Medium on the

Activities of Guanidine and Choline

Treatment	Virus yield, PFU/cell		
	MEM[b]	ESG[c]	ESG/MEM
Control	248	141	0.57
0.3 mM guanidine[a]	0.17	0.11	0.65
0.3 mM guanidine[a] and 1.0 mM choline	62	33	0.53

[a] Added at 1.5 hours after infection.

[b] Cultures were incubated in reinforced Eagle's medium until infection and in MEM after infection.

[c] Cultures were incubated in ESG for 12 hours prior to infection as well as after infection.

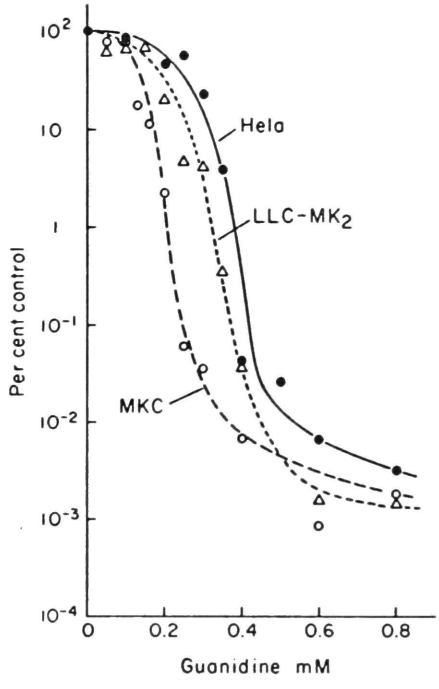

Figure 22. Inhibition of poliovirus yield by guanidine in HeLa cells, LLC-MK$_2$ cells and primary rhesus monkey kidney cells. Confluent monolayers of cells were infected at a multiplicity of 100 PFU/cell and treated with poliovirus antiserum as described in Materials and Methods. Guanidine was added at 1.5 hours after infection and all cultures were harvested at 8.0 hours after infection. Virus yields in untreated controls were as follows: HeLa cells, 270 PFU/cell; LLC-MK$_2$ cells, 290 PFU/cell; MKC, 180 PFU/cell.

Blocking of Guanidine Action by Choline

The ability of choline to block the inhibitory action of guanidine was examined in HeLa cells as a function of choline and guanidine concentrations. At 1.5 hours after infection guanidine and choline were added at several concentrations. Duplicate cultures were harvested at 8 hours after inoculation. Figure 23 shows that the inhibition caused by 0.3 or 0.4 mM guanidine was blocked almost completely by the addition of 10.0 mM choline to the culture medium, and 0.1 mM choline was sufficient to cause considerable blocking. Furthermore, the very marked inhibition caused by guanidine at a concentration as high as 1.0 mM could be blocked partially by choline at high concentrations. The highest choline concentrations employed might be somewhat toxic to cells, although we observed no indications of such toxicity during the 6-8 hours of choline treatment. Treatment of infected cultures with concentrations of choline up to 50 mM had no effect on the yield of poliovirus in the absence of guanidine.

A similar experiment was carried out using all three cell types, but utilizing 0.4 mM guanidine in all cases. Figure 24 shows that when poliovirus replication was more strongly inhibited by guanidine in a given cell type, a higher concentration of choline was required to obtain an equivalent degree of blocking. Similar results were obtained at different guanidine concentrations. Host cell variation in sensitivity to the inhibitory action of guanidine appears to be the dominant factor in determining to what extent guanidine action may be blocked by a guanidine antagonist at a given concentration.

Kinetics of Reversion of Guanidine Action by Choline

Effect of Concentration of Choline. To determine the kinetics of poliovirus multiplication after reversion of guanidine inhibition by choline, infected HeLa cell monolayers were divided into two groups. One group received 1.6 ml MEM one hour after infection plus 0.2 ml each of the appropriate solutions to give final concentrations of 0.4 mM guanidine and either 10 or 0.1 mM choline. The other group received 1.6 ml MEM plus 0.4 ml PBS and served as a control. Duplicate samples were

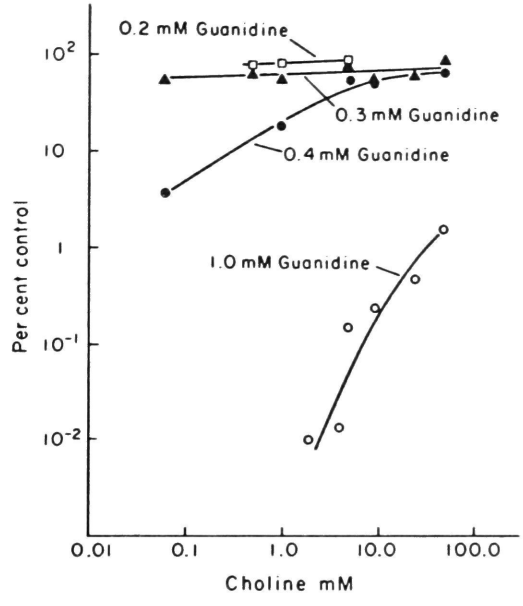

Figure 23. Relationship between concentrations of choline and extent of blocking of the inhibitory effect of guanidine on poliovirus multiplication in HeLa cells. Input multiplicity was 160 PFU/cell. Guanidine and choline were added at 1.5 hours, and all cultures were harvested at 8.0 hours after intection. The virus yield in untreated controls was 305 PFU/cell. In the absence of added choline, 0.2 mM guanidine reduced virus yield to 60% of controls; 0.3 mM guanidine to 10%, and 0.4 mM guanidine to 0.5% of controls. One mM guanidine reduced the virus yield by at least 4 orders of magnitude, but residual input virus not neutralized by poliovirus antiserum prevented exact determination of the extent of inhibition.

