EFFECT OF POKEWEED ANTIVIRAL PROTEIN IN COMBINATION WITH GUANIDINE ON POLIOVIRUS MACROMOLECULAR SYNTHESIS

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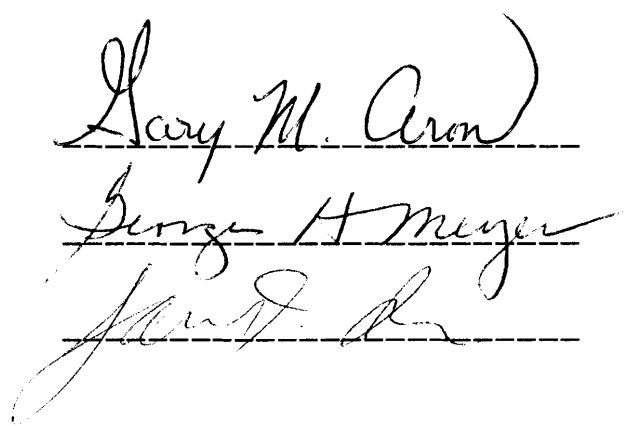

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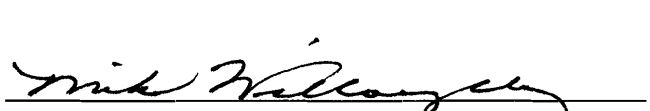

DEAN OF THE GRADUATE SCHOOL

EFFECT OF POKEWEED ANTIVIRAL PROTEIN IN COMBINATION WITH GUANIDINE ON POLIOVIRUS MACROMOLECULAR SYNTHESIS

THESIS

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INTRODUCTION

Poliovirus, a member of the *Picornaviridae*, has a plus-strand RNA genome, approximately 7,500 nucleotides in length, with a small viral protein, VPg, covalently attached to the 5′ end and a genetically encoded poly(A) tail at the 3′ end [23]. Poliovirion RNA has a molecular weight (Mw) of 2.5 x 10^6 and replicates in the cytoplasm of infected cells. Virus replication requires a virus-specific RNA-dependent RNA polymerase (3D, Table 1) that is not present in uninfected cells. The primary site of RNA replication in infected cells is a membrane-bound replication complex which is composed of a single, minus-strand RNA, several nascent chains of plus-strand RNA, and the viral RNA polymerase [12].

Poliovirus contains a plus-strand RNA but does not carry an RNA polymerase. Formation of polymerase(s) is a prerequisite for the synthesis of viral progeny RNA. Therefore, the parental RNA which encodes for the polymerase has to first function as mRNA. Replication of the viral RNA then proceeds in two distinct steps. First, the parental RNA serves as a template for the synthesis of minus-strand RNA, then the minus-strand serves as a template for the synthesis of new plus-strand RNA. This newly formed plus-strand RNA has three potential fates: (i) it can act as template for the synthesis of additional minus-strand RNAs, (ii) it may function as mRNA, or (iii) it may become incorporated into progeny virus particles [25].

Double stranded (RF-RNA) and partially double stranded (RI-RNA) viral RNAs have been isolated from poliovirus infected cells. These have

Table 1. Poliovirus proteins [a].

Protein	Sequence	Mw (x 10^3)	Function
NCVPOO	741-7361	247.5	Genomic translate
P1	744-3380	97.2	Capsid region &their precursors
VPO	744-1760	37.3	VP2 & VP4 precursor
VP4	744-947	7.3	Capsid protein
VP2	948-1760	29.9	Capsid protein
VP3	1761-2474	26.4	Capsid protein
VP1	2475-3380	33.5	Capsid protein
P2	3381-5105	64.9	Non-capsid central region
2A	3381-3832	16.7	Cleavage of NCVPOO into P1, P2, and P3; host shut-off
2BC	3833-5105	48	Unknown; viral RNA synthesis?
2B	3833-4123	10.7	Unknown; viral RNA synthesis?
2C	4124-5105	38	guanidine resistance
P3	5106-7361	84.2	Non-capsid region
3A	5107-5332	14	Precursor?
3AB	5291-5332	12	VPg carrier to initiate plus-strand RNA synthesis
3B	5332-5432	2.3	Genomic protein(VPg)
3CD	5433-7361	72.1	Proteinase cleavage of P1 into VP0, VP1, & VP3
3C	5433-5978	19.6	Proteinase cleavage of non-structural proteins
3D	5979-7361	52.4	RNA dependent RNA polymerase
3C′	5433-6430	36.4	Proteinase
3D′	6431-7361	35.6	?

[a] References [24, 25, 42].

been implicated as intermediates in the replication of viral RNA. RF-RNA is thought to be the product of the first step in viral RNA synthesis. Synthesis of minus-strand RNA is initiated using the parental RNA as template, and a double stranded RF-RNA molecule is formed. Plus-strand RNA synthesis is then initiated repeatedly on the minus-strand of the RF-RNA, which yields RI-RNA [25].

A stepwise model for the initiation of viral RNA synthesis has been difficult to determine. Two possible mechanisms for the initiation of nascent RNA chains have been proposed. One mechanism, based on *in vitro* synthesis of minus-strand RNAs, involves a template priming step that requires a "host factor" to act as a terminal uridylyltransferase which adds a small number of uridylate residues to the 3′ terminal poly(A) of virion plus-strand RNA. A poly(A)-oligo(U) hairpin then forms and acts as a primer for the elongation activity of the RNA-dependent RNA polymerase (3D, Table 1). A second mechanism for initiation of poliovirus RNA synthesis has been proposed in which either VPg, a polypeptide precursor to VPg, or a uridylated derivative of VPg acts as a primer for the viral RNA polymerase [14].

Nucleic acid sequencing of poliovirus RNA has revealed an open reading frame of 2,207 consecutive triplets which spans over 89% of the entire viral genome. Poliovirus RNA has the coding capacity for a polypeptide of 247,000 Mw. The length of the reading frame is strong evidence that poliovirus RNA codes for a polyprotein which represents the entire translatable information of the poliovirus genome. Further support

for a polyprotein is: (i) no other open reading frame longer than 78 triplets exists within the sequence, and (ii) all major viral polypeptides map in phase with the long reading frame. Thus, all of the known poliovirus proteins can originate by proteolytic cleavage from a single precursor polypeptide which has been designated NCVPOO [18, 19, 24]. Capsid proteins are located in the amino-terminal section (designated P1) of NCVPOO [17, 35]. Non-structural proteins are located in the central portion (designated P2) and 3′ portion (designated P3) of NCVPOO [50].

During poliovirus replication, the P1 section of NCVPOO is separated from the polyprotein during translation through a *cis* cleavage event catalyzed by a proteinase (2A, Table 1) which is located at the amino terminus of NCVPOO. After translation of the entire polyprotein reading frame, another proteinase (3CD, Table 1) is autocatalytically separated from the carboxyl end of the polyprotein. Capsid proteins VP0 (precursor to VP2 and VP4 capsid proteins) , VP3, and VP1 (Table 1) are then released from P1 by a proteinase-mediated cleavage (3CD). An autocatalytic cleavage most likely cleaves VP0 which yields VP2 and VP4. Cleavage is almost certainly occurring in completely assembled virions and it is difficult to imagine how a protease could access internalized capsid residues of VP0 [17, 18, 19, 27, 50].

The P2 central region of the genome is translated to form several proteins including a proteinase designated 2A and a protein responsible for guanidine resistance designated 2C [19, 37]. The P3 region of the genome is also translated to form several proteins. The most well understood of

the P3 proteins are two proteinases designated 3CD and 3C, the RNA-dependent RNA polymerase designated 3D, and the VPg carrier to initiate plus-strand RNA synthesis designated 3AB [14, 18, 19, 27, 50]. The poliovirus proteins are summarized in Table 1.

Poliovirus protein synthesis is similar to cellular protein synthesis with a few modifications. A "cap" structure, m^7GpppX (where X = any nucleotide), is present at the 5′ end of all eukaryotic cytoplasmic (nonorganelle) mRNAs and most eukaryotic viral mRNAs. Picornaviral and some plant mRNAs are notable exceptions to the cap structure [32, 43]. Eukaryotic initiation is a complex pattern that involves at least nine initiation factors. In general, elongation factor (eIF) 4A is a multimer that includes CPB (the cap binding protein) which recognizes the "cap" at the 5′ end of mRNA and unwinds any secondary structure that may exist in the first 15 bases of the mRNA. Unwinding of structure farther along the mRNA is accomplished by eIF 4A and eIF 4B. At some stage during this process, the 40S subunit and other initiation factors bind together [32].

Eukaryotic initiation proceeds through the formation of a ternary complex containing Met-$tRNA_i$, eIF 2 and GTP. The ternary complex associates directly with free 40S subunits. Binding of the 40S-ternary complex to mRNA depends on eIF 3 (as well as eIF 4F, 4A, and 4B). Junction of the 60S subunits with the initiation complex cannot occur until eIF 2 and eIF 3 have been released from the initiation complex, a function mediated by eIF 5. The 40S-60S joining reaction may also directly depend

on eIF 4C. Probably all of the remaining factors are released when the complete 80S ribosome is formed which leads to translation [32].

Within 30 to 60 m following the infection of cells by poliovirus, host protein synthesis is so abruptly and dramatically reduced, that this phenomenon has been termed the shut-off [25]. In poliovirus-infected cells, an early shut-off of host protein synthesis correlates with the cleavage of the p220 component of eukaryotic translation initiation factor 4F (eIF 4F). This is accomplished in conjunction with a poliovirus proteinase (2A, Table 1) and an undetermined cellular factor [18, 27, 44, 49]. The complex eIF 4F consists of three polypeptides, which include the p25 cap binding protein, p220 and eIF 4A. Although the biochemical function of the p220 subunit in the complex is not known, eIF 4F binds the 7-methylguanosine 5′-triphosphate cap group on mRNA and appears to mediate its binding to the 40S ribosomal subunit. Cleavage of p220 is thought to inactivate this function, thus inhibiting the translation of capped mRNA. The 5′ end of poliovirus RNA is uncapped, and translation is initiated by a cap-independent mechanism which does not require the eIF 4F cap-recognition system [49].

