INVESTIGATION OF THE ANTIVIRAL ACTIVITY OF THE TRITERPENOID COMPOUND, CICLOXOLONE SODIUM.

by

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SUMMARY

Cicloxolone sodium (CCX) and the closely related compound Carbenoxolone sodium (CBX) have been shown to inhibit the growth of HSV 1 and HSV 2 in Flow 2002 and BHK-21 cells (Dargan and Subak-Sharpe, 1985). The anti-HSV effect is characterised by the synthesis of poor quality progeny virus particles (Dargan and Subak-Sharpe, 1986a and b). This results in an elevated particle/p.f.u. ratio in the virus yield accompanied by a small reduction in the total number of virus particles made. Based on their analysis of protein synthesis in infected cells, Dargan and Subak-Sharpe have concluded that CCX and CBX disrupt the normal functioning of host cell membrane, affecting aspects of protein synthesis, transport and post-translational processing, particularly glycosylation and sulphation. This proposed antiviral action involving cell membranes implies that the effect of CCX is of a more general nature, suggesting that the replication of several unrelated virus groups would be affected by the drug. In order to examine the effect of CCX on the replication of a range of viruses, cell lines permissive for virus growth and resistant to CCX treatment had to be identified, to allow uncoupling of cytotoxic and antiviral effects. The aims of this thesis were: 1) to determine the tolerance of a range of cell lines to CCX, 2) to determine the antiviral range of CCX and 3) to more clearly elucidate the mode of action of CCX.

Twenty cell lines were studied for their sensitivity to CCX treatment. The survey revealed that cell lines differed in their tolerance to the drug and could be designated as Resistant, Intermediate or Sensitive on the basis of cell viability counts. Nine cell lines (Flow 2002, RK-13, Chang Liver, Detroit 532, HeLa, Hep2, MDBK, MDCK and BS-C-1), fell into the Resistant class, six into the Sensitive class (FG 293, XTC-2, HOOD, Reα, Re99 and PCEF), while five displayed Intermediate sensitivity (BHK-21, Flow 4000, MRC-5, Rabbit Epithelium and Rabbit Keratocytes). Although the viability of cells in the Resistant class remained high in the presence of CCX, the replication of cells was inhibited at high concentrations (200 and 300uM CCX). However, this should not contribute to any antiviral

effect as all virus dose-response experiments were performed in confluent monolayers. Most importantly, the effect on the cell lines used in the virus dose-response experiments ~~were~~ was shown to be fully reversible.

Using cell lines identified as resistant to CCX treatment as hosts, a range of viruses was examined for their sensitivity to CCX and placed into three general groupings depending on their response. The first class is composed of Vesicular Stomatitis virus (VSV), Influenza A and the Herpes viruses Herpes Simplex virus 1 and 2 (HSV 1/2), Equine Herpes virus (EHV-1) and Bovine Herpes virus (BHV-1), all of which exhibit a continuous drug dose-dependant reduction in virus infectivity. The extent of the reduction varies for individual viruses from 100 fold (Influenza A) to greater than 10,000 fold (BHV-1). For Varicella Zoster virus (VZV) and Human Cytomegalovirus (HCMV) only ED_{50} values could be ascertained. Since these values are lower than those obtained for HSV 1/2, it would seem likely that both these herpesviruses also belong to this class.

The second class contains Bunyamwera and Germiston virus, Poliovirus-1, Reovirus-3 and Adenovirus-5. Members in general, exhibit a less extensive total CCX response, infectivity being reduced by only 20-100 fold, even with 300uM CCX. All the dose-response curves reach their plateau level with concentrations between 100 and 200uM CCX. However, this second class falls into two sub-divisions: whereas both Adenovirus-5 and Reovirus-3 exhibit great sensitivity to low doses of drug and then reach their plateau values, Polio virus-1, Bunyamwera and Germiston are little affected by doses less than 50uM and only then respond to increased doses until a plateau is reached between 150-200uM CCX.

The third class, presently consists of only Semliki Forest virus (SFV), whose infectious virus yields are unaffected by CCX treatment when whole cell harvests are performed.

Representatives of each class were chosen for further investigation. However, VSV was the most extensively studied virus in this survey, as apart from the herpes viruses, this was most drastically affected by the drug. VSV infectious

yields from BS-C-1 cells are reduced -10,000 fold with 300uM CCX. The predominant anti-VSV effect is a reduction in particle production (typically -1000 fold), which is also accompanied by an elevation in particle/p.f.u. ratio (typically 10 fold). This elevation can be explained by the direct inactivation of VSV particles.

CCX had a progressive effect operating throughout the virus life cycle. To determine how CCX reduces the production of VSV particles, several stages in the virus life cycle were investigated. VSV secondary but not primary transcripts were progressively reduced with increasing CCX concentrations. Protein synthesis was affected in CCX treated VSV-infected cells: some bands decreased, as expected with increasing CCX (L, N and NS), while protein bands G, M and UK_3 increased with increasing CCX despite reduced levels of transcripts. Dargan and Subak-Sharpe (1986a and b) had shown that glycosylation was affected in HSV-infected BHK-21 cells. Comparative studies were performed with compounds known to disrupt glycosylation: tunicamycin, which blocks the addition of core sugar normally occurring in the ER and monensin, which disrupts sugar processing occurring in the Golgi apparatus. These studies revealed that CCX did not completely block sugar addition. Similarities emerged between monensin and CCX; both resulted in elevated levels of G and M protein bands inside cells and both had no significant effect on glucosamine incorporation. Johnson and Schlesinger (1980), showed that monensin caused dilation of Golgi membrane and that transport of the VSV G protein from the Golgi apparatus to the cell surface was blocked. VSV assembly normally occurs following interaction of the nucleocapsid with G inserted into the PM, this interaction being mediated by the M protein. Retention of the G protein at the Golgi membrane therefore effectively inhibits VSV assembly. Given the similarities between CCX and monensin in VSV infected BS-C-1 cells, it seems likely that this is also the explanation for the accumulation of these bands in CCX treated VSV infected cells, leading to inhibition of virus assembly. M and UK3 may co-localize with G or accumulate elsewhere in the cell. CCX can also affect the PM of cells and this may also contribute to reduced particle numbers by disrupting the

interaction mediated by the M protein between nucleocapsid and G protein necessary for VSV assembly and budding.

Similarities between monensin and CCX also emerged in the investigation of the class three virus SFV. Total infectious yields were not affected by CCX treatment. However, when infectious yields were divided into cell associated (CA) and cell released (CR) fractions the CA fraction increased with increasing CCX and increasing monensin with a concomitant decrease in the CR fraction. In addition, both monensin and CCX induced similar changes in SFV polypeptide synthesis in treated BS-C-1 cells, resulting in the accumulation of viral glycoproteins. EM analysis of SFV infected BS-C-1 cells treated with CCX or monensin revealed nuclecapsids associated with and budding into vacuoles, thus accounting for the increase in the CA fraction.

Dose response experiments, performed with monensin and CCX revealed that disruption of processing in the Golgi could in theory account for the total anti-Bunyavirus effect of CCX. However, glycosylation is not required for Poliovirus, Adenovirus or Reovirus infectivity and so disruption of glycoprotein processing cannot contribute to the antiviral effect of CCX in these cases. More detailed studies were performed with Reovirus-3 and Adenovirus-5 as these viruses were very sensitive to low doses of CCX.