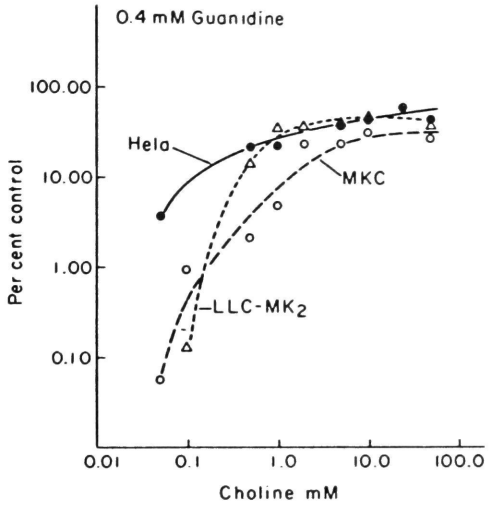

Figure 24. Blocking of the inhibitory effect of guanidine on poliovirus multiplication by choline in HeLa cells, LLC-MK_2 cells and primary rhesus monkey kidney cells. Input multiplicity was 100 PFU/cell. Guanidine (final concentration: 0.4 mM) and choline were added at 1.5 hours after infection. All cultures were harvested at 8.0 hours after infection. Virus yields in guanidine-treated cultures are expressed as percent of yields in untreated cultures of the same cell type, which were as follows: HeLa cells, 80 PFU/cell; LLC-MK_2 cells, 147 PFU/cell; MKC, 124 PFU/cell.

harvested at various times after infection. Figure 25, part A, shows that after addition of 10 mM choline to guanidine-treated cultures, the viral growth kinetics were indistinguishable from those in control cultures, except that the final virus yield was not quite as high as in controls. Figure 25, part B, shows that when 0.1 mM choline was added with guanidine, there was a delay before virus production was evident and the rate of production was less than that in controls. It cannot be determined from this experiment how long viral multiplication would continue under these conditions, but other experiments have indicated that after addition of small amounts of choline to guanidine-treated cultures, viral multiplication often continues for several hours after growth in control cultures has ceased. Thus, the 8-hour virus yields shown in Figures 23 and 24 may not represent the maximum yields of poliovirus in guanidine-treated cells in the presence of low concentrations of choline.

Effect of Time of Addition of Choline and Guanidine. The results of experiments in which the time of addition of choline was varied are shown in Figure 26. As noted above, when choline and guanidine were added simultaneously, the growth curve obtained was very similar to that in control cultures (Part A). Virus multiplication could first be detected at the same time as in control cultures, i.e. 2.5 hours after infection. In parts B and C, choline was added at thirty minutes and 1.5 hours after guanidine, respectively, and in both cases the latent period was increased by the time during which guanidine, alone, was in the culture medium before the addition of choline. In all cases the rate of virus increase was similar to that in control cultures. These results indicate that choline can promptly and effectively reverse the action of guanidine.

With this information available, experiments can be designed to determine whether the guanidine-sensitive process, i.e. the synthesis of viral RNA, is already in progress during the latter part of the latent period. Infected HeLa cell monolayers were divided into three groups. One group received 1.6 ml MEM plus 0.4 ml PBS and served as

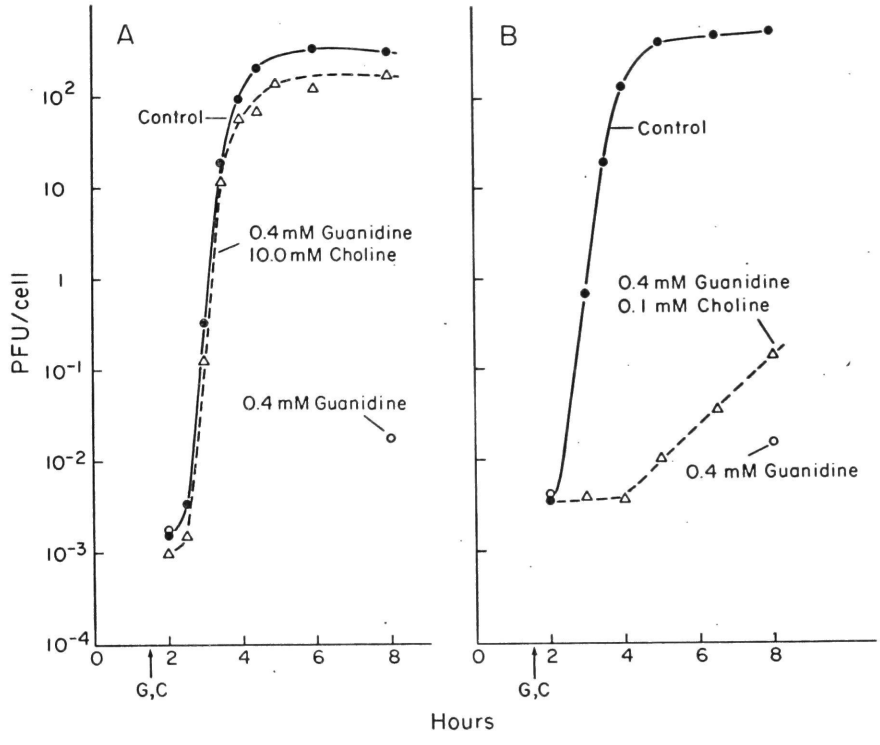

Figure 25. Kinetics of poliovirus multiplication in HeLa cells treated with guanidine and choline. Input multiplicity was 130 PFU/cell. Guanidine and choline were added at 1.5 hours after infection.