Viral infections are estimated to be responsible for more than 60% of the illnesses in developed countries, compared to only 15% which result from bacterial infections [40]. With the wide application of killed viral vaccines in the USA (1955) and of live viral vaccines in the USSR (1959), the incidence of viral infection has drastically declined. Although vaccination is the most effective protection against viral infection, vaccines

have yet to be developed against many viral diseases. Moreover, viral vaccines available today do not provide 100% immunization [40]. Consequently, there is a need for efficacious antiviral drugs.

The problems associated with the use of chemotherapeutic antiviral agents for the treatment of infectious disease include toxicity and the emergence of drug resistant strains. The use of two or more chemotherapeutic agents is known as combinational drug therapy. Combinational drug therapy has the advantages of reducing drug toxicity, as well as decreasing the number of drug resistance virus [8].

In this study, the effect of the pokeweed antiviral protein (PAP) in combination with guanidine was determined on poliovirus macromolecular synthesis. PAP is a broad spectrum antiviral agent effective against both RNA-containing viruses (e.g. influenza and poliovirus) and DNA-containing viruses (e.g. herpes simplex virus) [2, 21]. The antiviral activity of PAP is believed to be associated with its ability to inactivate eukaryotic ribosomes [21] and is classified as a ribosomal inactivating protein (RIP).

Ribosomal inactivating proteins (RIP) are grouped in two classes. Proteins belonging to the first class, such as ricin, consist of two identical disulfide bonded A and B subunits [11]. The proteins in this class are highly potent inhibitors of protein synthesis in cell-free systems [21], and are highly cytotoxic [11, 21]. Proteins which belong to the second class, such as PAP, consist of a single polypeptide chain of Mw of ~ 30K [11].

Proteins in this second class are also highly potent inhibitors of protein synthesis in cell-free systems [21], but are relatively non-cytotoxic [1]. *Trichosanthes* anti-HIV protein (Mw ~ 29K), also known as TAP 29, belongs to this second class of single-chain ribosome-inactivating proteins (SCRIP) [31]. MAP 30 (*Mirabilis* antiviral protein), is another SCRIP which shows negligible cytotoxicity [31].

The main effect of PAP on eukaryotic ribosomes is the inhibition of protein synthesis. This is due to the inhibition of translocation mediation of elongation factor 2 (elF-2) [13]. PAP is one of many RIP's whose mechanism of action is the removal of a single adenine residue from a conserved base sequence in 28S rRNA [11]. *In vivo* , PAP has been shown to inhibit cellular protein synthesis in uninfected cells over prolonged periods of time [1]. The cytotoxic potency of PAP is more pronounced in HeLa cell culture than in Vero cell culture [1].

The effect of PAP on protein synthesis in virus-infected cells is more rapid when compared to its effect on uninfected cells. Studies on the effect of PAP (3μM) in cell culture show little or no inhibition of protein synthesis following a 24 h incubation [1]. However, following infection of cells with either poliovirus or herpes simplex virus, PAP (3μM) rapidly inhibits protein synthesis within 3 h post-infection [2, 47]. Virus incubated with PAP and washed prior to infection showed little inhibition of viral yields which demonstrates that PAP does not interact with the virion [2, 47]. PAP's entry into the cell is dependent, however, on the integrity of

virus capsid proteins which induce permeability of the cell membrane to the antiviral protein [30].

The broad spectrum activity of the pokeweed antiviral protein is limited in clinical use although its cytotoxicity is relatively low. Attempts have been made to decrease the cytotoxic effects of PAP without a loss of antiviral activity. First, it has been suggested that RIP's might be chemically coupled to antibody specific for viral and cancer antigen for localized chemotherapy [21, 22, 36]. Second, synergistic combinations of PAP with other antiviral agents such as guanidine allows for the use of smaller doses of PAP [6].

Guanidine has been the most extensively studied inhibitor of poliovirus replication [25]. Guanidine inhibits the replication of poliovirus at concentrations as little as 1-2 μM [10]. The antiviral effects of guanidine occur at less than a thousand-fold the concentration required to inhibit cellular RNA and protein synthesis [25]. Guanidine does not decrease cellular protein synthesis in HeLa cells at concentrations of 100μM [10]. Other studies indicate that concentrations as high as 10mM guanidine do not inhibit cellular RNA or protein synthesis *in vitro* [25].

Although the mechanism of action of guanidine has been the subject of intensive study, the exact mechanism of its antiviral activity is still not known [25]. Guanidine is thought to inhibit the replication of poliovirus by the following four mechanisms: (i) rapid inhibition of the initiation of RNA synthesis, (ii) inhibition of the release of membrane dependent plus-

strand 35S RNA from the replication complex, (iii) interference with the movement of newly synthesized membranes from their place of formation (the rough endoplasmic reticulum) to smooth viral specific vesicles and, (iv) prevention of the association of procapsids and the poliovirus protein 2C (formerly NCVPX) with the replication complex [25]. Guanidine may also affect the capsid precursor, VP0, which would, in turn, exert an indirect effect on RNA transcription due to a polarity effect. That is, improper folding of the coat precursor in the presence of guanidine may lead to an inactive configuration of the polymerase protein whose translation follows that of the coat precursor [26]. The major antiviral effect of guanidine appears to be blockage of synthesis of viral RNA, particularly the production of single-stranded RNA. The specific site of inhibition appears to be the initiation step of RNA synthesis [37].

Combinational drug interactions have been studied extensively in antibacterial and anticancer research, but has only recently gained popularity in antiviral research. With the advent of combinational drug therapy, the problem of a standard method of measurement arose by which to evaluate the drug interactions. The terms synergy, zero-interaction and antagonism have been used in the literature without agreement on their precise definition [15]. Berenbaum, in a recent review on synergy states that there is no need to define more than three classes of interactions: (i) zero-interaction or summation, the expected effects are a combination of each of the individual dose responses; (ii) synergy, the expected effect is greater than zero-interaction; and (iii) antagonism, the expected effect is

less than zero-interaction [5]. Concepts presented in this study are based on these three classes of interactions.

Four methods to calculate zero-interaction are reported to be valid [5, 8]. These include: (i) the fractional product method [48], (ii) the median effect principle [8], (iii) the fractional inhibitory concentration method [16], and (iv) the original isobologram method developed by Loewe [33]. Berenbaum considers the isobologram method by Loewe to be the most valid of the strictly empirical methods [4]. The isobologram is valid for all combinations of agents independent of their mechanisms of action or nature of their dose-response curves [5].

Combinational indices were calculated in this study according to the following formula [8] :

$$(3)\quad CI = (D_1)/(ED_{50})_1 + (D_2)/(ED_{50})_2 = 1$$

where D_1 and D_2 represent the dose of drugs 1 and 2, and $(ED_{50})_{1 \text{ and } 2}$ represent the 50% effective dose of drugs 1 and 2, respectively. The combinational index reveals by inspection whether the interaction of the drugs has a synergistic, antagonistic or summated effect [7]. Synergistic combinations of drugs have an index less than 1, summated drug combinations have an index equal to 1 and the index of antagonistic drug combinations is greater than 1 [7]. This formula can be used for all drugs and their combinations which yield a monotonic dose-response curve [5].

The determination of synergy and antagonism in this study was based on a modification of the median effect principle for the calculation of combinational indices. Combinational indices were calculated on the basis of the percent effective dose using both the observed, and expected effect of the drugs [7, 8]. This method determines zero-interaction for drug combinations irrespective of the individual drugs dose-response curves or mechanisms of action.

This study investigates the effect of synergistic and antagonistic combinations of PAP and guanidine on poliovirus macromolecular synthesis We confirmed reports by others [6] that high combinations of PAP and guanidine are antagonistic; whereas, low combinations are synergistic against the replication of poliovirus. The results suggest that the mechanism of action for synergy is a block in the processing of the capsid precursor protein, P1. In contrast, the antagonistic drug combination causes the complete shut-off of RNA synthesis which leads to an absence of viral protein synthesis.

MATERIALS AND METHODS

Cell cultures. H-HeLa cells were obtained from C. J. Gauntt (U.T. San Antonio, Texas). Cells were grown and maintained in 75 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) at 37°C and 34°C, respectively, in Eagle's minimum essential medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 0.075% $NaHCO_3$, 2mM glutamine, 50U of penicillin, 50 µg of streptomycin and 2.5 µg of fungizone per ml. HeLa cells grown in tissue culture plates (Flow Laboratories, Inc., McLean, VA) were grown at 37°C and maintained at 34°C in MEM supplemented with 10% serum containing 0.225% $NaHCO_3$, 2mM glutamine, 50U of penicillin, 50 µg of streptomycin and 2.5 µg of fungizone per ml in a 5% CO_2 atmosphere.

Virus production and assay. Attenuated poliovirus type 1 was obtained from B. Sagik (The University of Texas at San Antonio, San Antonio, Texas). Three day old HeLa cell monolayers in 75cm^2 flasks which contained ≈ 6 x 10^6 cells were washed 2X with Earle's balanced salt solution (EBSS) and infected with a total of 3.2 x 10^6 PFU's for a multiplicity of infection (MOI) equal to ≈ 2 PFU/cell. The cells were incubated at 37°C with shaking at 10 min intervals. After infection, the cells were incubated at 34°C in 6 ml of MEM which contained 10% serum and 0.225% $NaHCO_3$ until 4+ cytopathic effect (CPE) was observed. Infected cells were disrupted with three freeze-thaw cycles at -80°C and the cell debris removed by centrifugation at 3000 rpm for 15 min at 6°C. The

supernatent containing the virus was stored in 6ml aliquots at -80°C. Virus titer was determined by an agar cell plaque method [9] according to the following protocol. Serial ten-fold dilutions of stock virus suspensions were prepared in EBSS and kept in an ice bath at 4°C. Aliquots which contained 0.2 ml from each dilution were used to inoculate confluent 24 h HeLa cell monolayers in 60 mm tissue culture dishes. For virus adsorption, the infected cells were incubated for 1 h at 37°C in a 5% CO_2 atmosphere with shaking at 10 min intervals. After adsorption, the cells were washed with EBSS and overlayed with 5 ml of MEM containing 1% purified agar (Difco Laboratories, Detroit, MI). After 48 h at 34°C, the cells were stained with 3 ml of a 0.1% solution of neutral red in EBSS. Plaques were counted after an additional 8-24 h incubation period at 34°C.