It was shown that the plateau obtained in the dose-reponse curves is not due to the selection of resistant virus. It seems likely therefore, that CCX affects a function either host or virus specified which enhances but is not essential for virus infectivity. The anti-Reovirus-3 effect of CCX was of two types. At low CCX concentrations, the drug predominantly operated by lowering the quality of virus particles produced and at higher concentrations by inhibiting virus assembly (with quality unaffected). Protein synthesis in Reovirus-3 infected BS-C-1 cells was progressively reduced with increasing CCX, this almost certainly contributing to reduced particle numbers. Structural polypeptides of Reovirus-3 produced from drug treated cells have not been analysed and may explain the effect on virus quality at low CCX concentrations.

As evidenced by the reduction in particle number and

almost unchanged particle/p.f.u. ratio of Adenovirus-5 yields from CCX treated infected HeLa cells, the anti-Adenovirus effect of CCX operates by inhibiting particle production. Polypeptides induced in CCX treated Adenovirus infected cells were progressively reduced (with the exception of low m.wt. bands 13 and 14K, which increased),in the presence of increasing CCX, at least contributing to the observed reduction in particle numbers. However, other factors may also play a role such as a direct effect on the nuclear membrane where virus assembly takes place.

ABBREVIATIONS

A	adenine
ATP	adenosine-5'-triphosphate
bp	base pairs
C	cytosine
CTP	cytosine-5'-triphosphate
Ci	Curie
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	any 2'-deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
DNase	deoxyribonuclease
GlcNAc	N-acetyl-glucosamine
G	guanine
GTP	guanosine-5'-triphosphate
h	hour(s)
K	$x10^3$
min.	minutes
m.o.i.	multiplicity of infection
m.wt.	molecular weight
p.f.u.	plaque forming units
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
T	thymine
ts	temperature sensitive
U	uridine
UV	ultra violet
V	volts
ER	endoplasmic reticulum
CCX	cicloxolone sodium

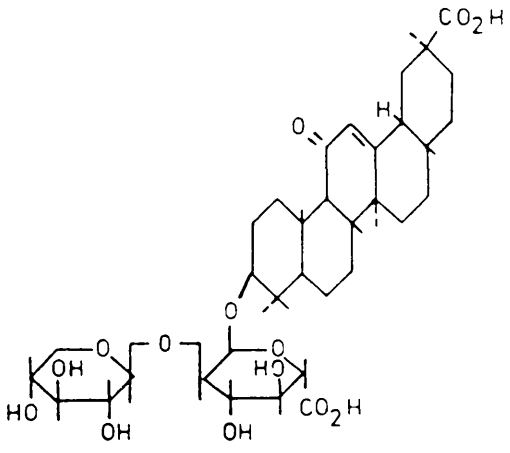
1. INTRODUCTION

This thesis is concerned with the determination of the antiviral activity spectrum of the triterpenoid compound, Cicloxolone Sodium (CCX) (Fig 1), and shedding further light on its mode of action.

A successful antiviral compound must affect either one or more specific virus-encoded functions or a cellular function required for the growth of the virus but not essential for the cell. This principle is most clearly illustrated with reference to known antiviral agents, the viruses they affect and the specific targets within the life cycles of each of these viruses.

1.1. A REVIEW OF ANTIVIRAL CHEMOTHERAPY

This review will be split into four main sections : section one - compounds which affect early pre-synthetic steps in the virus life cycle; section two - compounds which affect transcription and/or processing of mRNA; section three - compounds which affect protein synthesis and/or post-translational modifications and section four - compounds which affect DNA and/or RNA replication. Some compounds have multiple targets and will therefore belong to more than one section. Such compounds will be discussed in turn in each section, with reference to the viruses affected by the action of the compound against that particular target.



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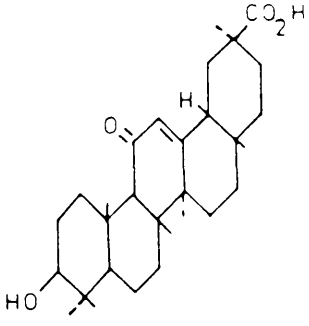

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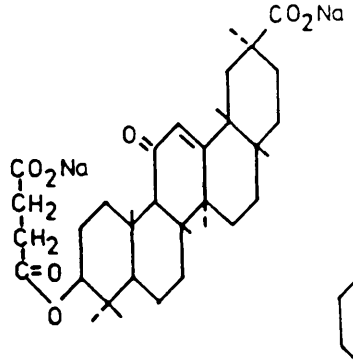

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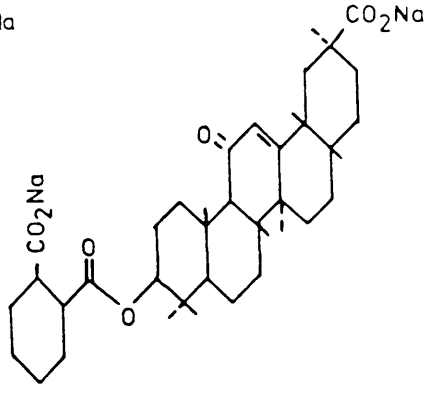


Cicloxolone sodium

Figure 1

Structure and derivation of Cicloxolone Sodium.

1.1.1. COMPOUNDS WHICH AFFECT EARLY PRE-SYNTHETIC STEPS IN THE VIRUS LIFE CYCLE

Any virus infecting a cell, binds to the surface membrane, enters the cell and must shed at least part of its protein coat, before the synthetic steps e.g. DNA/RNA synthesis, protein synthesis, in the life cycle can occur. Agents which have been shown to effect these pre-synthetic steps are shown in Table 1.

OLIGOPEPTIDES

The penetration of both paramyxo and orthomyxoviruses into a cell, requires the cleavage of certain of their glycoproteins, which activates the fusion of the cellular and viral membranes. A deeper understanding of this process led to the design of oligopeptides as anti-paramyxo and orthomyxovirus agents.

PARAMYXOVIRUS

Two glycoproteins HN_A and F_A are associated with the paramyxovirus envelope. The HN protein (m.wt. 65-70K), is present on the surface as a dimer, held together by disulphide bonds in the hydrophilic region and hydrophobic bonds at the base of the protein (Scheid et al., 1978). The isolated HN protein has receptor binding activity which is manifested by haemagglutination when the erythrocyte is used as the target cell, and neuraminidase activity which is capable of destroying the receptors for the virus (Scheid et al., 1972; Scheid and Choppin, 1973).Thus the HN protein is responsible for the first step in infection, the adsorption of the virus to the cell. Because neuraminic acid containing macromolecules that can act as receptors for paramyxo or orthomyxoviruses are ubiquitous on the surfaces of vertebrate cells, the adsorption of these viruses to receptors is not a step in replication that plays an important role in determining host range or tropism, in contrast to the situation with some other viruses, such as picornaviruses with which virus-receptor interactions play a decisive role in host cell specificity (Crowell, 1980). Thus the HN protein of paramyxoviruses, although it mediates the important step of virus adsorption, is not a significant factor in determining host range, tropism and virulence. In contrast, the other viral surface protein, F, plays a crucial role in these viral properties.

The evidence for the biological activity of the F protein came initially from studies showing that proteolytic cleavage of a precursor protein Fo, to yield two polypeptides F_1 and F_2 , activated virus-induced cell fusion and haemolysis, and the initiation of infection of the step of virus penetration , which is accomplished by fusion of the viral and cell membranes (Homma and Ohuchi, 1973; Scheid and Choppin, 1974, 1976, 1977). Each of these biological activities, virus penetration, cell fusion and haemolysis reflects the ability of the F protein to cause membrane fusion. The proteolytic cleavage that activates the F protein is normally accomplished by a host protease and some cells lack an enzyme to cleave the F protein of certain viruses. For example, bovine kidney (MDBK) or mouse fibroblast (L) cells lack an enzyme capable of cleaving the Sendai virus Fo. These cells produce non-infectious virions that are incapable of causing cell fusion and haemolysis. These biological properties can be activated *in vitro* by cleavage of the F protein with trypsin. Mutants have been isolated that require different proteases from wild type (wt) viruses for activation, and that exhibit a different host range at levels of both cultured cells and the chick embryo (Scheid and Choppin, 1976).