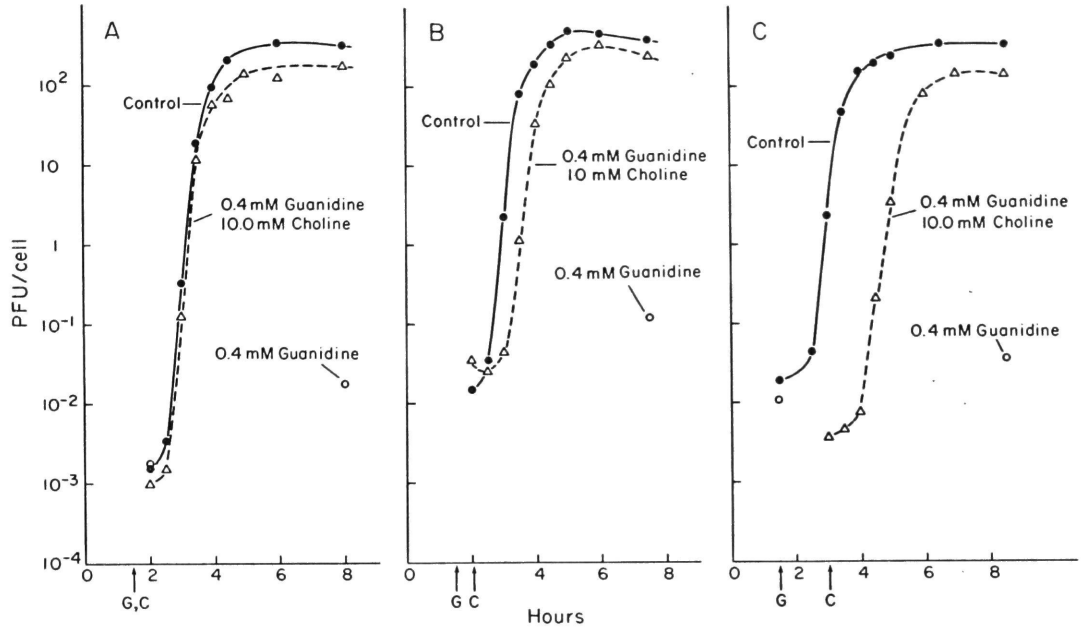

Figure 26. Relationship between time of addition of choline and kinetics of poliovirus multiplication in guanidine-treated HeLa cells. Input multiplicity was as follows: Part A: 130 PFU/cell; Part B: 85 PFU/cell; Part C: 110 PFU/cell. Guanidine was added at 1.5 hours after infection. In Part A choline was added at 1.5 hours after infection; in Part B at 2 hours after infection, and in Part C at 3 hours after infection.

a control. The others received 1.6 ml MEM plus 0.2 ml each of the appropriate solutions to give final concentrations of 0.4 mM guanidine and 10 mM choline. Figure 27A shows that when guanidine and choline were both added 1.5 hours after infection, choline permitted onset of virus multiplication with essentially normal kinetics without any lengthening of the latent period. However, when guanidine was added one hour after infection, followed by choline one-half hour later, there was a delay of one-half hour in the onset of multiplication. This clearly shows that the guanidine-sensitive process was already in progress between one and 1.5 hours after infection, which agrees with previous results obtained by a different technique (Eggers et al., 1965). Figure 27 demonstrates again that choline acts promptly and effectively.

Effect of Cell Type. In the two monkey cell cultures, the kinetics of virus production were similar to those observed in HeLa cells during choline-mediated reversal of the guanidine block. Figure 28 shows that in all three cell types when guanidine was added 1.5 hours after infection and choline at 3 hours after infection, the latent period was increased by 1.5 hours. Again, the rates of virus production were in all cases similar to those in untreated controls. In both LLC-MK_2 cells and primary monkey kidney cells a final concentration of 25 mM choline was used to insure maximum reversal of guanidine effect. These experiments again demonstrate the rapidity of choline action as well as the high degree of reversal of the guanidine effect that was possible when fairly high levels of choline were used. The somewhat lower virus yields obtained in cultures treated with both guanidine and choline, compared with those in untreated controls, probably indicate the inability of choline to reverse the action of guanidine at every site in the infected cell, a common finding in drug action. The similarity of the kinetics of reversal in the three cell types suggests that the mechanism of choline reversal of guanidine action is similar in all cell types.

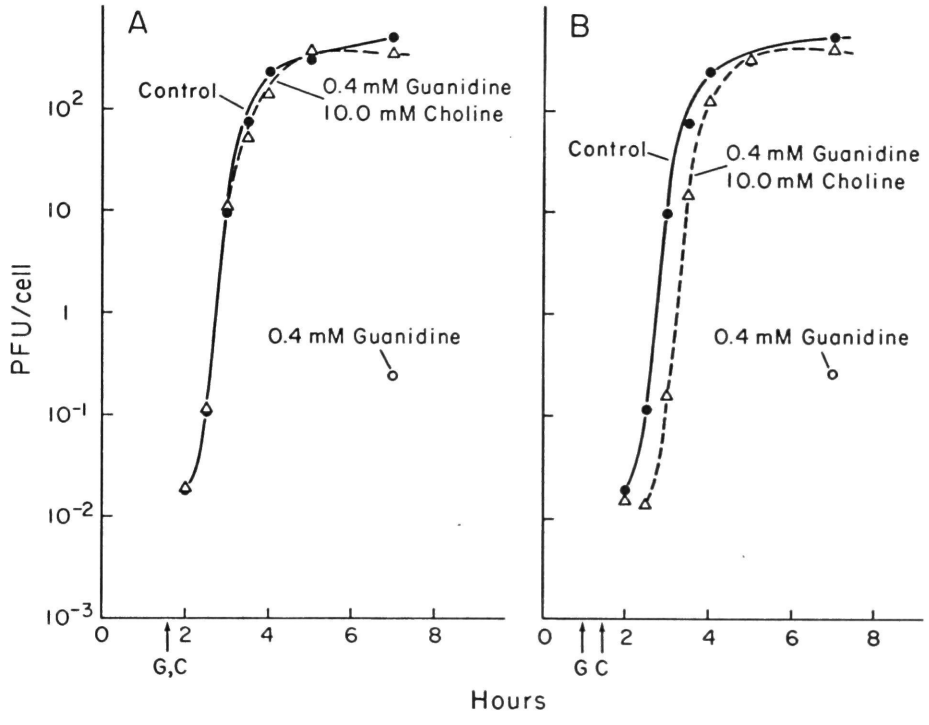

Figure 27. Time of onset of guanidine-sensitive process in poliovirus multiplication determined with the aid of choline. Input multiplicity was 180 PFU/cell. Guanidine was added at 1.5 hours after infection in Part A and at 1.0 hour after infection in Part B. Choline was added to both sets of cultures at 1.5 hours after infection.