Antivirals. Pokeweed antiviral protein (PAP) was obtained from James D. Irvin (Southwest Texas State University, San Marcos, Texas). PAP was extracted from the spring leaves of the pokeweed plant *Phytolacca americana* and purified with ammonium sulfate fractionation followed by ion exchange chromatography [20]. PAP was sterilized by filtration through 0.45μM membrane filters (Type HA, Millipore Corp., Bedford, MA) and stored at -20°C. Guanidine was purchased from the Sigma Chemical Co. (St. Louis, MO).

Combinational drug experiments. HeLa cell monolayers in 24-well plates (Flow Laboratories Inc., Hamden, CN) were infected with virus in the presence of either PAP, guanidine, or combinations of the antivirals according to the following protocol. Sterile stock solutions of PAP and

guanidine were prepared in EBSS at four times the final concentration. To prepare solutions which contained combinations of PAP and guanidine, equal volumes of each drug were mixed together and the resulting solution was mixed with an equal volume of virus which contained 1.2 x 10^8 PFU/ml. For solutions which contained either PAP or guanidine alone, four-fold concentrations of the antivirals were mixed with equal volumes of EBSS to yield twice the final drug concentration. Solutions which contained each antiviral were then mixed with an equal volume of virus which contained 1.2 x 10^8 PFU/ml. For infection of cells, 0.1 ml volumes of the antiviral plus virus mixtures were added to cell monolayers which contained 6 x 10^5 cells for an MOI of 10 PFU/cell. For mock infection, 0.1 ml volumes of solutions which contained the antivirals at the final concentration were added to cell monolayers. Thus, antivirals were added simultaneously with virus to cell monolayers. In addition, cells were also pre-incubated in the presence of the appropriate antiviral for 1 h prior to infection. Infected cell monolayers were incubated in the presence of virus at 37°C for 1 h with shaking at 10 min intervals. After virus adsorption, monolayers were washed with EBSS, supplied with 1 ml maintenance medium containing antivirals singly or in combination, and incubated at 34°C. Virus was harvested at 8 h infection p.i. and yields determined by an agar cell plaque method [9].

Combinational drug analysis. A combinational index was calculated on the basis of viral yields from cells infected in the presence of PAP, guanidine or combinations of the antivirals using the following equation:

$$(1) \quad CI = \%I_{D_1}/\%I_{D_{1+2}} + \%I_{D_2}/\%I_{D_{1+2}}$$

where %I is equal to the percent inhibition, D_1 and D_2 represent the individual doses of drugs 1 and 2, and D_{1+2} represents the combination of drug doses 1 and 2. The percent viral inhibition in the CI equation, was an average value obtained from three separate experiments.

The mean percent inhibition for a single dose of antiviral was determined by calculation of the average inhibition produced by that dose in each experiment used. A combinational index prime (CI*) was calculated by dividing the mean value for a single drug dose by the percent inhibition for each drug combination using the following equation:

$$(2) \quad CI^* = \%M_{D_1}/\%I_{D_{1+2}} + \%M_D/\%I_{D_{1+2}}$$

where %M is equal to the percent inhibition of the mean dose.

RNA synthesis. HeLa cell monolayers in 24-well plates were infected with virus (MOI = 100 PFU/cell) in the presence of PAP, guanidine or combinations of the antivirals as previously described. At the indicated times p.i., maintenance medium was removed, duplicate cell monolayers were washed twice with EBSS and MEM which contained 10% serum, 0.225% $NaHCO_3$, 2 mM glutamine and ^{3}H-uridine (5Ci/mol) was added. For the determination of viral RNA synthesis, actinomycin D (2 μg/ml) was added following virus adsorption. Following incorporation at 34°C for 1 h at the indicated times, cell monolayers were washed twice

with EBSS, solubilized with 0.1 N KOH and precipitated with 20% trichloracetic acid (TCA). The precipitates were collected on Millipore filters (Type HA, Millipore Corp., Bedford, MA) and washed with 5% TCA. The filters were oven dried and placed in scintillation counting fluid consisting of toluene and 0.5% 2,5-diphenyloxazole (PPO). Radioactivity was determined by using a Beckman LS-100 liquid scintillation counter.

Total protein synthesis. HeLa cell monolayers in 24-well plates were infected with virus (MOI = 100 PFU/cell) in the presence of PAP, guanidine or combinations of the antivirals as previously described. At the indicated times p.i., maintenance medium was removed, duplicate cell monolayers were washed twice with EBSS and 1ml of Eagle's MEM without leucine (Flow Laboratories, Inc., Mclean, VA) which contained 1% serum, 0.15% $NaHCO_3$, 2 mM glutamine and 0.15 µCi/ml of [^{14}C]-leucine was added to each well. Following incorporation at 34°C for 1 h at the indicated times, cell monolayers were washed twice with EBSS, solubilized with 1 ml of 0.1 N KOH and precipitated with 20% trichloroacetic acid (TCA). The precipitates were collected on Millipore filters (Type HA, Millipore Corp., Bedford, MA) and washed with 5% TCA. The filters were then oven dried and placed in scintillation counting fluid consisting of toluene and 0.5% 2,5-diphenyloxazole (PPO). Radioactivity was determined by using a Beckman LS-100 liquid scintillation counter.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). HeLa cells were pretreated with PAP in combination with

guanidine as previously described. Cells were infected with a MOI of 100 PFU/cell in the presence of the antiviral agents. After virus adsorption, monolayers were supplied with 1 ml of maintenance medium containing antivirals singly or in combination. In addition, cells were infected and maintained in the absence of the antivirals as a virus control. At various times postinfection, treated and non-treated cells were pulsed for 1 h with [^{35}S]-methionine (1μCi/ml, ICN Biomedicals, Inc., Irvine, CA 92715) contained in MEM without methionine (Flow Laboratories, Rockville, MD) in either the presence or absence of the antivirals. Cells were then washed with EBSS and dissolved in 0.5 ml of treatment buffer which contained 0.06 M Tris-hydrochloride (pH 6.8) , 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol. Cell extracts were then sonicated three times for a total of 15 sec with a Biosonik IV sonicator, boiled 2.5 min, and stored at -80°C. Before gel electrophoresis, cell extracts were thawed, heated for 5 min at 90°C, and 0.025 ml of 0.005% bromophenol blue added to each sample.

The method of Laemmli [29] was employed for SDS gel electrophoresis with the following modifications. The running gel contained 12% pro-page solution (Amresco, Solon, OH 44139) and the stacking gel contained 4% gene-page solution (Amresco, Solon, OH 44139). The standards obtained from Diversified Biotech (Newton Centre, MA 02159) used for molecular weight markers were cytochrome C (12.4 K) , beta lactoglobulin (18.4 K) , carbonic anhydrase (29 K) , lactate dehydrogenase (36 K) , ovalalbumin (43 K) , glutamate dehydrogenase (55 K) and phosphorylase b (95.5 K). The standards and cell extracts of 5-10 μl were loaded into separate wells on the gels. Electrophoresis took place

at a constant voltage of 170 volts for 3 h using a SE280C Tall Mighty Small Vertical Slab Unit (Hoefer Scientific Instruments, San Francisco 94107) and a PS500 XT DC power supply (Hoefer Scientific Instruments, San Francisco, CA 94107). The gels were stained overnight with 0.125% Coomassie Blue R-250 in 50% methanol and 10% acetic acid. The gels were destained with 50% methanol and 10% acetic acid for 2 h followed by 10% methanol and 10% acetic acid overnight in a Model 222 Slab Gel Diffusion Destainer (Bio Rad Hoefer Laboratories, San Francisco, CA). Gels were dried with a Dry Gel Jr. (Hoefer Scientific Instruments, San Francisco, CA) for 1 h and then placed in contact with Kodak X-omat AR film (Eastman Kodak Company, Rochester, NY). A Dupont Coronex Lightning Plus intensifying screen (Sigma, St. Louis, MO 63178) was placed over the film to decrease background. The film was exposed to the dried gels for 48-72 h at -80°C.

RESULTS

Poliovirus multiplication. The effect of synergistic and antagonistic drug combinations on the multiplication of poliovirus was determined. The percent inhibition of poliovirus yields (observed ED_X) and zero-interaction values (expected ED_X) are shown in Table 2. Low concentrations of PAP (0.04 and 0.07 μM) in combination with guanidine (9.80, 2.50 and 1.25 μM) yielded combinational indices (CI) less than 1 and were synergistic. The greatest synergistic effect observed (CI of 0.28) was obtained with the lowest concentration of PAP and guanidine used in this study (0.04 and 1.25 μM, respectively). High concentrations of PAP (0.25 and 0.125 μM) in combination with guanidine (35.0, 15.0 and 9.80 μM) were antagonistic yielding combinational indices greater than 1. The greatest antagonistic effect observed (CI of 1.51) was obtained with the highest concentration of PAP and guanidine used in this study (0.25 and 35.0 μM, respectively). Combinational index prime (CI*) values (Table 2) did not change the class of interaction (e.g. synergy, summation or antagonism). This indicates that deviations which occurred in the calculation of zero-interaction values for drug combinations were small. In subsequent experiments, PAP and guanidine were used at concentrations which yielded the greatest antagonistic effect (0.25 PAP and 35.0 μM guanidine) and the greatest synergistic effect (0.04 PAP and 1.25 μM guanidine).

Poliovirus RNA synthesis. To ascertain if synergistic and antagonistic drug combinations inhibited viral RNA synthesis, the ability of

infected cells to incorporate ^{3}H-uridine in the presence of the antiviral agents was determined. The data in Fig. 1 shows the effect of PAP, guanidine and combinations of the antivirals on viral RNA synthesis. The antagonistic drug combination (0.25 μM PAP and 35.0 μM guanidine) completely inhibited the synthesis of viral RNA. High concentrations of PAP (0.25 μM) and guanidine (35.0 μM) alone were also as effective in the inhibition of viral RNA synthesis as was the antagonistic drug combination. In contrast, viral RNA synthesis was not inhibited in the presence of the synergistic drug combination (0.04 μM PAP and 1.25 μM guanidine) or in the presence of either PAP (0.04 μM) alone or guanidine (1.25 μM) alone. The data suggests that the antiviral effect of the synergistic drug combination is not a result of an inhibition of viral RNA synthesis. The inhibition of viral RNA synthesis by the antagonistic drug combination may be due to either an inhibition of protein synthesis by PAP, a block in the processing of specific proteins (e.g. RNA-dependent RNA polymerase) or the inhibition by guanidine of plus-strand RNA synthesis.