The proteolytic cleavage of Fo, m.wt. 65K, yields two disulfide linked polypeptides (F_1 and F_2), with m.wt. of 50K and 15K respectively (Scheid *et al.*, 1972; Homma and Ohuchi,1973; Scheid and Choppin, 1974, 1976, 1977). The C terminus of Fo, which is the C terminus of the F_1 polypeptide, is embedded in the viral membrane, and the activating cleavage generates a new N terminus on F_1 (Scheid and Choppin, 1977; Gething *et al.*, 1978). The original N terminus of Fo is the N terminus of F_2, and F_2 is linked to F_1 by disulphide bonds.

The new N terminal region of F_1, is highly conserved among different viruses. The amino acid sequences of three different paramyxoviruses (Scheid *et al.*, 1978; Gething, White and Waterfield, 1978; Richardson, Scheid and Choppin, 1980) are shown in Table 2. In the first twenty amino acids there are only six positions at which differences occur among the three viruses shown in Table 2, and in each case one hydrophobic amino acid is substituted for another. This

region is also highly hydrophobic; there are no charged amino acids in the first twenty residues in each virus, and the first charged amino acid detected does not appear until residue 26.

The findings that the biological activities of the F protein (virus penetration, virus-induced cell fusion and haemolysis), appeared when the new N-terminus on the F_1 polypeptide was generated, and that the primary structure of this region is highly conserved and furthermore hydrophobic, suggested that this region of the molecule was involved in the membrane fusing activity of the protein (Scheid and Choppin, 1977; Scheid et al., 1978). The highly hydrophobic nature of this region raised the possibility that it could interact with the lipid bilayer of the target membrane (Scheid et al., 1978; Gething, White and Waterfield, 1978; Richardson, Scheid and Choppin, 1980;). Such a hydrophobic interaction could result in the F_1 polypeptide facilitating fusion by bringing the bilayers of the cell and viral membrane together i.e. the N-terminus of F_1 would be inserted into the cell membrane while its C -terminus was anchored in the viral membrane. This hypothesis was supported by the finding of a conformational change in the F protein upon cleavage .

The characteristics of the F protein discussed therefore suggested that the N-terminus of F_1 was directly involved in membrane fusing activity. On the basis of these findings, Choppin, Richardson and Scheid (1984), synthesised oligopeptides that mimicked the N-terminal region of the F_1 polypeptide, designed to competitively inhibit the action of the protein. A large number of oligopeptides were synthesised, using the sequence of the N-terminus of Sendai virus as the primary model, but varying several parameters, including the sequence and steric configuration of the amino acids and various substitutions on both the N and C-terminal amino acids of the oligopeptides (Richardson, Scheid and Choppin, 1980, 1981; Richardson and Choppin, 1983; Choppin Richardson and Scheid, 1983). Some of these oligopeptides were tested against measles, canine distemper, SV5 and Sendai viruses, and oligopeptides with the appropriate structure were found to inhibit the membrane fusing activity of the F protein, as manifested by virus penetration and

virus-induced cell fusion and haemolysis. Although cell fusion and haemolysis were also inhibited, the most sensitive and efficient method of quantitating inhibitory activity, was to employ a plaque assay to determine inhibition of infectivity at the level of virus penetration. African green monkey kidney (CV-1) cells were used for most assays, and in each experiment the ability of various concentrations of the oligopeptide to inhibit plaque formation was determined. Dose-response curves were obtained and 50% effective inhibitory concentrations were derived from these curves. It is very important to note that these experiments were performed under conditions in which the F protein is cleaved i.e. the oligopeptides do not act as protease inhibitors but as inhibitors of the membrane fusing activity of the F protein (Richardson, Scheid and Choppin, 1980, 1981; Richardson and Choppin, 1983; Choppin, Richardson and Scheid, 1983). Measles virus was found to be the most sensitive to inhibition by the oligopeptides, and therefore it was chosen for extensive structure-activity studies, which yielded the following information:
1.)Oligopeptides with the correct amino acid sequence are highly active specific inhibitors. The longer the oligopeptide the more effective it is.

2.)The addition of certain hydrophobic groups to the N-terminal amino acid significantly increased activity e.g. carbobenzoxy group (Z), t-butylocarbonyl group.
3.)Esterfication of the C-terminal amino acid with a methyl group decreased activity.

4.)The steric configuration of the first and second amino acids was found to significantly affect activity e.g. Z-D-Phe-L-Phe-Gly was more active than Z-L-Phe-L-Phe-Gly- or Z-D-Phe-D-Phe-Gly. Table 3 shows the 50% effective concentrations derived from dose-response curves carried out with different oligopeptides which illustrate these points. The reason for the effects on the inhibitory activity of the additions at the termini of the oligopeptides is not yet clear, however the added groups may have an effect on the positioning of the oligopeptide at its site of action. The carbobenzoxy and dansyl groups add hydrophobicity to the N-terminus, and the esterification of the C-terminus decreases the polarity of the peptide. Such changes could

affect the orientation of the peptide at its site of action at the membrane. The effect of the steric configuration of the amino acids is also not completely understood. The observed greater inhibitory activity of an oligopeptide with D-phenylalanine at the N-terminus, as compared to the naturally occurring L-amino acid may in part be explained by protection of the peptide from proteolytic activity. However, this is probably not the only factor involved, because the same structure-activity relationship was found when inhibition of haemolysis was used to assay activity, and proteolytic digestion of the peptide is less likely to occur in a short period of time in a haemolysis assay than in a plaque assay in cultured cells which requires several days. It therefore appears that the steric configuration of the terminal amino acids has an effect on inhibitory activity per se.

Three approaches were adopted to determine the precise site of action of the oligopeptides. Firstly, oligopeptides labelled with ^{3}H or ^{125}I were reacted at $4^{o}C$ with purified virus, mock-infected cells or infected cells, and after washing, samples were assayed for radioactivity to quantitate binding. Such studies suggested that oligopeptides bound to cells, whether infected or uninfected, but not to virus (Choppin, Richardson and Scheid, 1983; Richardson, Scheid and Choppin, 1983). Secondly oligopeptides, such as Z-PPGCK, were synthesised that could bind irreversibly. Z-PPGCK was preincubated with monkey erythrocytes (rbc), or rbc plus adsorbed measles virus, or purified measles virus, and after extensive washing, haemolysis assays were performed (Richardson, Scheid and Choppin, 1983). It was found that Z-PPGCK irreversibly inhibited haemolysis when it was pre-incubated with rbc, or rbc plus virus, but not when it was pre-incubated with virus alone, indicating again that the oligopeptides act on the cell and not on the virus. The third approach, involved the use of an oligopeptide inhibitor containing an azido group as a photoaffinity probe i.e. Z-D-Phe-L-Phe-(azido)Phe(Z-APPP). This peptide retained activity when the azido group was added. rbc or measles virus were incubated with this peptide and exposed to ultraviolet light for various times to cross link the

oligopeptide *in situ.* The samples were then washed extensively, and haemolysis assays carried out. Pre-treatment of virus with Z-APPP had no effect, but pre-treatment of rbc with this oligopeptide inhibited haemolysis (Richardson, Scheid and Choppin, 1983). These studies therefore show, that the site of action is on the target cell membrane, not on virus. The results further suggest, that the inhibitors compete with the N-terminus of the F_1 polypeptide for a site on the membrane.