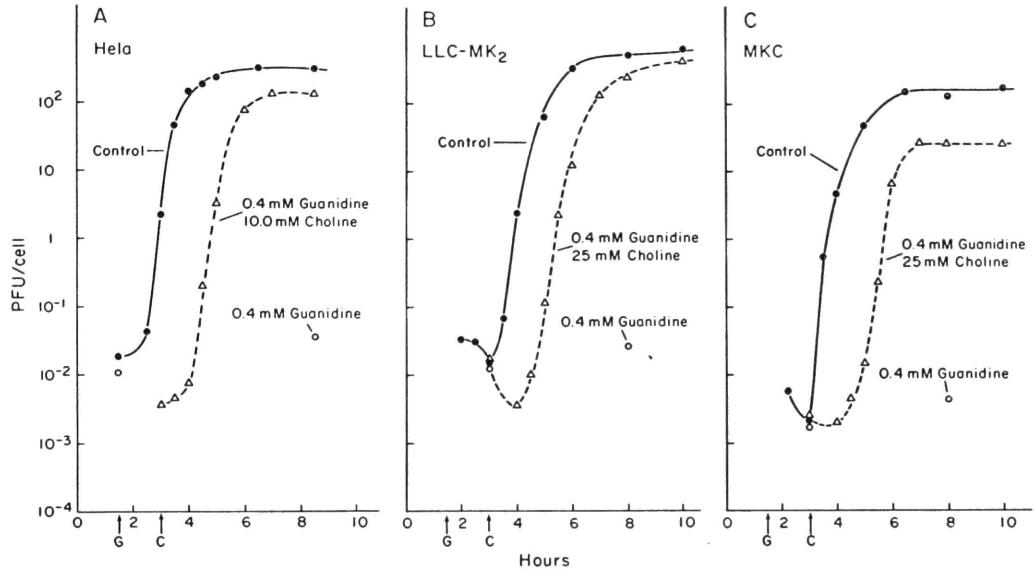

Figure 28. Kinetics of poliovirus multiplication in HeLa cells, LLC-MK_2 cells and primary cultures of rhesus monkey kidney cells after reversion of guanidine inhibition of poliovirus multiplication. Input multiplicities were as follows: HeLa cells, 110 PFU/cell; LLC-MK_2 cells, 30 PFU/cell; MKC, 130 PFU/cell. Guanidine and choline were added at 1.5 and 3.0 hours, respectively; control cultures received PBS at 1.5 hours after infection.

Dimethylethanolamine (DME) as a Guanidine Antagonist

Philipson et al. (1966) noted that, on a molar basis, DME was more active than choline in blocking the inhibitory activity of guanidine in poliovirus type 1-infected HeLa cells. We investigated the kinetics of poliovirus type 2 multiplication in HeLa cells treated simultaneously with 0.4 mM guanidine and either 0.1 mM choline or 0.1 mM DME. For comparison, the results obtained with 0.1 mM choline are shown again in Figure 29. Even though the virus multiplied, the rate of increase was slow. In contrast, almost complete blocking of guanidine action was achieved with 0.1 mM DME. The rates of virus production in control and guanidine plus DME-treated cultures were indistinguishable, but there was a short delay before the onset of multiplication. A somewhat shorter, but still detectable delay was observed when higher concentrations of DME such as 10 mM were used. We have also observed a delay in onset of replication in poliovirus-infected LLC-MK_2 cells treated simultaneously with 0.4 mM guanidine and 0.1 mM DME. This might be due to a slower entry of DME into the cell or some preliminary biochemical transformation required before DME can act. Philipson et al. (1966) noted that KB cells, a fibroblastic human cell line, are unable to utilize DME as effectively as choline in reversing the inhibitory action of guanidine. It is possible that different cell types possess varying capacities to make DME available in the form needed to reverse guanidine inhibition.

Synergism Between Different Guanidine Antagonists

Philipson et al. (1966) investigated the ability of DME to act synergistically with certain amino acids in blocking the poliovirus-inhibitory activity of guanidine, and obtained evidence for such action. We have done similar experiments with choline and methionine. Figure 30 demonstrates the synergistic action of choline and methionine in reversing the poliovirus-inhibitory activity of 1.0 mM guanidine in HeLa cells. However, we were unable to demonstrate synergism between choline and DME in poliovirus-infected cells treated with 0.6 mM guanidine (Figure 31).

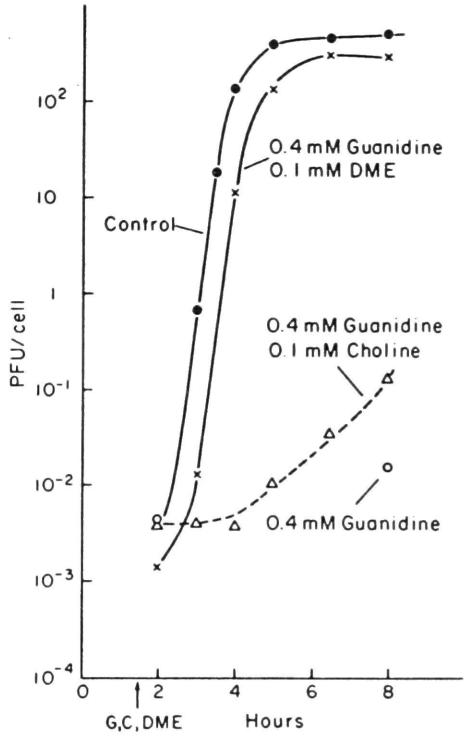

Figure 29. Kinetics of poliovirus multiplication in HeLa cells after blocking the inhibitory effect of guanidine with choline or dimethylethanolamine (DME) at low concentrations. Input multiplicity was 130 PFU/cell. At 1.5 hours after infection guanidine and choline or DME were added while control cultures received PBS.