Protein synthesis. The effect of synergistic and antagonistic drug combinations on protein synthesis was determined by the ability of infected cells to incorporate ^{14}C-leucine in the presence of the antiviral agents. The data in Fig. 2 shows the effect of PAP, guanidine and combinations of the antivirals on protein synthesis. Protein synthesis was inhibited by 50% and 100% in the presence of PAP alone at both low (0.04 μM) and high (0.25 μM) concentrations, respectively. In contrast, protein synthesis was found to increase by 50% and 100% in the presence of both low (1.25 μM) and high (35.0 μM) concentrations of guanidine, respectively. Although,

protein synthesis in the presence of the synergistic drug combination was similar to that observed with PAP (0.04 μM) alone. protein synthesis was greater in the presence of the antagonistic drug combination when compared to PAP (0.25 μM) alone. No correlation was observed between virus yields (Fig. 2, shown in parenthesis) and the inhibition of protein synthesis. For example, although both PAP (0.04 μM) alone and the antagonistic drug combination inhibited protein synthesis by 50%, virus yields were 96% and 1%, respectively. The data suggests that the synergistic and antagonistic effect of the antivirals may not be due to an overall inhibition of viral protein synthesis. It is possible that the presence of the drug combinations may result in a failure to cleave and/or process poliovirus polyproteins. The increase observed in protein synthesis in the presence of guanidine may simply be due to a failure to shut-off cell protein synthesis. SDS polyacrylamide gel electrophoresis of infected cell extracts was performed to determine if the antivirals had an effect on the processing of poliovirus polyproteins or the shut-off of cell protein synthesis.

Poliovirus protein synthesis. To determine the effect of synergistic and antagonistic drug combinations on the synthesis and processing of specific viral proteins, treated and untreated infected cells were pulsed for 1 h with ^{35}S-methionine at 3, 5, and 7 h p.i. Cell extracts from infected cells pulsed in the presence of the antivirals (Fig. 3) or pulsed in the absence of the antivirals (Fig. 4) were analyzed using SDS polyacrylamide gel electrophoresis.

In the presence of both PAP (0.04 μM) alone at low concentration and guanidine (1.25 μM) alone at low concentration, viral proteins 3AB and 3C were absent. A significant decrease in the amount of viral proteins was observed in the presence of the synergistic drug combination (0.04 μM PAP and 1.25 μM guanidine) when compared to viral proteins detected in the presence of either PAP (0.04 μM) or guanidine (1.25 μM) alone at low concentration. In particular, a reduction in the amount of viral proteins 3D, VP0 and VP3 was observed at 5 and 7 h p.i. Proteins 2C, 3C, 2A, 3A and 3AB were absent at 7 h p.i. (Fig. 3). The data shows that both PAP and guanidine alone at low concentration inhibit the processing of the same specific poliovirus polyproteins. Furthermore, when the antivirals are present in combination, additional poliovirus proteins are blocked.

In the presence of PAP (0.25 μM) alone at high concentration, a block in the processing of poliovirus polyproteins was observed as early as 3 h p.i. The appearance of most viral proteins was blocked at 7 h p.i. with the exception of proteins P1, P3, 3CD, 3D, P2, VP0, VP3, VP1, and VP2. Viral proteins synthesis was inhibited in the presence of guanidine (35.0 μM) alone at high concentration which resulted in the failure to shut-off of cell protein synthesis (Fig. 3). Both viral and cellular protein synthesis was inhibited in the presence of the antagonistic drug combination (0.25 μM PAP and 35.0 μM guanidine). The data suggests that PAP (0.25 μM) at high concentration is not as effective in the inhibition of poliovirus protein synthesis when compared to its use in combination with guanidine.

The data in Fig. 4 shows poliovirus protein synthesis when the antivirals were absent during the ^{35}S-methionine pulse. Viral proteins 3C and 3AB appear at 5 and 7 h p.i. upon removal of either PAP or guanidine alone at low concentration. Similarly, viral proteins 2C, 3C, 2A, 3A and 3AB are detected upon removal of the synergistic drug combination. Viral proteins P1, P3, 3CD, 3D, VP0, 2C, VP1, VP2, VP3, 2A and 3A were unblocked at 7 h p.i. upon removal of PAP (0.25 μM) alone at high concentration. Upon removal of guanidine (35.0 μM) alone at high concentration, several viral proteins appeared. When the antagonistic drug combination was removed, however, only protein 3CD was unblocked. The data suggests that the effect of PAP and guanidine alone and in combination on poliovirus polyprotein synthesis and processing is, at least partially reversible.

Poliovirus protein synthesis at immediate early and early post infection. To determine if the removal of the antivirals is completely reversible, cells were treated with the antiviral agents either immediate early (from 1 h prior to infection to 1 h p.i.) or early following infection (from 2 - 4 h p.i.) and the drugs were removed for 7 h and 4 h, respectively. The infected cells were pulsed for 1 h with ^{35}S-methionine at 7 h p.i. and analyzed for viral proteins by SDS polyacrylamide gel electrophoresis (Fig. 5). Viral yields were also determined and are shown in Table 3. Gel profiles following removal of the antivirals immediate early and early were similar to those profiles observed in the absence of the antiviral agents. However, the amount of viral protein 3D was observed to decrease and protein 2A was blocked upon removal of either

guanidine (35.0 μM) alone or the antagonistic drug combination (Fig. 5). Virus yields following removal of guanidine (35.0 μM) alone and the antagonistic drug combination were 94% and 90%, respectively (Table 3). No significant reduction in virus yields were observed following the removal of the antivirals when compared to control virus yields. The data suggests that the antiviral effect of the synergistic and antagonistic drug combinations, as well as the antivirals when present alone, is completely reversible.

Table 2. Effects of PAP, guanidine and combinations of the two inhibitors on the multiplication of poliovirus.

Dose		Expected ED_x	Observed ED_x	Combinational Indexes	
{P}[a]	{G}[b]	{P}+{G}	{P+G}	CI[c]	CI*[d]
0.250	-	-	53%	-	-
-	35.00	-	96%	-	-
0.250	35.00	149%	>99%	1.51	1.54
0.250	-	-	53%	-	-
-	9.80	-	27%	-	-
0.250	9.80	80%	76%	1.051	1.061
0.125	-	-	50%	-	-
-	15.00	-	36%	-	-
0.125	15.00	86%	78%	1.102	1.012
0.125	-	-	50%	-	-
-	9.80	-	27%	-	-
0.125	9.80	77%	74%	1.041	1.027
0.070	-	-	23%	-	-
-	9.80	-	21%	-	-
0.070	9.80	44%	59%	.695	.849
0.070	-	-	23%	-	-
-	2.50	-	37%	-	-
0.070	2.50	27%	37%	.730	.811
0.040	-	-	4%	-	-
-	9.80	-	21%	-	-
0.040	9.80	25%	35%	.714	.874
0.040	-	-	4%	-	-
-	1.25	-	4%	-	-
0.040	1.25	8%	29%	.280	.291

[a] {P}, pokeweed antiviral protein, μg/ml

[b] {G}, guanidine, μg/ml

[c] CI, Sum of the expected effective dose for each antiviral / the observed effective dose for the combination of inhibitors

[d] CI*, Sum of mean values of the expected effective dose for each antiviral / the observed effective dose for the antiviral combination

Fig. 1. Effect of combinations of PAP and guanidine on viral RNA synthesis. HeLa cell monolayers were infected with poliovirus at a MOI of 100 PFU/cell in the presence of either a synergistic drug combination : 0.04 μM PAP plus 1.25 μM guanidine (△) or an antagonistic drug combination : 0.25 μM PAP plus 35 μM guanidine (▲). Infected cells in the presence of individual inhibitors: 0.04 μM PAP (○); 0.25 μM PAP (●); 1.25 μM guanidine (❑); and 35 μM guanidine (■). At indicated times p.i., maintenance medium was replaced with MEM containing ^{3}H-uridine and the infected cells were incubated for 1 h at 34°C. The rate of incorporation of ^{3}H-uridine into TCA-precipitable material was determined as described in Materials and Methods. Actinomycin D at a final concentration of 2 μM was added to all cell monolayers at 1 h p.i. Results are presented as percent RNA synthesis in virus infected cells without inhibitor is 100%. Yields of poliovirus are shown in parenthesis.