ORTHOMYXOVIRUSES

Influenza viruses (myxoviruses), also possess two glycoproteins that form spike-like projections on their surface; however, unlike paramyxoviruses, the haemagglutinating and neuraminidase functions of influenza viruses reside on separate proteins designated HA and NA respectively. The influenza virus HA protein serves two functions; receptor binding and penetration. Like the F protein of paramyxoviruses, the HA protein of influenza virus is cleaved by a host cell protease to yield two disulfide bonded subunits (Lazarowitz, Compans and Choppin, 1971). This cleavage has no effect on haemagglutinating activity (Lazarowitz, Goldberg and Choppin, 1973), but it activates the infectivity of the virus (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975) at the level of viral penetration. Furthermore, not only is the membrane fusing activity of the HA protein activated by cleavage, as is the F protein but also the amino acid sequence of the new N-terminus generated on the HA_2 polypeptide by cleavage, resembles that of the paramyxovirus F_1, except that glycine is the N-terminal amino acid. The structural and functional similarities between the HA and F polypeptides, led Choppin, Richardson and Scheid (1983), to synthesise oligopeptides that mimicked the N-terminus of the HA_2 polypeptide, and to test their ability to inhibit influenza virus replication. With influenza virus also, inhibition was obtained that was amino acid sequence specific. As shown in Table 4, oligopeptides that mimicked the N-terminal sequence of the HA_2 polypeptide of influenza A and B viruses (Z-Gly-L-Leu-L-Phe-Gly and Z-Gly-D-Phe-L-Phe-Gly, respectively) were active against influenza virus, but inactive against measles virus, whereas the reverse was true

with Z-D-Phe-L-Phe_Gly, which resembles the N-terminus of paramyxovirus.

Although oligopeptides of this type have proved successful against paramyxo and orthomyxoviruses *in vitro*, good *in vivo* efficacy has not been reported.

LEUPEPTIN

The peptide leupeptin (acetyl-L-leucyl-L-argininal) is produced by actinomycetes, which is known to inhibit specifically trypsin, plasmin, papain, kallikrein and cathepsin B (Aoyagi and Umezawa, 1975). Recently leupeptin was shown to suppress the replication of influenza (A/swine/1976/31 H1N1) and also the development of fatal pneumonia in mice co-infected with the virus and staphylococcus aureus (Tashiro, Klenk and Rott, 1987). As stated previously, the post-translational proteolytic cleavage of the viral haemagglutinin glycoprotein by cellular proteases is essential for infectivity, spread of the virus in the host organism and pathogenicity (Rott and Klenk, 1986). In a host cell where an appropriate protease for cleavage is not present, and infectious virus cannot be produced, infectivity can be recovered by trypsin treatment of non-infectious virus *in vitro* (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975). It has been shown that Staphylococcus aureus, which is most frequently isolated from patients with influenza pneumonia (Stuart-Harris, Schild and Oxford, 1985), secretes serine proteases that activate the infectivity of several influenza A viruses by proteolytic cleavage of the haemagglutinin (Tashiro *et al.*, 1987a and b). The presence of the bacterial enzyme enables the virus to undergo multiple replication cycles in host cells that do not contain an activating protease. Co-infection of mice with influenza A and S. Aureus, resulted in extensive lesions and an enormous increase of virus titre in the lung and death of the mice.

Leupeptin inhibits this activation by staphylococcal protease both in cell culture and in mice (Tashiro, Klenk and Rott, 1987). If chicken embryo cells (CE) were infected with influenza A (swine/1976/, H1N1), at an m.o.i. of 10^{-2} p.f.u./cell, progeny virus grew efficiently only in the presence of staphylococcal proteases (Tashiro *et al.*, 1987a

and b). Leupeptin could prevent multistep replication of virus when added in addition to staphylococcal proteases, the inhibitory effect dependent on the leupeptin dose: 100ug/ml leupeptin resulted in a 5 log reduction in virus titre, as determined by haemagglutination.

Mice co-infected with the virus and Staphylococcus aureus were treated with leupeptin several times a day over a 6 day period. Compared with control animals there was a significant reduction in infectious virus titre in the lung, reduction in the level of lung lesions and an improved survival rate.

Unlike the oligopeptides discussed earlier, leupeptin was proposed to act by directly inhibiting protease activity. Several lines of evidence supported this proposal. Levels of bacteria in mice treated or untreated with leupeptin were equal and although inhibition of bacterial protease production cannot be ruled out, it is pertinent that the infectivity of virus isolated from mice treated with leupeptin could be enhanced by trypsin treatment. This was not the case for virus isolated from untreated mice. Since the haemagglutinin of influenza virus has an arginine at its cleavage site, leupeptin would be expected to inhibit cleavage activation by the bacterial proteases, and polyacrylamide gel electrophoresis has shown this to be the case (Tashiro, Klenk and Rott, 1987). Whether this inhibition is selective for cleavage by staphylococcal enzymes, or affects cellular proteases also remains to be determined. Preliminary experiments confirmed that prolonged intranasal administration was not toxic and protected mice from developing severe pneumonia and death (Tashiro, Klenk and Rott, 1987). These observations suggest a possible way for theraputic treatment of influenza pneumonia by leupeptin or similar trypsin inhibitors, particularly in infections with bacteria which are resistant to antibiotics.

AMANTIDINE AND RIMANTIDINE

Amantidine (1-aminoadamantane hydrochloride, Fig 2a), selectively inhibits influenza A viruses (Davies et al., 1964). Many clinical trials have been conducted in man which show amantidine to be effective both prophylactically and therapeutically against influenza A but not B (Oxford and Galbraith, 1980; Galbraith, 1985). The drug was licensed in 1976 for use against all influenza A subtypes, although its use has been limited, except in the Eastern block. Rimantidine (alpha-methyl-1-adamantane methylamine hydrochloride, Fig 2a), is as effective as amantidine, but with fewer side effects (Dolin et al., 1982), and this drug has been used extensively in the U.S.S.R.

While amantidine resistant variants can readily be obtained from tissue culture (Cochran et al., 1965; Appleyard, 1977), or from laboratory animals (Oxford, Logan and Potter, 1970), there is little information regarding the occurence of amantidine resistant viruses in the human population or their emergence as a result of clinical use of the drug. Therefore, information regarding the mode of action of amantidine has been obtained by investigation of resistant mutants isolated by passage in the presence of amantidine in tissue culture. Amantidine has been shown to exert two different actions depending on the concentration used (Hay and Zambon, 1984; Hay et al., 1986).

INHIBITION BY HIGH CONCENTRATIONS (0.5mM)

The inhibition of virus infection by high concentrations of amantidine, of the order 0.5mM, is not specific to influenza virus; it is seen with a number of enveloped RNA viruses which enter cells by endocytosis (Wallbank, Matter and Klinikowski, 1966; Skehel, Hay and Armstrong, 1978; Helenius et al., 1980). Nor is such inhibition a property peculiar to amantidine, since a variety of amines, including for example, methylamine and ammonium chloride exhibit similar effects (Jensen and Liu, 1963; Helenius, Marsh and White, 1982; Yoshimura et al., 1982; Hay and Zambon, 1984). These high drug concentrations are cytotoxic and from the clinical standpoint do not reflect the highly restricted spectrum of antiviral activity in vivo.

At these concentrations, the drugs cause an elevation

in the pH of endosomes. Many viruses, including influenza enter cells via receptor mediated endocytosis. The subsequent fusing of viral and endosomal membranes, initiating virus infection is pH dependent, lying between 5 and 6 depending on the virus strain. Therefore raising the pH in these compartments, successfully blocks the interaction between viral and cellular membrane and so blocks initiation of infection.