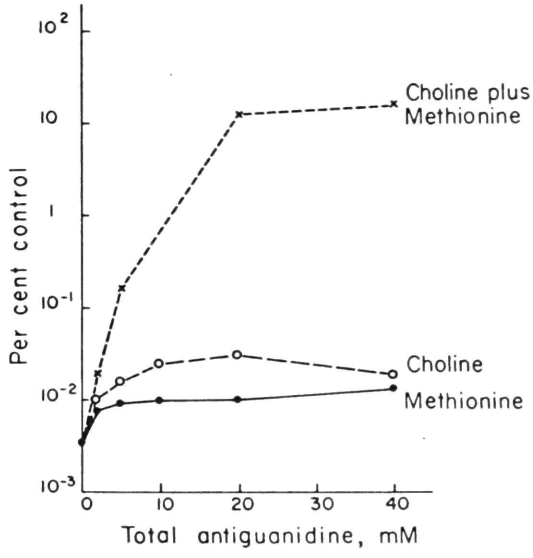

Figure 30. Synergism between methionine and choline in blocking the virus-inhibitory action of guanidine in HeLa cells. Input multiplicity was 65 PFU/cell. At 1.5 hours after infection, guanidine was added to a final concentration of 1.0 mM. At 2.0 hours after infection, choline and methionine were added to give the final concentrations shown. In the mixtures of choline and methionine, these compounds were used in equal concentrations.

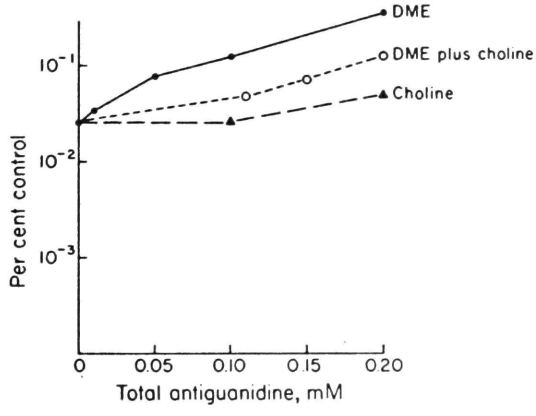

Figure 31. Lack of synergism between choline and dimethylethanolamine (DME) in blocking the virus-inhibitory action of guanidine in HeLa cells. Input multiplicity was 30 PFU/cell. At 1.5 hours after infection guanidine was added to a final concentration of 0.6 mM, and choline and DME were added to give the concentrations indicated. Where choline and DME were added to the same culture, the choline concentration was always 0.1 mM, whereas DME concentration was 0.01, 0.05, or 0.1 mM.

Other experiments utilizing different concentrations of guanidine, choline and DME also failed to demonstrate a synergistic effect. Dinter and Bengtsson (1964) have shown that the most active guanidine antagonists among the amino acids in lactalbumin hydrolysate do not even show additive activity when used together. These results suggest that guanidine antagonists may be separated into several classes of compounds with similar structure on the basis of their lack of ability to act synergistically with each other.

Action of Guanidine Antagonists in Blocking the Multiplication of Guanidine-dependent Poliovirus in the Presence of Guanidine

If guanidine antagonists block the inhibitory action of guanidine on the guanidine-sensitive virus, then they should also suppress the promoting effect of guanidine on a guanidine-dependent strain, unless guanidine acts at entirely different receptor sites in the two situations. Both Lwoff and Lwoff (1965) and Loddo et al. (1966) found that the poliovirus-supporting action of 0.2 to 0.4 mM guanidine could be blocked by guanidine antagonists. Philipson et al. (1966), using 1.0 mM guanidine and a series of guanidine antagonists, could not detect any effect on the multiplication of poliovirus type 1. However, even high concentrations of guanidine antagonists could not be expected to block measurably the action of 1.0 mM guanidine. We investigated the effect of several guanidine antagonists on the replication of guanidine-dependent poliovirus type 1 in HeLa cells in the presence of 0.4 mM guanidine. Our results, shown in Table VI, indicate that guanidine antagonists are very effective in blocking the promoting effect of guanidine on the growth of guanidine-dependent mutants.

Choline as an Antiguanidine - Possible Modes of Action

Several hypotheses have been proposed for the mechanism of action of guanidine antagonists. Most of the compounds with known activity are capable of donating either a methyl or ethyl group, and therefore a methylation mechanism was considered by several investigators. Both Lwoff (1965) and Philipson and co-workers (1966) tested the hypothesis

Table VI

Inhibition of Guanidine-dependent Poliovirus Type 1 Replication in the Presence of 0.4 mM Guanidine

Guanidine, mM[a]	Guanidine Antagonist[a]	Virus yield PFU/cell[b]	% of control
0	0	1.3	1.00
0.4	0	130.0	100.
0.4	0.1 mM choline	65.2	50.
0.4	10.0 mM choline	2.3	1.75
0.4	0.1 mM DME	5.4	4.08
0.4	10.0 mM DME	2.3	1.92
0.4	10.0 mM methionine	9.0	6.92

[a] Added at 1.5 hours after infection.

[b] Harvested at 8 hours after infection.

that choline or methionine inactivates guanidine by participating in its methylation. Methylguanidine is indeed very much less active in inhibiting poliovirus multiplication than is guanidine, but neither investigator could obtain evidence for the production of methylguanidine in infected cells treated with guanidine and ^{3}H-choline labeled in the methyl groups. Philipson and co-workers (1966) also reported that folic acid analogs, which inhibit dihydrofolate reductase, an enzyme used in de novo synthesis of methyl groups, had no effect on the blocking of guanidine action. Loddo and co-workers (1966) noted that some of the active antiguanidine compounds, such as trimethylamine, are not known to be methyl donors. At the same time, betaine, a very active methyl donor, is completely inactive as a guanidine antagonist. They therefore concluded that, if methylation or ethylation was involved in the blocking of guanidine inhibition, it must not involve a known biological methylating system.

Another hypothesis for the mechanism of action of choline is that it promotes the exit of guanidine from the cell. To test this hypothesis the following experiment was performed. HeLa cells were grown in monolayers in 60 mm tissue culture dishes containing 3 coverslips each. Cells were incubated for 4 hours in the presence of 0.01 mM ^{14}C-guanidine, 0.8 μCi/plate, washed twice with warm PBS, and 2.0 ml MEM or MEM containing either 10 mM choline or 1.0 mM unlabeled guanidine was added. After incubating for various lengths of time, the coverslips were harvested by dipping in a beaker containing warm PBS, drained by touching one edge to a paper towel and dried in coverslip racks. The radioactivity was determined by counting in glass scintillation vials in toluene-Liquifluor scintillator as described in Materials and Methods. As shown in Figure 32, after removal of labeled guanidine from the culture fluids, cells incubated in unlabeled guanidine lost their labeled guanidine somewhat faster than cells incubated in MEM alone, but cells incubated in choline retained the label for a longer period of time.