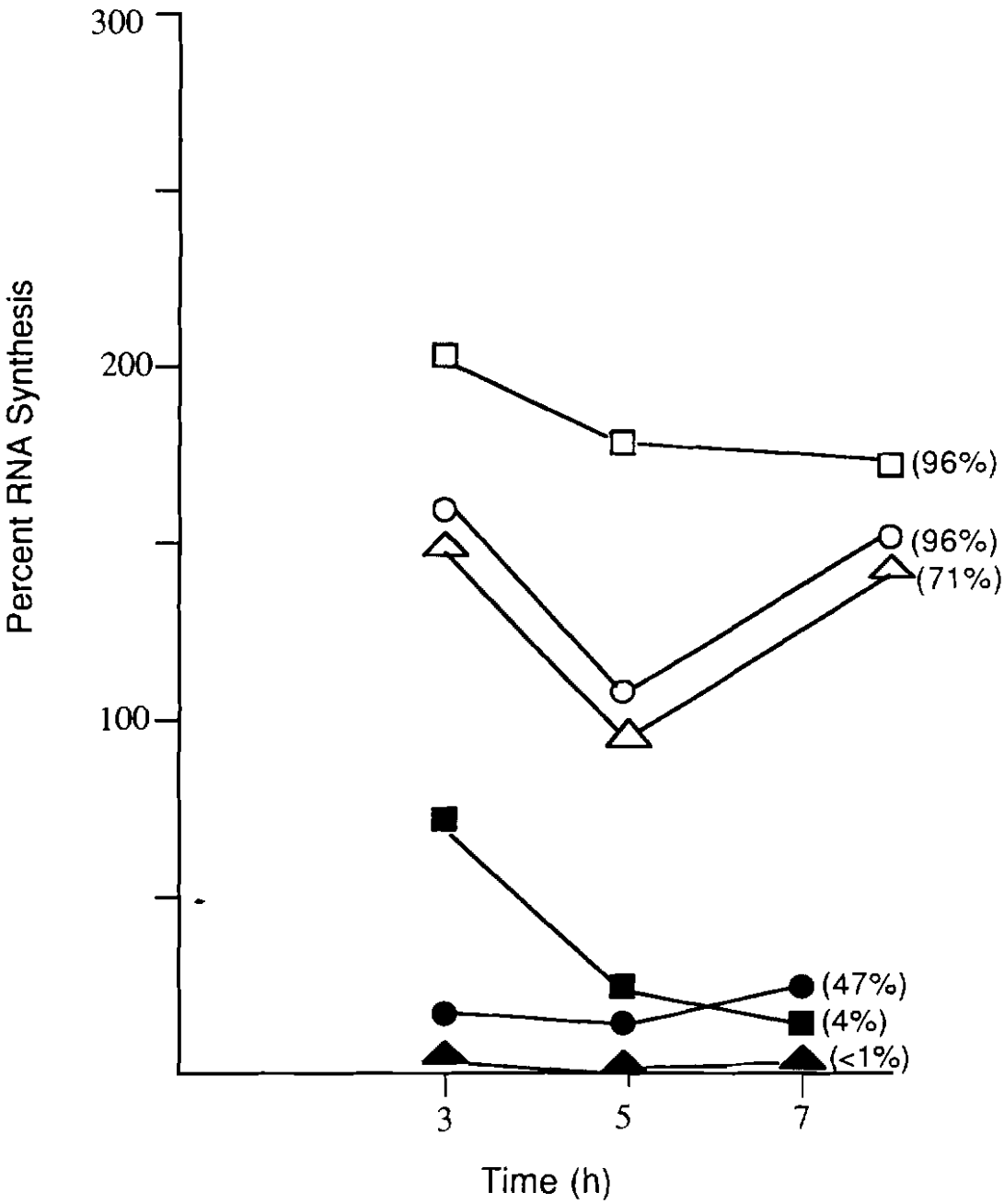
300
200
100
Percent RNA Synthesis
(96%)
(96%)
(71%)
(47%)
(4%)
(<1%)
3
5
7
Time (h)

Fig. 2. Effect of combinations of PAP and guanidine on protein synthesis. HeLa cell monolayers were infected with poliovirus at a MOI of 100 PFU/cell in the presence of either a synergistic drug combination : 0.04 μM PAP plus 1.25 μM guanidine (△) or an antagonistic drug combination : 0.25 μM PAP plus 35 μM guanidine (▲). Infected cells in the presence of individual inhibitors: 0.04 μM PAP (○); 0.25 μM PAP (●); 1.25 μM guanidine (❑); and 35 μM guanidine (■). At indicated times p.i.. maintenance medium was replaced with MEM containing ^{14}C-leucine and the infected cells were incubated for 1 h at 34°C. The rate of incorporation of ^{14}C-leu into TCA-precipitable material was determined as described in Materials and Methods. Results are presented as percent protein synthesis in virus infected cells minus inhibitor is 100%. Yields of poliovirus are shown in parenthesis.

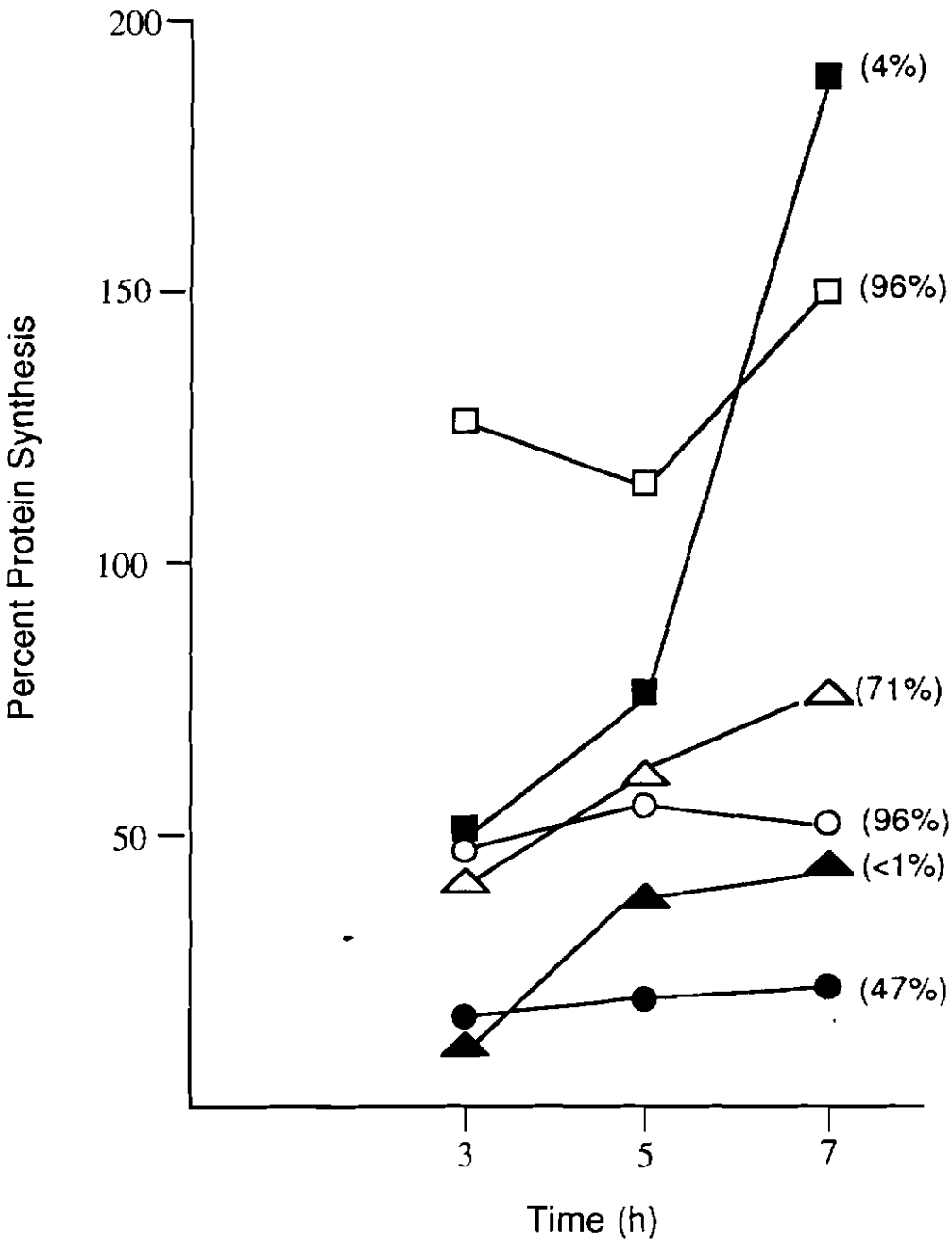
200
150
100
50
Percent Protein Synthesis
(4%)
(96%)
(71%)
(96%)
(<1%)
(47%)
3
5
7
Time (h)

Fig. 3. Autoradiograph of poliovirus proteins synthesized in the presence of PAP and guanidine. HeLa cell monolayers were pretreated with antivirals for 1 h prior to infection. Monolayers were then infected with poliovirus at a MOI of 100 PFU/cell in the presence of either a synergistic combination (0.04 μM PAP and 1.25 μM guanidine), antagonistic combination (0.25 μM PAP and 35 μM guanidine) or antivirals alone. Cells were then washed and supplied with maintenance medium containing antivirals singly or in combination. Proteins from treated and untreated infected cells were pulsed with ^{35}S-methionine in the presence of the antivirals for 1 h at 3, 5 and 7 h p.i. (A, B and C, respectively) and then analyzed using SDS polyacrylamide gel electrophoresis as described in Materials and Methods. V_O represents the virus control lane. The remaining lanes represent the synergistic drug combination, antagonistic drug combination or antivirals alone. The right column represents a standard poliovirus protein profile found in infected cells.