Influenza virus resistance to high concentrations of amantadine, maps to the haemagglutinin (HA) gene (Hay et al., 1986). As mentioned previously, influenza virus (orthomyxovirus) penetration is dependant on the cleavage of HA by a protease, activating the fusion of viral and cellular membranes. Resistant mutants may be explained by a change in the structure of HA which allows fusion to proceed at a higher pH. The nature and location of such mutations suggests a lowering of the energy barrier to the conformational change leading to the fusion active state (Daniels et al., 1985).

SELECTIVE ACTION OF LOW CONCENTRATIONS

The influenza A specific action of low concentrations of amantadine correlates with selective activity in vivo, optimum inhibition varying from 0.3uM to 5uM (Hay et al., 1986). The stage of virus replication inhibited varies between virus strains. Human influenza A viruses for example, A/Singaporel/57, H2N2, appear to be inhibited at an early stage since drug must be present prior to infection and primary transcription is inhibited (Hay and Zambon, 1984). This is in contrast with data obtained from two avian strains, Rostock H7N1 and Weybridge, where early events are not affected, and virus inhibition occurs when the drug is added later suggesting that a late stage of virus replication, probably assembly is blocked (Hay and Zambon, 1984; Hay et al., 1986). However, all amantadine resistant variants following passage in 5uM amantadine, contained a mutation in their M gene, leading to a single amino acid substitution in the M2 protein, whereas less than a quarter of such mutants had a change in the amino acid sequence of the HA (Hay et al., 1985). Resistance is consistently transferred with M2 and the optimum inhibitory concentration reflects the parental M gene. However, in addition the HA

gene modifies the degree of susceptibilty and influences which stage (early or late) is inhibited. Amantidine treatment of Rostock-infected cells greatly reduced the membrane expression and antigenicity of HA; an effect which is relieved in resistant mutants (Hay et al., 1986). Therefore, both the HA and M2 proteins are implicated, suggesting that amantidine interferes with their functions or with interactions between the two proteins, in one case leading to a block in the initial stages of the virus life cycle and in the other inhibiting later stages in infection, most probably assembly.

ARILDONE AND RELATED COMPOUNDS, WIN 51711 AND WIN 52084

Arildone (Fig 2b), is a lipophilic B-diketone, which displays some selectivity against a number of RNA and DNA viruses in cell culture (Diana et al., 1977). It has been shown to have particular effect against poliovirus both in cell culture (McSharry, Caliguiri and Eggers, 1979) and in mice (McKinlay et al., 1982). Arildone blocks cell mediated uncoating (Fig 3b) and also protects the virus capsid from inactivation by host and alkali (McSharry et al., 1979). Unfortunately the rapid metabolism of this drug in man, makes it unlikely to be useful as a chemotherapeutic agent (Eggers, 1985). A structure-activity based programme of research, starting from arildone, led to the development of compounds WIN 51711 and WIN 52084 (Fig 2b), which inhibit picornavirus replication in tissue culture and in animal models of human enterovirus disease (Smith et al., 1986). Disappointingly, no significant effect was observed when WIN 51711 was evaluated clinically using a human rhinovirus challenge in man (Diana and Otto, 1985).

X-ray diffraction studies of WIN 51711 and WIN 52084 bound to crystallised human rhinovirus 14 (HRV-14), revealed the site of attachment and information regarding the mode of action (Smith et al., 1986). The 3-dimensional structure of HRV 14 has been described in atomic detail (Rossman, 1985). The three larger structural viral proteins (VP1, VP2 and VP3), form the exterior of the viral capsid, while VP4 is at the interface between the capsid and the RNA. VP1, VP2 and VP3 are each folded into an 8 stranded B-barrel and are organised in the capsid with a pseudo T=3 surface lattice (Caspar and Klug, 1962).Four types of neutralizing antibody binding sites were found at insertions in the B-barrel structure, on the extreme surface of the virus, surrounding a 25 A deep canyon, on the viral surface. Each canyon encircles one of the 12 vertices of the virus and has been proposed to be the site of receptor binding (Rossmann, 1985). VP1 is the major structural contributor to the walls and the floor of the canyon. The canyon floor is formed by relatively conserved sequences of known picornaviruses, while the neutralizing antibody sites are at hypervariable regions of the viral capsid proteins. The compounds WIN 51711 and WIN 52084, consist of a 3-methylisoxazole group

that inserts itself into the hydrophobic interior of the VP1 B-barrel and a 4-oxazolinylphenoxyl (OP) group that covers the entrance to an ion channel in the floor of the canyon (Fig 3a). The functioning of the antiviral compounds might be compared to that of a nicotinamide adenine dinucleotide (NAD) cofactor in dehydrogenases (Adams, 1973; Holbrook et al., 1975). The adenine end of NAD binds into a hydrophobic pocket and enhances protein stability just as it is envisaged that the methylisoxazole group of the WIN compounds might increase viral stability by filling the empty hydrophobic pocket within the VP1 B-barrel (Argos et al., 1979). The nicotinamide of NAD is functionally necessary for enzymic catalysis whereas the OP group of the WIN compounds perhaps blocks ions from entering the virion through the pore on the floor of the canyon, thus decreasing the permeability of the virion to caesium ions. Alternatively, the WIN compounds might lock the conformation of VP1 such that the virus cannot disassemble by collapsing the hydrophobic pocket (Fig 3b).

That the binding of these compounds is responsible for their antiviral activity is supported by two lines of evidence. Firstly, the different effects of compounds against individual human rhinovirus (e.g. HRV 2 and HRV 14), can be explained in terms of the amino acid composition of the structural viral proteins in their viral capsid. For example, the minimum inhibitory concentration (MIC) (concentration which reduces plaque numbers by 50% in cell culture), of WIN 51711 for HRV 14 is 0.4uM compared with 3.8uM for HRV 2 (Smith et al., 1986). The most significant difference in the WIN binding pocket between HRV 14 and HRV 2 is that Val^{188} and Val^{191} are replaced by leucine. This is likely to cause steric hindrance to the insertion of the isoxazole group and aliphatic chain into the hydrophobic pocket which would account for the increase in MIC. Secondly, the antiviral activity of the compounds can also be altered by modifications to the structure, including the length of the aliphatic chain and substitutions in the oxazoline and phenolic rings. The effects of these modifications are shown in Table 5. Any decrease in MIC (i.e. improvement in antiviral activity), in each case can be explained in terms of improved binding.

LENGTH OF ALIPHATIC CHAIN

The optimal length of the aliphatic chain $(CH_2)_n$ for HRV 14 inhibition is when n is either 5 or 7 (Table 5). Less than 5 would probably reduce the hydrophobic binding within the pocket and cause steric hindrance to the isoxazole group in the more restricted portions of the binding site; n greater than 7 would cause severe steric hindrance as the isoxazole group is pushed further into the hydrophobic pocket and the aliphatic chain is severely buckled; n even would require cis bonds in the aliphatic chain to keep the isoxazole group (assuming the same binding of the OP group) out of sterically hindered positions.

SUBSTITUTION ON THE OXAZOLINE GROUP

The asymmetric carbon atom in the oxazoline ring of WIN 52084 is clearly important because the S isomer is ten times more active than the R isomer (Table 5). The result can be explained by improved hydrophobic interactions of the S methyl group with a hydrophobic pocket formed by Leu^{106} and Ser^{107}, which would stabilise the conformation of the oxazoline ring thereby allowing for a favourable orientation for hydrogen bonding to Asn^{219}. The R methyl group would be expected to have no positive effect on hydrophobic interactions consistent with similar MIC values obtained for the R methyl compound and the desmethyl compound WIN 51711.