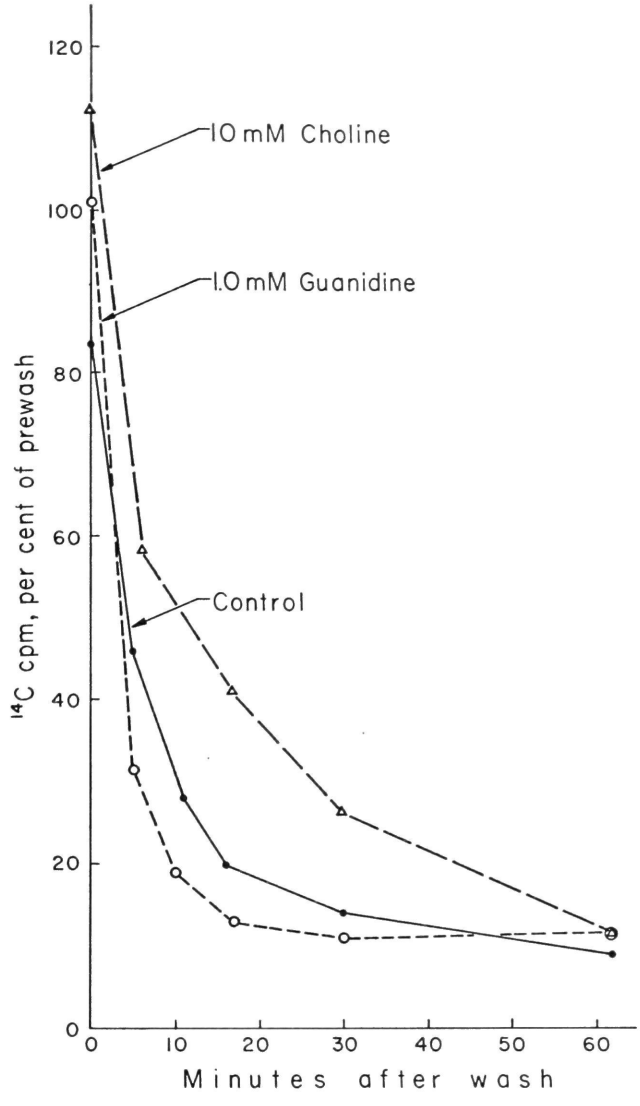

Figure 32. Rate of exit of ^{14}C-guanidine from HeLa cells incubated with or without choline. Each determination represents the mean of ^{14}C radioactivity from 6 coverslips. Prewash values were determined by removing coverslips from tissue culture dishes still containing ^{14}C-guanidine after 4.0 hours of incubation. They were dipped in warm PBS, drained and dried in the same manner as experimental coverslips.

Discussion

The results presented in this chapter indicate that there are significant differences in the sensitivity of poliovirus biosynthesis to inhibition by guanidine in various cell types. Of the three cell types examined, sensitivity increases in the order: HeLa cells, LLC-MK_2 cells, and primary rhesus monkey kidney cells. More choline is required to produce an equivalent degree of blocking or reversal in cells more strongly inhibited by guanidine.

Kinetic experiments with all three cell types show that choline at high concentrations is capable of blocking the action of simultaneously added 0.4 mM guanidine without measurable delay. The rate of virus multiplication in such cultures is indistinguishable from that in control cultures which had received neither guanidine nor choline.

When choline is added after guanidine, measurable virus production begins after a delay that depends on the time in the growth cycle when guanidine was added and on the kinetics of virus multiplication in a particular cell type. These two factors determine the extent to which virus biosynthetic events have progressed before guanidine addition halts viral RNA synthesis. When choline is added to such cultures, the delay observed is that which would be expected if choline released the guanidine inhibition immediately. The kinetics of virus multiplication after choline treatment of guanidine-inhibited cultures correspond closely to the time course of virus increase observed after washing guanidine out of cells (Eggers et al., 1965). The rapidity of the antiguanidine action of choline is striking and places restraints on any hypothesis on its mode of action.

The activity of choline cannot be explained by the hypothesis that it promotes the exit of guanidine from the cell, since experiments on the rate of exit of ^{14}C-guanidine from HeLa cells have shown that choline actually delays somewhat the exit of guanidine from cells. Choline could prolong the cellular retention of guanidine in several ways by acting either at the plasma membrane or intracellularly, but we have no information on the mechanism involved.

Our experiments with guanidine-dependent poliovirus indicate that guanidine antagonists block the poliovirus-supporting activity of guanidine, as well as its inhibitory activity. This confirms earlier studies by Lwoff and Lwoff (1965) and by Loddo et al. (1966). The fact that Philipson et al. (1966) could not demonstrate an effect of guanidine antagonists on the multiplication of guanidine-dependent poliovirus in the presence of 1.0 mM guanidine may be explained by the relatively high concentration of guanidine used.

Results of experiments utilizing pairs of antiguanidine compounds simultaneously suggest that these compounds fall into several classes. Amino acids apparently form one such class on the basis that they are incapable of synergistic action with one another (Dinter and Bengtsson, 1964), but can act synergistically with choline or DME (Philipson et al., 1966; see Figure 30). Our experiments indicate that choline and DME, which are closely related structurally, may be members of another class, since they also are incapable of synergistic action with each other. Taken together, these observations suggest that amino acids and compounds structurally related to choline act at different sites in reversing guanidine action.

6. GENERAL DISCUSSION

Chapter 6

General Discussion

The studies described in this dissertation were undertaken in an effort to further the understanding of the biochemistry of poliovirus replication. We have demonstrated a progressive increase in the smooth membranes with which viral RNA synthesis is associated; these membranes probably correspond to the rapidly proliferating smooth membrane-bounded cisternae seen by electron microscopy of infected cells. The accumulation continues at late times after infection, when cellular nucleic acid and protein syntheses have declined markedly, and viral RNA synthesis has also declined. Viral protein products have accumulated by this time. Guanidine, which stops viral RNA synthesis, has been shown by Dales and co-workers (1965) to prevent the proliferation of the small cisternae; we have shown that guanidine prevents the great increase in membranes in Fraction 2. Yet under these same conditions of infection, guanidine does not abolish the increased incorporation of choline into cellular membranes. Thus, although increased incorporation of precursors into membranes, and the accumulation of smooth microsomal membranes are undoubtedly associated in the normal course of poliovirus infection, they are not inseparably linked to each other.