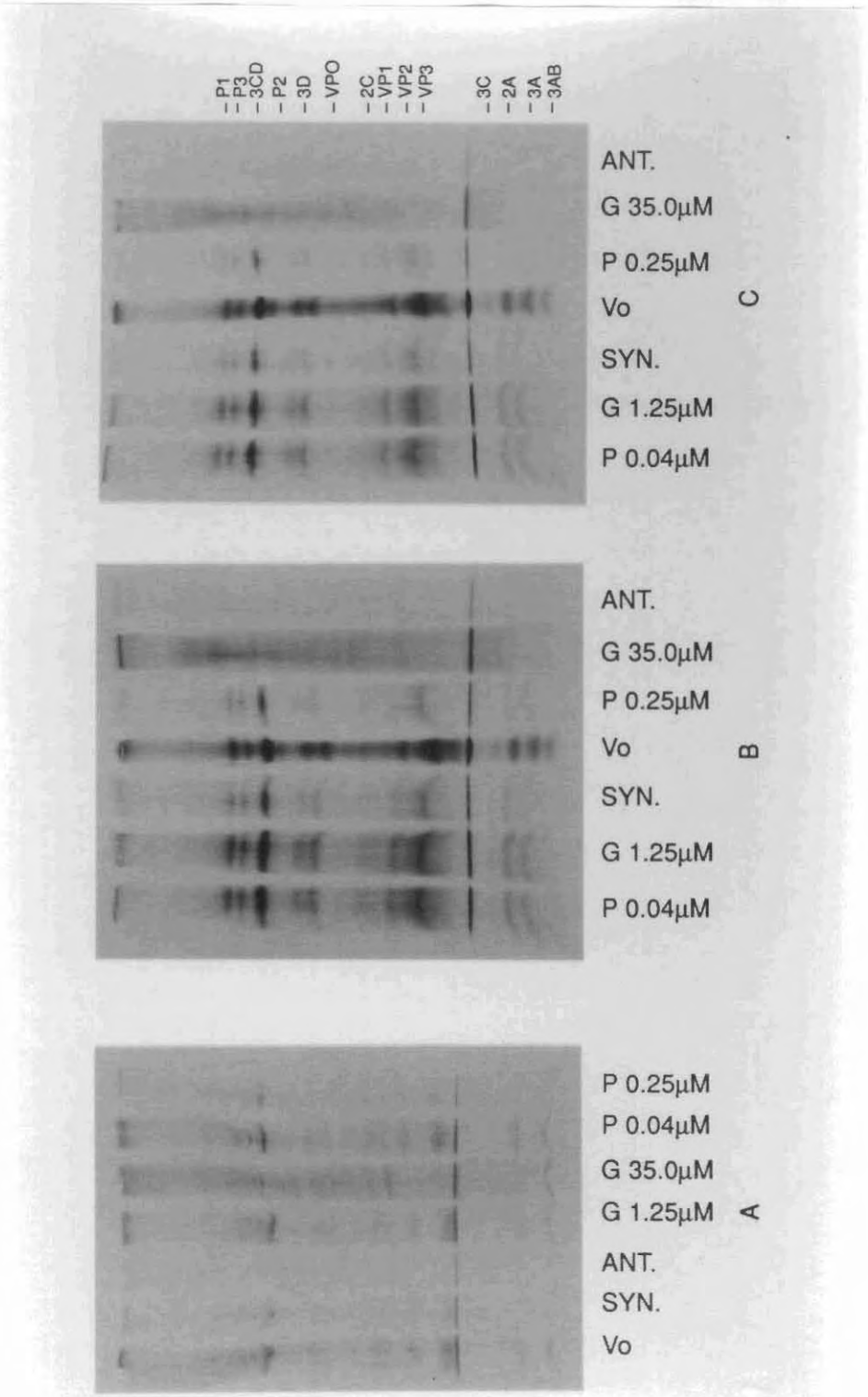
P1
P3
3CD
P2
3D
VPO
2C
VP1
VP2
VP3
3C
2A
3A
3AB
ANT.
G 35.0µM
P 0.25µM
Vo
SYN.
G 1.25µM
P 0.04µM
C
ANT.
G 35.0µM
P 0.25µM
Vo
SYN.
G 1.25µM
P 0.04µM
B
P 0.25µM
P 0.04µM
G 35.0µM
G 1.25µM
ANT.
SYN.
Vo
A

Fig. 4. Autoradiograph of poliovirus proteins synthesized in the presence of PAP and guanidine. HeLa cell monolayers were pretreated with antivirals for 1 h prior to infection. Monolayers were then infected with poliovirus at a MOI of 100 PFU/cell in the presence of either a synergistic combination (0.04 μM PAP and 1.25 μM guanidine), antagonistic combination (0.25 μM PAP and 35 μM guanidine) or antivirals alone. Cells were then washed and supplied with maintenance medium containing antivirals singly or in combination. Proteins from treated and untreated infected cells were pulsed with ^{35}S-methionine in the absence of the antivirals for 1 h at 3, 5 and 7 h p.i. (A, B and C, respectively) and then analyzed using SDS polyacrylamide gel electrophoresis as described in Materials and Methods. V_o represents the virus control lane. The remaining lanes represent the synergistic drug combination, antagonistic drug combination or antivirals alone. The right column represents a standard poliovirus protein profile found in infected cells.

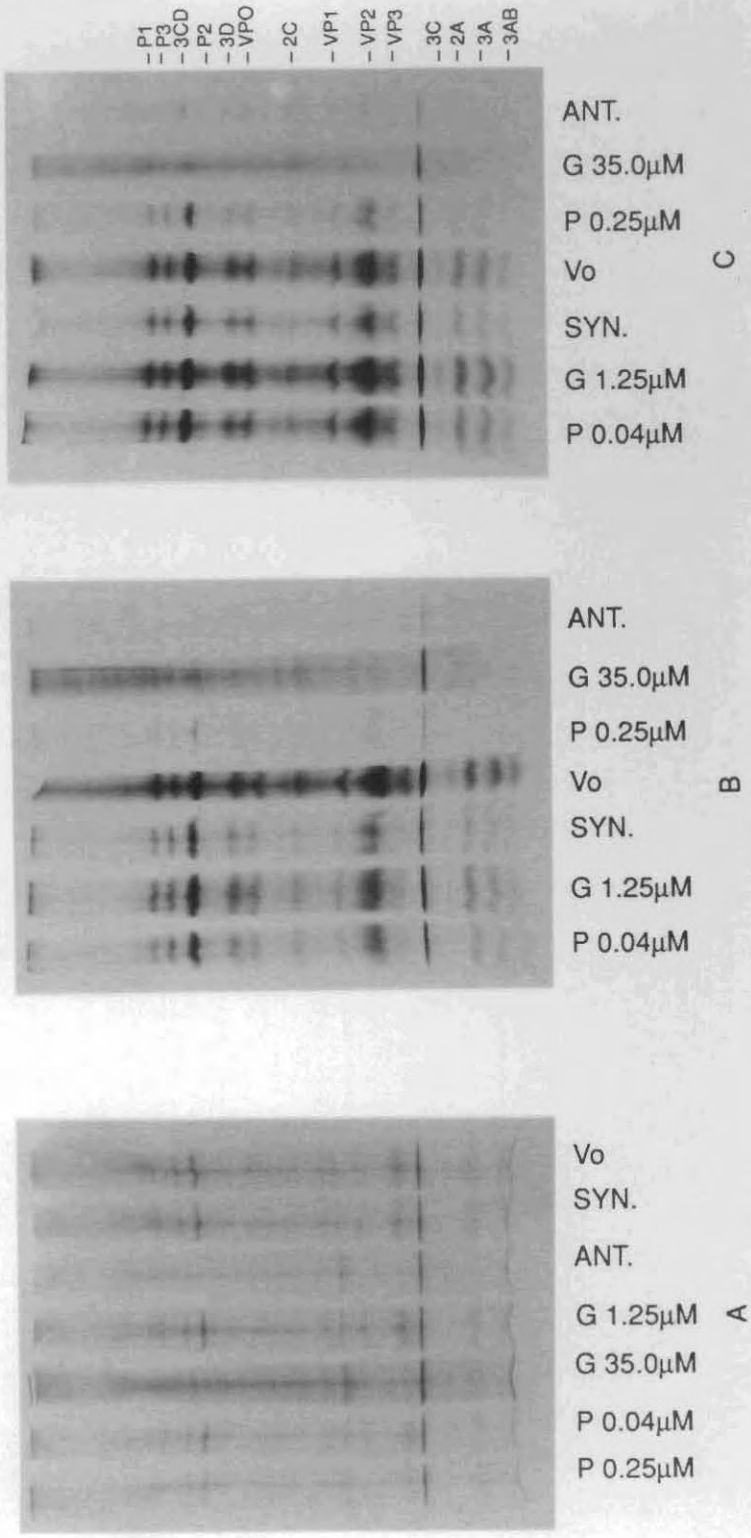
P1
P3
3CD
P2
3D
VPO
2C
VP1
VP2
VP3
3C
2A
3A
3AB
ANT.
G 35.0μM
P 0.25μM
Vo
SYN.
G 1.25μM
P 0.04μM
C
ANT.
G 35.0μM
P 0.25μM
Vo
SYN.
G 1.25μM
P 0.04μM
B
Vo
SYN.
ANT.
G 1.25μM
G 35.0μM
P 0.04μM
P 0.25μM
A

Fig. 5. Autoradiograph of poliovirus proteins synthesized either in the immediate early (1 h prior to infection to 1 h p.i.) presence of PAP and guanidine or in the early (from 2-4 h p.i.) presence of PAP and guanidine (A and B, respectively). HeLa cell monolayers were infected with poliovirus at a MOI of 100 PFU/cell. Proteins from treated and untreated infected cells were pulsed with ^{35}S-methionine for 1 h at 7 h p.i. and then analyzed using SDS polyacrylamide gel electrophoresis as described in Materials and Methods. V_0 represents the virus control lane. The remaining lanes represent the synergistic combination (0.04 μM PAP and 1.25 μM guanidine), antagonistic combination (0.25 μM PAP and 35 μM guanidine) or antivirals alone. The right column represents a standard poliovirus protein profile found in infected cells.

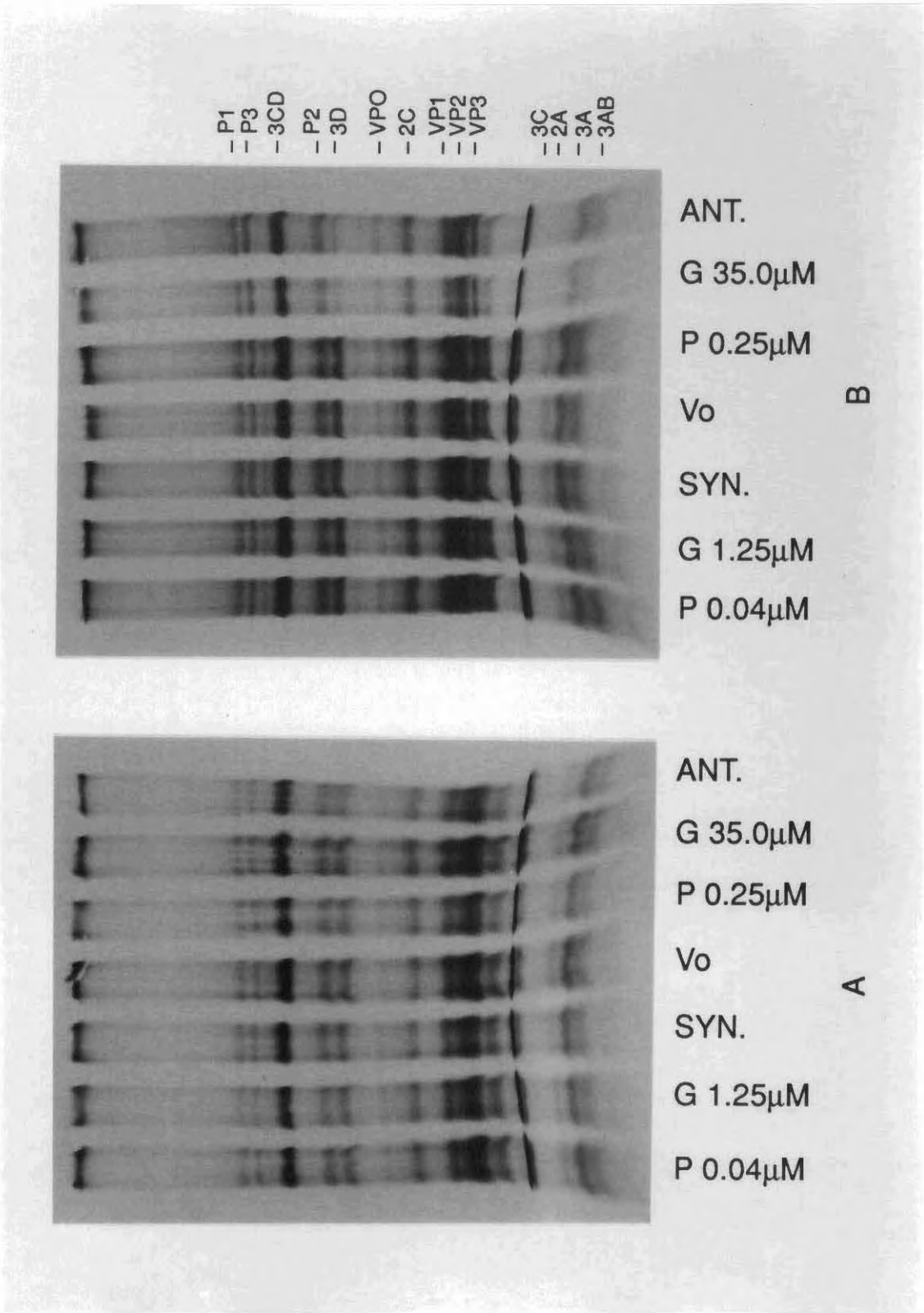
P1
P3
3CD
P2
3D
VPO
2C
VP1
VP2
VP3
3C
2A
3A
3AB
ANT.
G 35.0µM
P 0.25µM
Vo
SYN.
G 1.25µM
P 0.04µM
B
ANT.
G 35.0µM
P 0.25µM
Vo
SYN.
G 1.25µM
P 0.04µM
A