SUBSTITUTIONS ON THE PHENOLIC GROUP

The antiviral activity can be improved by introducing suitable additional hydrophobic groups onto the phenyl group (Table 5). Larger hydrophobic groups in position 2 increase the efficacy against HRV 2 possibly because of a better interaction with a phenylalanine which replaces the Leu^{116} of HRV 14. Larger hydrophobic groups have little effect when the compounds are tested on HRV 14 where these aromatic groups are absent. (Comparative observations are available only when the aliphatic chain is of length n=5). The explanation of the MIC observations is at present entirely qualitative, but could be quantitated using energy calculations of various compounds bound to the conformationally changed virion and by checking the resultant predicted binding mode with X-ray diffraction observations, thereby comparing directly, binding and antiviral activity.

This study amounted to the first description of the interaction of an antiviral drug with a virion at atomic resolution. The results offered plausible mechanisms for the biological activity of this class of compounds and raised the possibility of antiviral drug design for rhinoviruses as well as other picornaviruses.

FLAVANOIDS AND CHALCONES

DICHLOROFLAVAN, CHALCONE AND RMI-15,731

Dichloroflavan (4',6-Dichloroflavan), chalcone (4'-Ethoxy-2'-hydroxy-4, 6'-dimethoxychalcone), and RMI-15,731 (1-(5-Tetradeayloxy-2-furanyl)-ethanone) (Fig 2c), all exhibit antirhinovirus activity in cell culture (Ishitsuka, Ninomiya and Suhara, 1986). A comparative study was performed with these three compounds to elucidate their mode of action. Interaction with the viral capsid protein was suggested because different serotypes of human rhinovirus (HRV), varied widely in their susceptibility to the compounds and the major differences between serotypes are in the the capsid proteins. In addition, all three compounds can directly inactivate HRV, but infectivity can be restored to the original level by removal of the bound compounds with chloroform.

The specific interaction of with HRV was confirmed by using radiolabelled chalcone. After exposing the HRV type 2 to [^{3}H] chalcone, the amount of the chalcone bound to the virus was assayed following centrifugation through a sucrose gradient. A distinct peak of [^{3}H] chalcone was observed at the position of the virus particles, indicating that the chalcone bound to the virus.This binding of radiolabelled chalcone could be completely inhibited by unlabelled chalcone and dichloroflavan, and to a lesser extent by RMI. The infectivity of the peak fractions could be shown only after extraction of chalcone with chloroform.(Ishitsuka, Ninomiya and Suhara, 1986).

HRV type 2 subtypes, which are resistant to chalcone, dichloroflavan or RMI, were isolated by serial passage of the virus in tissue culture in the presence of sub-lethal concentrations of these compounds. HRV sublines resistant to both dichloroflavan and RMI showed cross-resistance to chalcone and vice versa. These results again suggest that the modes of action of dichloroflavan and RMI are essentially the same as that of chalcone. However, since the sublines resistant to each of dichloroflavan and RMI were not cross resistant, the interaction site for dichloroflavan must be different from that for RMI. Chalcone binds to intact HRV type 2 particles at a specific site not found on particles degraded by treatment at pH 5 or 56^{o}C, or on viruses insensitive to the agents (Ninomiya et al., 1984).

Dichloroflavan and RMI competed with chalcone for binding at this specific site, indicating that their binding sites were very similar to that for chalcone.

Evidence against the conclusion that chalcone, dichloroflavan and RMI have the same mode of action is that these three agents differ significantly in their antiviral spectrum in HRV serotypes (Ninomiya et al., 1985). It is thought that the conformation of the binding site on the capsid may slightly differ between HRV serotypes. Some difference at the binding site was suggested by the fact that chalcone could bind to the HRV subline resistant to dichloroflavan, and that there was no cross resistance between dichloroflavan and RMI. The capsid conformation on some serotypes may favour one compound while that on other serotypes favours the other compounds.

Instability at acidic pH's or 56^{o}C is the most characteristic feature that distinguishes HRVs from other human picornaviruses (Stott and Killington, 1972; Newman, Rowlands and Brown, 1973). Exposure to acid pH or heating at 56^{o}C caused alteration in size, conformation and antigenicity of the virus (Korant et al., 1972, 1975; Lonberg-Holm and Yin, 1973). HRV bound to chalcone resists these alterations, through stabilisation of the virus conformation (Ninomiya et al., 1984). Infection of cells with rhinovirus is accompanied by the same type of alteration of the uncoating process as that observed by treatment at pH5 (Lonberg-Holm and Korant, 1972). Therefore, it seems likely that HRV, when bound to any of these compounds, is not able to undergo the normal process of uncoating in infected cells.

The mode of action is therefore the same as that proposed for arildone and related compounds. However, the activity of chalcone, DCF or RMI appears to be confined to rhinoviruses, while arildone, WIN 51711 and WIN 52084 display activity against a range of DNA and RNA viruses, particularly picornaviruses. X-ray diffraction studies of HRV bound to chalcone, DCF or RMI would yield valuable information regarding the mode of action and perhaps reveal the basis for this selectivity.

So far the results of clinical trials have proved disappointing. Rhinovirus 9 is particularly sensitive to

dichloroflavan, however, volunteers infected with this virus were not protected by oral administration of the compound (Phillpotts et al., 1983). Similarly, chalcone and the pro-drug (which was inactive but absorbed and then converted to the active form by loss of a phosphate group), also had no effect when administered orally to volunteers. These disappointing results, may be due to the fact that concentrations of flavan and chalcone attained in the nasal mucosal cells were not sufficiently high to afford protection (Phillpotts and Tyrrell, 1985), emphasising the need to explore better ways for their delivery (e.g. by fine particle aerosols).

FLAVONE AND ENVIROXIME

Flavone and enviroxime (Fig 2d), also exhibit activity against a wide range of rhinoviruses (Ishitsuka, Ninomiya and Suhara, 1986). Their mode of action is however unclear. It has been reported that flavone and enviroxime inhibit processes before the initiation of viral RNA synthesis (Ishitsuka et al., 1982; Delong and Reed, 1980). Unlike chalcone, dichloroflavan and RMI, flavone and enviroxime do not directly inactivate HRV particles and continue to be active against virus sublines resistant to chalcone, dichloroflavan and RMI. HRV sublines resistant to flavone or enviroxime could not be isolated (Ishitsuka, Ninomiya and Suhara, 1986). The protein conformation with which these two agents associate may be important for virus replication to an extent that amino acid sequences at the interacting sites are genetically conserved and that mutation of these sites is lethal. An alternative explanation is that these two agents affect host cell functions or factors which are essential for viral relication.

In 1981, Phillpotts et al., reported that enviroxime was effective prophylactically in trials against rhinovirus 9 in man, when delivered by mouth and nasal spray, with fewer colds and cold symptoms noted. However, adverse side effects, principally nausea and vomiting, attributed to the oral enviroxime, were unacceptably frequent. Intranasal therapy also proved ineffective, even prophylactically against rhinovirus type 4 (Levandowski et al., 1982) or type 39 (Hayden and Gwaltney, 1982).