Production of Cellular Membranes

Membrane components have been shown to be synthesized and inserted into membranes at independent rates. In rats, microsomal membrane synthesis accelerates in the fetal liver at the end of gestation, while microsomal enzyme activities are increased later, in the early postnatal period (Dallner et al., 1965a,b). The rates of increase in activities of different enzymes also vary (Dallner et al., 1966b). The turnover rates of membrane lipids and proteins in young rats have been found to differ. Different rates have been found for fatty acids and for the glycerol backbone of phospholipids, and for two different enzymes (Omura et al., 1967). These data are consistent with the hypothesis of Dallner, Siekevitz and Palade (Dallner et al., 1966b) that membranes are

synthesized in a multistep assembly process. They are dynamic structures, constantly being renewed and modified to fit the cell's needs.

This implies independent control mechanisms for the production of membrane constituents. There is evidence that in some bacteria the control processes are not tightly coupled. In Bacillus subtilis mutants which require glycerol for the synthesis of phospholipids, proteins may be synthesized and incorporated into the membranes of the bacteria at nearly normal rates without net synthesis of phospholipids. This leads to a diminution of the phospholipid/protein ratio and an increase in the buoyant density of the membranes (Mindich, 1970a,b). It was also shown that cells treated with chloramphenicol and supplied with exogenous glycerol produced membranes with a lipid content 15-30% higher than membranes from untreated cells. However, in this case, the incorporation of labeled glycerol into membranes stopped within an hour after administration of chloramphenicol, suggesting that some protein synthesis is necessary for continued synthesis of lipids. There is suggestive evidence that this may also be true in mammals. Tzur and Shapiro (1964) found that in isolated microsomal preparations from rat liver, synthesis of lipids from L-α-glycerophosphate-1-C^{14} was greatly stimulated by addition of protein or glycoprotein to the incubation mixture. They postulated that lipid synthesis might be limited by the amount of acceptor protein available. Schiefer (1969) demonstrated inhibition of incorporation of choline into rat liver membranes after administration of cycloheximide to the intact animal.

Synthesis of Membrane Components in Picornavirus-infected Cells

The synthesis and incorporation of membrane phospholipids are accelerated in picornavirus-infected cells at a time when cellular protein synthesis is greatly diminished. In fact, L cells which have been infected with the large plaque variant of mengovirus continue to incorporate labeled choline into membranes at an enhanced rate compared to uninfected cells at a time when 60 to 80% of the cells are undergoing cytopathic degeneration as measured by staining with 0.05% erythrocin (Amako and

Dales, 1967a,b). Thus, picornavirus-infected cells provide another example of independent control of the synthesis of membrane constituents. A virus product apparently triggers the synthesis of membrane phospholipids, and many of these become components of the smooth-walled vesicles with which viral RNA synthesis is associated. Not all of the components of the smooth membranes in infected cells are, however, newly synthesized. Their phospholipids derive both from preexisting and newly synthesized molecules (Amako and Dales, 1967b). There is probably a preexisting pool of cellular membrane proteins which are incorporated into membranes after picornavirus infection, since synthesis of new proteins from cellular messenger RNA is greatly reduced. That the smooth membranes of Fraction 2 contain cellular proteins has been demonstrated. If cells are labeled with ^{3}H-amino acids prior to infection, label sediments with the membranes of this fraction, and polyacrylamide gel electrophoresis reveals many peptide peaks (Caliguiri, unpublished observations).

There is evidence, however, that most of the smooth membranes in Fraction 2 of infected cells represent a structure found in significant quantities only in infected cells. Very little membranous material from uninfected cells is found at the density of these smooth membranes and the phospholipid/protein ratio is lower than that of the equivalent fraction from infected cells. The phospholipid content of membranes in Fraction 2 of infected and uninfected cells differs (See Table II, Chapter 3). Some viral proteins are associated with these membranes of infected cells. When actinomycin D-treated cells are labeled for 1.5 hours with ^{35}S-methionine or ^{3}H-amino acids after infection, only a small percentage of the incorporated label sediments in this fraction, but the label which is found here is quite firmly bound. A significant amount of label remains with the membranes when they are pelleted and recentrifuged in a continuous sucrose gradient (Caliguiri and Mosser, submitted for publication). The major labeled proteins in this fraction migrate with the major capsid proteins of poliovirus in polyacrylamide gels. The precursor protein (VPO) which is cleaved to form VP2 and VP4 is also found in this fraction as are some larger proteins which probably

represent other primary and secondary products of poliovirus protein synthesis. The electrophoresis pattern of protein is consistent with the hypothesis that all newly labeled proteins are viral in origin. Finally, there is associated with the smooth membranes from Fraction 2 of infected cells a new enzyme activity, the viral RNA polymerase.

Our results are compatible with the hypothesis that membrane components are synthesized and assembled in the rough endoplasmic reticulum, and that the components of the smooth endoplasmic reticulum are derived from rough surfaced membranes (Dallner et al., 1965a,b; 1966a; Orrenius, 1965; Orrenius and Erickson, 1966). We have found that very short pulses of ^{3}H-choline (e.g. 3 minutes) label predominantly the pellicle fraction whose major constituent is rough microsomes. After longer periods of labeling, such as 30 minutes, a substantial amount of radioactivity is found in the smooth membrane fraction of infected cells which contains proliferating cisternae.

Pulse-chase studies with glycerol-2-^{3}H have confirmed that radioactivity incorporated into the rough endoplasmic reticulum can be chased into the smooth membranes in infected cells, suggesting a precursor-product relationship.