Table 3. Effects of PAP, guanidine and combinations of the two inhibitors on the multiplication of poliovirus at immediate early and early p.i.

Dose		Time [c]	Titer [d]	% Yield [e]	EDx	
{P}[a]	{G}[b]	IE/E	pfu/ml		Observed	Expected
0.250	-	IE	1.05×10^9	98%	2%	-
-	35.00	IE	1.01×10^9	94%	6%	-
0.250	35.00	IE	9.88×10^8	92%	8%	8%
-	-	IE	1.08×10^9	100%	-	-
0.250	-	E	1.05×10^9	99%	1%	-
-	35.00	E	1.00×10^9	94%	6%	-
0.250	35.00	E	9.63×10^9	90%	10%	7%
-	-	E	1.07×10^9	100%	-	-
0.040	-	IE	1.04×10^9	97%	3%	-
-	1.250	IE	1.05×10^9	98%	2%	-
0.040	1.250	IE	1.01×10^9	94%	6%	5%
-	-	IE	1.08×10^9	100%	-	-
0.040	-	E	1.04×10^9	97%	3%	-
-	1.250	E	1.05×10^9	99%	1%	-
0.040	1.250	E	1.03×10^9	96%	4%	4%
-	-	E	1.07×10^9	100%	-	-

[a] {P}, pokeweed antiviral protein, µg/ml

[b] {G}, guanidine, µg/ml

[c] Drug incubation time, IE (from 1 h prior to infection to 1 h p.i.) vs E (from 2-4 h p.i.)

[d] Titer, Average number of plaques / volume of virus plated x virus dilution

[e] % Yield, titer (virus with drug) / virus control x 100

DISCUSSION

The present study reports the effect of pokeweed antiviral protein (PAP) in combination with guanidine on poliovirus macromolecular synthesis. The object of this study is to determine a mechanism of action for the synergistic effect of the antivirals on the multiplication of poliovirus. A model of the effect of the antivirals on poliovirus macromolecular synthesis is illustrated (Fig. 6).

The use of chemotherapeutic agents at reduced concentrations without a reduction in viral inhibition is advantageous in the treatment of viral infection where drug toxicity is a problem [7]. In addition to reducing drug toxicity, combined drug chemotherapy can lead to a reduction in the emergence of resistant strains and its study may lead to an understanding of viral replication. Although PAP has been shown to inhibit poliovirus multiplication by 90% at a concentration of 2 μM [30], PAP is cytotoxic at concentrations as small as 0.5 μM during a 48 h incubation [1]. The synergistic (0.04 μM PAP and 1.25 μM guanidine) and antagonistic (0.25 μM PAP and 35.0 μM guanidine) drug combinations used in this study are not cytotoxic during a five day incubation [6]. The antagonistic drug combination inhibited viral yields by greater than 99%; whereas, the synergistic drug combination showed greater efficacy for reducing poliovirus multiplication. Specifically, each antiviral at low concentration reduced virus yields by 4%; whereas, in combination yields were reduced by 29% (Table 2). These results agree with prior studies on the effect of combinations of PAP and guanidine on poliovirus multiplication [6].

The pokeweed antiviral protein (PAP) is one of several ribosome inactivating proteins which inhibit protein synthesis by targeting the translocation mediation of elongation factor 2 [13]. Specifically, PAP removes a single adenine residue from a conserved base sequence in 28S rRNA [11]. PAP inhibited total protein synthesis in virus infected cells when used singly at either low or high concentration (Fig. 2). These results agree with previous studies which demonstrated PAP's inhibitory action on cell protein synthesis [1, 30, 45]. However, we found that virus protein synthesis was not completely inhibited but, only specific poliovirus proteins were absent when PAP was present. Furthermore, a greater number of poliovirus proteins were blocked when PAP was present at high concentration compared to those present with PAP at low concentration (Fig. 3). Studies with herpes simplex virus, an unrelated virus, revealed that no significant inhibition in the synthesis of the majority of HSV-1 viral infected-cell polypeptides occurred in the presence of PAP, however, the synthesis of individual infected cell polypeptides was reduced by 48 to > 99% [45].

PAP at low concentration completely blocked synthesis of viral proteins 3AB and 3C. In addition to 3C and 3AB, the synthesis of viral proteins 2C, 3A and 2A were completely blocked in the presence of PAP at high concentration. Also, proteins VP0, VP3 and 3D were present in reduced amounts in the presence of PAP at high concentration (Fig. 3). The absence of specific polyprotein end products may be due to a block in processing since polyproteins P1, P2 and P3 were present. In contrast, a

reduction in the amount of viral protein synthesis observed may be the result of an inhibition in the elongation of NCVP00 by PAP when present at high concentration. Indeed, the end products of P2 and P3, which are coded for at the 3' end of the message are present in reduced amounts when compared to the end products of P1 which are located at the 5' end of the message (Fig. 6). The fact that infectious virus was detected in the presence of PAP at high concentration (Table 2) suggests that the blocked viral proteins 2C, 2A, 3A, 3C and 3AB, are either not essential for virus multiplication or they may function internally. Poliovirus proteases have been postulated to function within polyproteins P2 and P3 [17, 27, 50, 51]. Clearly, the reduction observed in virus yields is most likely due to a decrease in synthesis of essential proteins VP0 and VP3 (capsid proteins). It is reasonable to postulate that a reduction in the synthesis of a protein which acts enzymatically (i.e. protein 3D) would have less of an impact on virus yields compared to a reduction in the synthesis of a structural protein.

Guanidine has long been considered the classic inhibitor of poliovirus plus-strand RNA synthesis [3, 10, 25, 26, 41, 46]. Guanidine hydrochloride acts as a protein denaturant at concentrations of 5.0 M or higher, but at millimolar levels (1 - 10 mM) it selectively blocks the growth of many picornaviruses, including poliovirus [10, 25, 37]. The concentration of guanidine used in this study (1.25 μM to 35.0 μM) effectively blocked the multiplication of poliovirus by 4% to 99%, respectively. Although viral protein 2C has been implicated as the antiviral target by guanidine [37, 38, 39], the role of protein 2C in viral RNA

synthesis remains unknown [37]. We found that viral protein and viral RNA synthesis was completely inhibited in the presence of both guanidine at high concentration and the antagonistic drug combination (Fig. 3). The results suggest that PAP blocks translation of cell message more effectively than viral message since cell protein was not detected in the presence of either PAP alone or in combination with guanidine (Fig. 3). The inhibition of RNA synthesis observed may be the result of a failure to synthesize plus-strand RNA from the parental RF-RNA since one would expect viral protein synthesis to occur if progeny plus-strands were present (Fig. 6). The complete inhibition of poliovirus macromolecular synthesis in the presence of the antagonistic drug combination was expected since guanidine prevents the synthesis of plus-strand RNA from parental RNA. The detection of polyproteins P1, P2 and P3 would imply that the elongation of the poliovirus polyprotein, NCVPOO, from progeny plus-strand RNA does occur in the presence of both PAP and guanidine. This suggests that PAP acts at a step following the action of guanidine. Specifically, PAP does not inhibit the translation of parental RNA but rather affects the translation of progeny RNA. Drugs which act independently of each other or have different modes of action are defined as being mutually nonexclusive [8]. The results suggest that guanidine and PAP at high concentration target different steps in poliovirus multiplication and act as mutually nonexclusive drugs.

It was surprising to find that PAP and guanidine at low concentration had similar effects on poliovirus protein synthesis. Both antiviral agents blocked viral proteins 3AB and 3C. Protein 3AB is the smallest VPg-

containing polypeptide and is a likely candidate to function as a donor of VPg (3B) to a membrane-associated poliovirus RNA-synthesizing complex [14]. The covalent attachment of viral protein, VPg (3B), to viral RNA is an absolute requirement for initiation of plus-strand RNA synthesis and genome replication *in vivo* [14]. VPg is located in the 5' untranslated region of the poliovirus RNA molecule and is responsible for the initiation of plus-strand RNA synthesis from both progeny and parental RF's (replicative form of RNA). Thus, if protein 3AB is blocked, one would expect viral RNA synthesis to decrease or be inhibited. In fact, PAP has been reported to block the synthesis of specific HSV-1 polypeptides required for viral DNA synthesis [45]. Surprisingly, viral RNA synthesis was found to increase in the presence of either PAP or guanidine at low concentration (Fig. 1). A block in viral protein 3AB leading to the absence of VPg (3B) would result in a failure to synthesize plus-strands from progeny RF-RNA, but not from the parental RF-RNA which already contains VPg (3B). An inhibition of plus-strand RNA synthesis from progeny RF's due to the absence of VPg may result in excessive plus-strand synthesis from the parental RF-RNA. Minus-strand RNA synthesis would also occur as a result of the conversion of plus-strands to progeny RF-RNA.