COMPOUND	ANTIVIRAL SPECTRUM	TARGET/S	PROPOSED MODE OF ACTION
OLIGOPEPTIDES	PARAMYXO AND ORTHOMYXOVIRUSES	ON CELL MEMBRANE, INTERACTION SITE FOR VIRAL GP	PREVENTS INTERACTION OF VIRAL GLYCOPROTEIN AND CELL MEMBRANE, WHICH ACTIVATES FUSION OF VIRAL AND CELLULAR MEMBRANES THUS FACILITATING PENETRATION
LEUPEPTIN	INFLUENZA A	S.AUREUS PROTEASE (CELLULAR?)	INHIBITS PROTEASE ACTIVITY THEREBY PREVENTING CLEAVAGE OF VIRAL HA, NECESSARY TO ACTIVATE FUSION AND PENETRATION OF VIRUS
E-AMINO CAPROIC ACID	INFLUENZA	CELLULAR PROTEASE	AS LEUPEPTIN
AMANTIDINE, RIMANTIDINE	A RANGE OF ENVELOPED RNA VIRUSES (HIGH CONC. 0.5 uM) HUMAN INFLUENZA A (LOW CONC. 0.3uM)	CELLULAR ENDOSOMAL COMPARTMENT M AND HA PROTEINS	RAISES pH OF ENDOSOMAL COMPARTMENTS THEREBY BLOCKING pH DEPENDENT FUSION OF VIRAL AND CELLULAR MEMBRANES; BLOCKS PENETRATION NOT CLEAR; PROBABLY INTERFERES WITH FUNCTION/S OR INTERACTION BETWEEN THESE PROTEINS; BLOCKS EARLY EVENT (PENETRATION?) BEFORE PRIMARY TRANSCRIPTION
ARILDONE, WIN 51711, WIN 52084	PICORNAVIRUSES	VIRUS PARTICLE	BINDS TO VIRUS, BLOCKING UNCOATING OF VIRUS
DICHLORO-FLAVAN, CHALCONE AND RMI	RHINOVIRUSES	VIRUS PARTICLE	BINDS TO VIRUS, BLOCKING UNCOATING OF VIRUS
FLAVONE, ENVIROXINE	RHINOVIRUSES	?	NOT CLEAR; ACTS BEFORE INITIATION OF RNA SYNTHESIS

Table 1

Compounds which affect early pre-synthetic steps in the virus life cycle: their antiviral spectrum and proposed mode of action.

※Antiviral spectrum: viruses affected by the action of the compound against the particular target shown.

References: Oligopeptides (Choppin, Richardson and Scheid, 1983, 1984); Leupeptin and e-amino caproic acid (Tashiro, Klenk and Rott, 1987); Amantadine/Rimantidine (Hay and Zambon, 1984; Hay et al., 1986); Arildone/WIN 51711/WIN 52084 (McSharry et al., 1979; Smith et al., 1986); Dichloroflavan/Chalcone/RMI/Flavone/Enviroxime (Ishitsuka, Ninomiya and Suhara, 1986).

	1				5				10
Sendai	Phe	Phe	Gly	Ala	Val	Ile	Gly	Thr	Ile	Ala
SV5	Phe	Ala	Gly	Val	Val	Ile	Gly	Leu	Ala	Ala
NDV	Phe	Ile	Gly	Ala	Ile	Ile	Gly	Gly	Val	Ala

	11				15				20
Sendai	Leu	Gly	Val	Ala	Thr	Ala	Ala	Gln	Ile	Thr
SV5	Leu	Gly	Val	Ala	Thr	Ala	Ala	Gln	Val	Thr
NDV	Leu	Gly	Val	Ala	Thr	Ala	Ala	Gln	Ile	Thr

Peptide	50% Effective Concentration uM
Z-D-Phe-L-Phe-Gly-D-Ala-D-Val-D-Ile-Gly	0.02
Z-D-Phe-L-Phe-Gly	0.20
Z-D-Phe-L-Phe	28
Z-L-Phe-L-Ser	141
Z-Gly-L-Phe-L-Phe	530
Z-D-Phe-L-Phe-Gly	0.20
DNS-D-Phe-L-Phe-Gly	0.34
t-BOC-D-Phe-L-Phe-Gly	2.0
D-Phe-L-Phe-Gly	180
Z-D-Phe-L-Phe-Gly(methyl ester)	20
Z-D-Phe-L-Phe-Gly	0.20
Z-D-Phe-D-Phe-Gly	10
Z-L-Phe-L-Phe-Gly	23
Z-D-Phe-L-Phe-Gly(chloromethylketone)	0.20
Z-D-Phe-L-Phe-L(azido) Phe	0.28

Table 2

N-terminal amino acid sequences of the F polypeptides of three paramyxoviruses.

Underlining indicates residues that differ from the residue in that position in Sendai virus (from Chopin et al., 1983).

Table 3

Inhibition by oligopeptides of plaque formation by Measles virus (from Chopin et al., 1983).

Z indicates a carbobenzoxy group; DNS, a dansyl group; t-BOC, a tertiary butoxylcarbonyl group.

PEPTIDE	50% EFFECTIVE CONCENTRATION uM	
	INFLUENZA A	MEASLES
Z-GLY-L-LEU-L-PHE-GLY	20	1000
Z-GLY-L-PHE-L-PHE-GLY	53	870
Z-GLY-D-PHE-L-PHE-GLY	23	-
Z-D-PHE-L-PHE-GLY	290	0.2

COMPOUND	Z1	Z2	POSITION 2	POSITION 3	n	HRV14	HRV2
OPTIMUM LENGTH OF ALIPHATIC CHAIN							
WIN 53670	H	H	H	H	4	Inactive	7.3
WIN 52035	H	H	H	H	5	0.7	1.1
WIN 53337	H	H	H	H	6	2.9	2.1
WIN 51711	H	H	H	H	7	0.4	3.5
WIN 53455	H	H	H	H	8	3.9	5.9
SUBSTITUTION OF THE OXAZOLINE GROUP							
WIN 51711	H	H	H	H	7	0.4	3.8
WIN 52084 S/R	H	CH3	H	H	7	0.06	0.11
WIN 52084 S	H	CH3	H	H	7	0.04	0.08
WIN 52084 R	H	CH3	H	H	7	0.4	1.7
WIN 52193	CH3	CH3	H	H	7	0.1	1.5
SUBSTITUTIONS ON THE PHENOLIC GROUP							
WIN 52035	H	H	H	H	5	1.2	1.0
WIN 54089	H	H	CH3	H	5	0.8	0.09
WIN 53338	H	H	CL	H	5	2.5	0.06
WIN 54274	H	H	H	CH3	5	1.6	1.2
WIN 54090	H	H	H	CL	5	0.3	0.9

Table 4

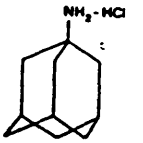
Comparison of the inhibition of Influenza A and Measles viruses by different oligopeptides (Chopin et al., 1983).

- NOT DETERMINED

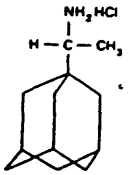
Table 5

Effect of modifying the structure of WIN 51711 and WIN 52084 on anti-HRV 14 and anti-HRV 2 activity: the numbers shown are "minimal inhibitory concentrations (MIC)", which is defined as the concentration which reduces plaque numbers by 50% in cell culture (from Smith et al., 1986).