Three hypotheses may be considered to explain the increased incorporation of ^{3}H-choline into phospholipids after picornavirus infection: (1) a decrease in the size of the intracellular pool of choline in soluble form, (2) an increased rate of turnover without change in net phospholipid synthesis, and (3) an increased rate of synthesis without a concomitant increase in the rate of breakdown.

Pool size changes have not been ruled out, but we consider such changes unlikely to explain all the results. In considering hypotheses 2 and 3, the available results suggest that the rate of phospholipid synthesis must exceed the rate of degradation, since the phospholipid content of infected cells (Amako and Dales, 1967b) and the phospholipid/protein ratio of infected cells (See Chapter 3, Table I, totals) increase as a result of infection. If the last hypothesis is true,

then infected cells either utilize preexisting cellular proteins and/or viral proteins for formation of membranes, or they make relatively lipid-rich membranes. There is evidence that the last alternative is correct to some extent. The phospholipid/protein ratio of the total of the fractions from infected cells increases with time after infection. Most likely, the proteins from preexisting cellular pools are also being inserted into these membranes but the rate is lower than the rate of insertion of phospholipids.

Blocking of Guanidine Action by Choline

There is good evidence that exogenous choline is utilized rapidly and efficiently by cells in making membranes (Plagemann, 1968). Infected cells incorporate labeled choline at an even faster rate for the same purpose. Since most of the guanidine blockers could also be used in the synthesis of membranes, do they block guanidine action in this way? For a variety of reasons we consider that this is probably not the mode of action of these compounds.

First, the inhibitory action of guanidine can be blocked extremely rapidly. Choline blocks or reverses guanidine inhibition without a measurable delay. The kinetics of virus multiplication after reversal of guanidine inhibition with dimethylethanolamine show a slight but significant delay. This may indicate that some sort of biochemical change must be made in the molecule before it is active or that dimethylethanolamine enters the cell more slowly than choline, but it does not necessarily point towards incorporation in a cellular structure.

Second, guanidine blockers, which enable the replication of drug-sensitive virus in the presence of guanidine, prevent the replication of drug-dependent virus under such conditions. This result is not compatible with a view that the blockers act by promoting synthesis of cellular membranes essential for poliovirus multiplication.

Finally, if we concentrate on choline, there are some apparent paradoxes in the concentrations we find to be active in blocking the inhibitory action of guanidine, and quantities known to be present in

the cell or taken up from the medium. Almost all intracellular choline is found in two locations; in a soluble pool which does not exchange readily with the extracellular medium (Amako and Dales, 1967b; Plagemann, 1968), or incorporated into cellular membranes. Plagemann (1968) found that almost all of the soluble choline pool in Novikoff rat hepatoma cells was in the form of phosphorylcholine. This is an intermediate in the formation of phosphatidylcholine, which is incorporated into membranes (Kennedy, 1957). We estimate from preliminary data that the total concentration of choline in all acid-soluble forms is probably on the order of 1.0 mM or higher. It is noteworthy that 1.0 mM choline, added to poliovirus-infected cells treated with 0.4 mM guanidine is capable of reversing to a large extent the inhibitory effect of guanidine (See Chapter 5, Figure 24). This suggests that added choline is needed in an intracellular state different from that of choline which makes up the bulk of the soluble pool. It is possible that choline is needed in an unphosphorylated state.

The problem of choline activity is further complicated by the fact that, in uninfected Novikoff rat hepatoma cells (Plagemann, 1968) or L cells (Amako and Dales, 1967b) a concentration of 0.01 to 0.06 mM choline is saturating for uptake into the cells over a period of one hour. The concentrations required for marked blocking of guanidine action generally exceed this level, yet choline added at adequate concentrations is active within a very short time interval. It is possible that choline uptake varies widely with the cell strain used and is faster in all three cell types used in our studies, but this is unlikely. Or, perhaps at very high choline concentrations a large amount of choline enters cells by simple diffusion.

If choline blocked the inhibitory activity of guanidine by participating in the synthesis of new membranes, we might expect to see a further increase in choline incorporation after treatment of infected cells with guanidine. This has not been observed in the present experiments (See Chapter 4).

The possibilities that the action of guanidine is blocked via a methylation or ethylation reaction, or that choline ejects guanidine from the cell were discussed earlier (Chapter 5), and are considered unlikely.

The available evidence is more in accord with the possibility that both guanidine and guanidine antagonists act by binding to a viral or cellular component which is important in viral RNA replication. This might be the viral polymerase or some cellular cofactor or membrane site necessary for its proper functioning. The interactions of these compounds with viral or cellular components have the characteristics of weak bonds. Guanidine is very difficult to localize within the cell. It can be washed out of cells effectively merely by changing the medium bathing the cells. We have attempted to demonstrate the binding of labeled guanidine to viral or cellular components by equilibrium dialysis without success. Since it is a charged molecule, it is likely that guanidine forms transient associations at many intracellular sites, and perhaps only a few of these sites are involved in poliovirus RNA synthesis.

It is tempting to speculate that guanidine affects poliovirus RNA synthesis by binding to the smooth membranes with which this process is associated. The differing sensitivities to guanidine in different cell types indicate that a cellular component is involved in some way, and we can easily envision the positively charged guanidine molecules interacting with the negatively charged membrane phospholipids. There is evidence that another strongly negatively charged molecule involved in viral RNA synthesis, the viral nucleic acid, is not the site with which guanidine interacts. The genome of a guanidine-sensitive virus may be replicated and therefore rescued from cells by the RNA polymerase of a guanidine-resistant mutant (Agol and Shirman, 1964; Cords and Holland, 1964; Ikegami et al., 1964; Wecker and Lederhilger, 1964).

Choline and related compounds could block or reverse the action of guanidine by similar associations with a receptor site or sites. Molecular dissimilarity between choline-like guanidine antagonists and guanidine make it improbable that the antagonists and guanidine have identical

receptor sites. Also, the phenomenon of synergism between classes of guanidine antagonists leads us to postulate separate sites for the action of different groups of guanidine antagonists. We speculate that the binding of a guanidine antagonist at particular intracellular receptors contiguous to the critical guanidine binding sites alters the micro-environment and thereby displaces guanidine from its site of action.

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