Viral proteinases 3C, 2A and 3CD are responsible for the processing of poliovirus precursor polypeptides [50, 51]. The data shows that either PAP or guanidine alone at low concentration inhibits the efficient processing of polyprotein P3 which leads to the formation of proteins 3AB and 3C (Fig. 6). This indicates that the presence of viral proteins 3AB and

3C are not essential for the production of infectious progeny virus since virus yields were high (96%) in the presence of either PAP or guanidine at low concentration (Table 2). It is also possible that viral proteinases may be functional in polypeptides prior to their processing [17, 27, 50, 51].

In the presence of the synergistic drug combination, not only were viral proteins 3AB and 3C blocked, but proteins 2C, 2A, and 3A failed to appear. Viral protein 2C has been implicated in replication because of its association with membrane-bound viral replication complexes and the demonstration that a guanidine-dependent trait maps within 2C [38]. Since viral RNA synthesis increased in the absence of protein 2C (Fig. 1), either 2C is a nonessential protein or it functions internally. Viral protein 2A is involved in the proteolytic cleavage of the eukaryotic translation initiation factor eIF-4F which is responsible for the rapid shut-off of host cell protein synthesis [18, 27]. The ability to produce infectious virus in the absence of protein 2A suggests that viral protein synthesis dependent shut-off is not essential for viral multiplication. The inhibition of cell protein synthesis observed is most likely due to a direct effect of PAP on the translation of cell message.

The effect of the synergistic drug combination is similar to the effect of PAP at high concentration. However, virus yields and the amount of essential viral proteins VP0, VP3 and 3D was less in the presence of high PAP when compared to the synergistic drug combination (Table 2, Fig. 3). The absence of proteases 2A and 3C could lead to a reduction in the processing of proteins P2 and P3. This, in turn, may account for the

absence or reduction observed in P2 and P3 end products. An absence of end products from polyproteins P2 and P3 did not result in complete inhibition of virus multiplication. However, a reduction of essential capsid proteins would cause a decrease in the encapsidation of plus-strand RNA. These results suggest that the mechanism of action of the synergistic drug combination causes a block in the processing of polyprotein P1 (Fig. 6).

The inhibitory action of PAP (3 μM) on cellular protein synthesis has been found to be partially reversible [1]. Viral protein synthesis following removal of the antivirals was similar to viral protein synthesis observed in the absence of the antiviral agents (Fig. 4 and 5). Furthermore, no significant reduction in yields was observed in the presence of the antivirals when compared to viral yields observed in the absence of the antivirals (Table 3). Clearly, the effect of the synergistic and antagonistic drug combinations as well as the antivirals alone is completely reversible. This is surprising since PAP's mechanism of action involves the removal of a single adenine residue from a conserved base sequence in 28S rRNA [11] which, in turn, leads to an inhibition in elongation [13]. It may be that a replacement of the adenine residue restores elongation activity. Indeed, the degradation of uncapped, nonencapsidated RNA could lead to pools of purines and pyrimidines which may serve as "substitute" bases in repair.

The inhibition of poliovirus infection has been found to be reversible with other antiviral compounds. The pyrimidine analog Py-11 (2-amino-4,6-dichloropyrimidine) is a potent reversible inhibitor of poliovirus

growth. Py-11 specifically interferes with viral assembly by impairing the cleavage of the capsid protein precursor P1 which is similar to the synergistic mechanism described in this study. The effect of Py-11 is completely reversible simply by removing the drug [28]. The effect of Brefeldin A, a fungal metabolite that blocks transport of newly synthesized viral proteins from the endoplasmic reticulum (unlike PAP or guanidine), was reversible when it was removed from the culture medium as late as 3 h p.i. [34].

The present study shows that the antiviral effect of PAP and guanidine at low concentration on poliovirus macromolecular synthesis is identical. Both antiviral agents block the appearance of viral proteins 3AB and 3C which suggests they inhibit the processing of polyprotein P3. In contrast, PAP and guanidine at high concentration acted at different sites in viral macromolecular synthesis. Specifically, guanidine blocked the synthesis of plus-strand RNA from parental RF-RNA; whereas, PAP inhibited the elongation of NCVP00 and processing of polyproteins P1, P2 and P3. In the presence of the antagonistic drug combination, guanidine acts prior to PAP causing a complete shut-off of RNA synthesis which results in an absence of viral protein synthesis. In the presence of the synergistic drug combination, proteins 2C, 2A, and 3A, in addition to 3AB and 3C, were completely blocked. Also, essential capsid proteins VP0 and VP3, which are end products of polyprotein P1, were present in reduced amounts. The data suggests that the mechanism of action for synergy is a block in the processing of the capsid precursor protein, P1.

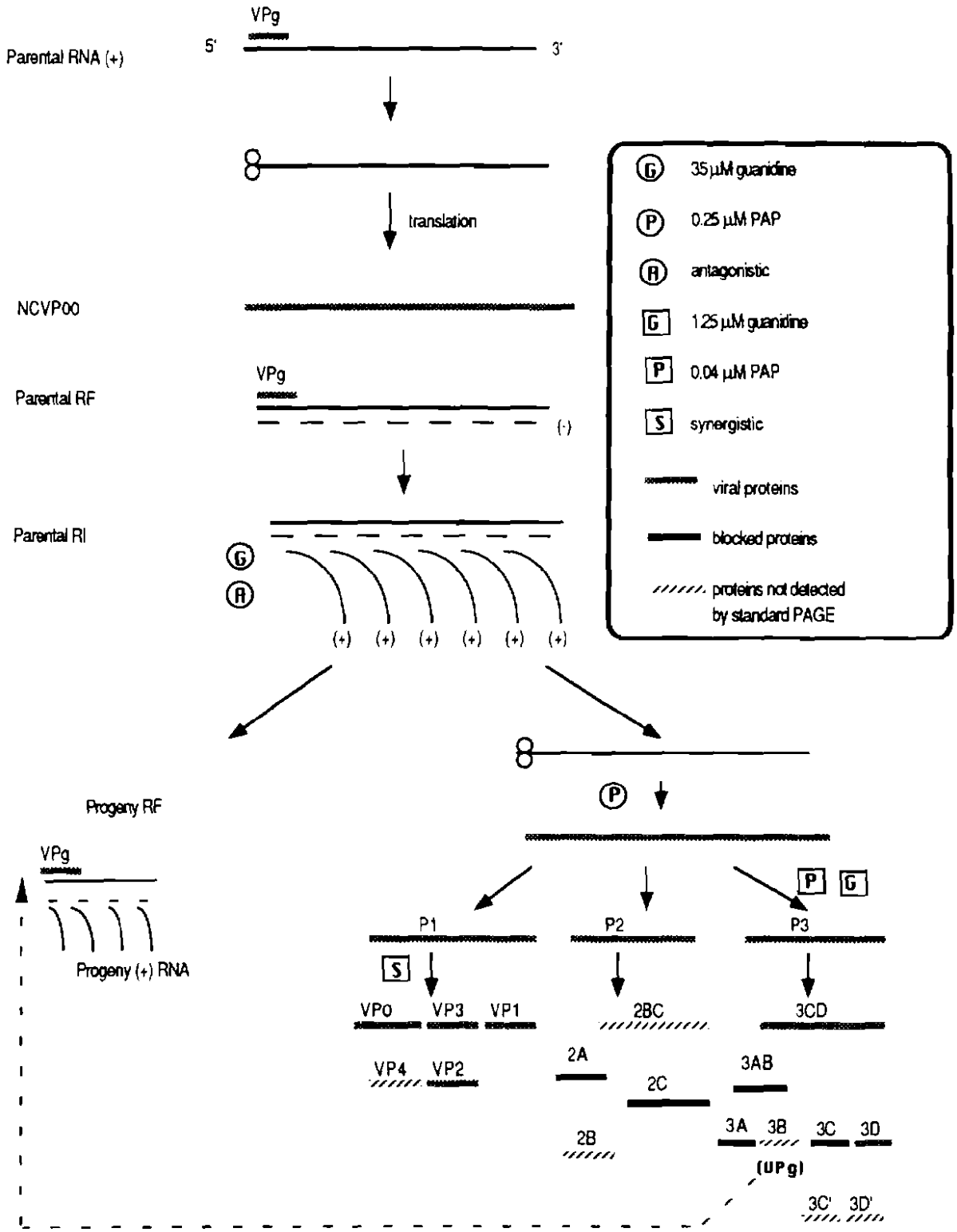

FIG. 6. A model for the antiviral sites of action on poliovirus macromolecular synthesis by guanidine, PAP, synergistic and antagonistic drug combinations.

SUMMARY

Viral RNA synthesis, total protein synthesis and the synthesis of specific poliovirus proteins were determined in the presence of synergistic (low) and antagonistic (high) combinations of PAP and guanidine. Viral proteins 3AB and 3C were blocked in the presence of either guanidine or PAP at low concentration. In the presence of the synergistic drug combination, proteins 2C, 2A and 3A, in addition to proteins 3AB and 3C failed to appear. Also, proteins 3D, VP0 and VP3 were present in reduced amount. The mechanism of action of the synergistic drug combination may involve an inhibition in the processing of poliovirus polyprotein P1 which is the precursor protein for essential capsid endproducts VP0 and VP3.

Viral RNA synthesis was stimulated in the presence of either PAP or guanidine alone at low concentration and in the presence of the synergistic drug combination. This may be due to the absence of protein VPg (3B) which has a regulatory function on RNA synthesis. In contrast, viral RNA synthesis was inhibited almost completely in the presence of either PAP or guanidine at high concentration and in the presence of the antagonistic drug combination. The antagonistic drug combination resulted in the failure to synthesize plus-strand RNA from the parental RF-RNA which led to a complete absence of viral protein synthesis.

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