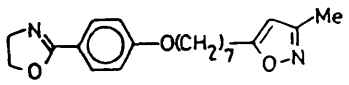
a



1-Adamantanamine hydrochloride (Amantadine)

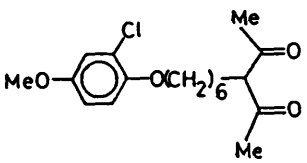


Rimantadine

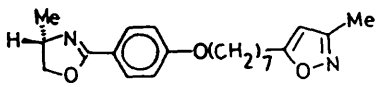


WIN 51711

b



Arildone

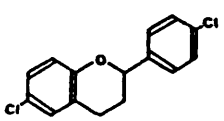


WIN 52084

Ro 09-0410

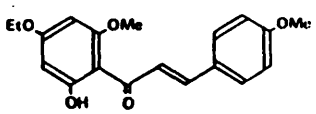
(4'-Ethoxy-2'-hydroxy-4,6'-dimethoxychalcone)

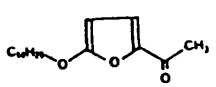
c



Dichloroflavan

(4',6-Dichloroflavan)

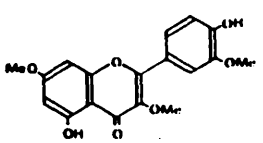




RMI-15,731

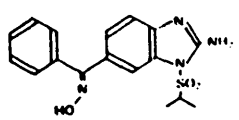
(1-(5-Tetradecyloxy-2-furanyl)-ethanone)

d



Ro 09-0179

(4'5-Dihydroxy-3,3'7-trimethoxyflavone)



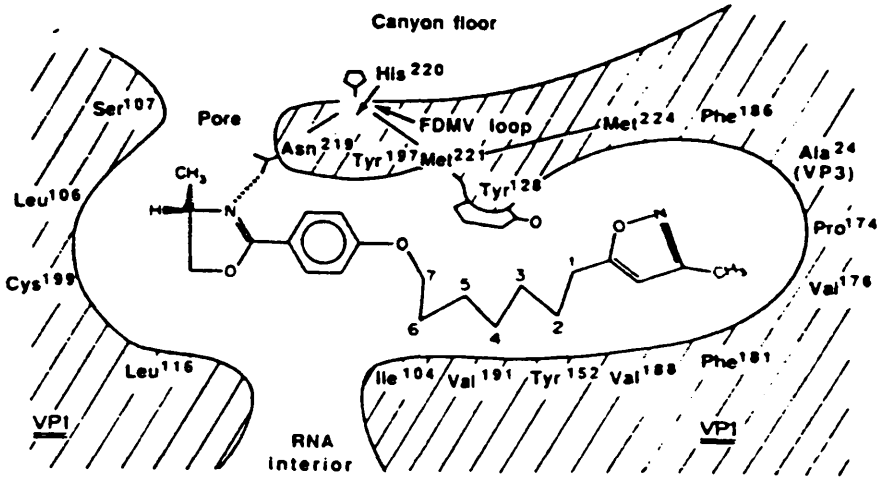
Enviroxime

(Anti-6-[(hydroxyimino)phenylmethyl]-1-[(1-methylethyl)sulfonylimidazol-2-amine)

Figure 2

Structure of compounds which affect early pre-synthetic steps in the virus life cycle.

a


Canyon floor
His 220
Ser 107
Pore
FDMV loop
Met 224
Phe 186
Asn 219
Tyr 197
Met 221
Ala 24
(VP3)
Tyr 128
Leu 106
Pro 174
Cys 199
Val 176
Leu 116
Ile 104
Val 191
Tyr 152
Val 188
Phe 181
VP1
RNA
Interior
VP1

b

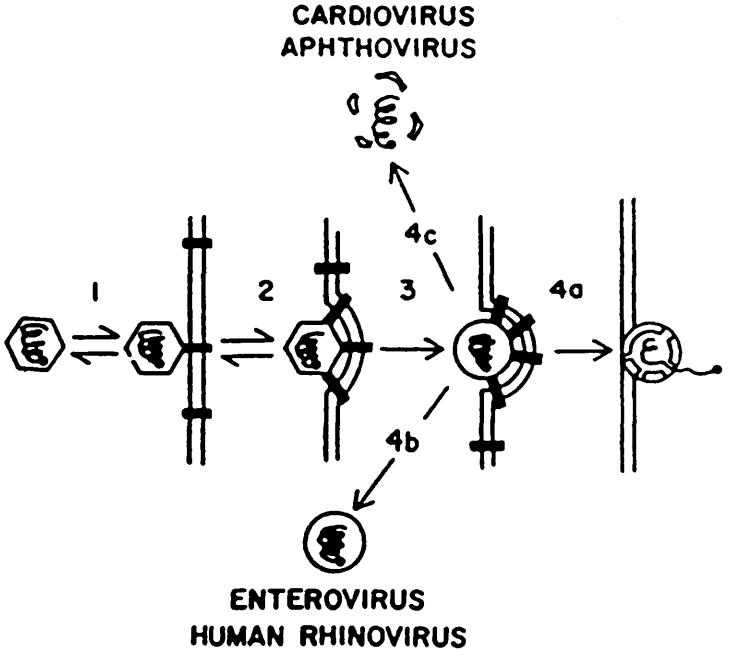

CARDIOVIRUS
APHTHOVIRUS
4c
1
2
3
4a
4b
ENTEROVIRUS
HUMAN RHINOVIRUS

Figure 3a

Diagrammatic representation of WIN compound binding site (from Smith et al., 1986).

Figure 3b

Model for attachment of picornaviruses, dismantling of the protective protein shell, and delivery of the RNA to a site where translation can begin.

1: Loose reversible attachment of antireceptor on the virion surface to one of the membrane-associated receptor units drifting in the liquid plasma membrane.

2: Attachment becomes tighter as mobile receptor units are recruited from the membrane surface: this process simultaneously draws the membrane around the virion to begin the invagination process.

3: Conformational alteration to the protein shell as indicated by susceptibility of the particle to digestion with proteases and loss of VP4 to form "A particles".

4a: Entry of particle, or delivery of the RNA genome, into the cytoplasm; this process is accompanied by many abortive events including elution or sloughing into the medium of A particles in the cases of polio, coxsackie, echo and human rhinoviruses (step 4b); or release of free RNA and 12-14S protein subunits in the case of cardio and apthoviruses, whose shells are less stable (step 4c). (from Boulanger and Lonberg-Holm, 1981).

1.1.2. COMPOUNDS WHICH AFFECT TRANSCRIPTION AND/OR PROCESSING OF mRNA

RIBAVIRIN

Ribavirin (Fig 4a) was first synthesised in 1972, and was reported to have activity against a range of RNA and DNA viruses *in vitro* (Sidwell *et al*., 1972). (Table 7). Its mode of action is not entirely clear and cannot be elucidated from resistant mutants, as these have not been isolated to date. There are several possible reasons for the inability to isolate mutants; the change required for relief from inhibition invariably leads to a lethal mutation in the virus (perhaps this locus has multiple functions); there may be several targets for drug action such that simultaneous mutations in several loci are required; or this may reflect a lack of selective toxicity in the mode of action.

Upon entering the cell, ribavirin is rapidly phosphorylated by cellular adenosine kinase to its' 5' monophosphate and then further phosphorylated to its' 5' triphosphate presumably by cellular enzymes. Ribavirin and its' 5' phosphate derivatives are unique in that the amido group which should be preferably coplanar with the triazole ring, can be rotated so that ribavirin resembles adenosine for phosphorylation by adenosine kinase, or as in ribavirin 5' triphosphate (RTP), the carbox-amido group can be rotated to resemble either GTP or ATP. The monophosphate form of ribavirin has been shown to inhibit IMP dehydrogenase, the enzyme responsible for the synthesis of GMP from IMP, resulting in reduced levels of GTP pools (Streeter *et al*., 1973). The triphosphate form of ribavirin is thought to inhibit the action of RNA polymerase and in the case of influenza RNA polymerase, RTP was shown to compete with both GTP and ATP, for the substrate site. Ribavirin has also been shown to have activity against a range of retroviruses, including HIV (Sidwell *et al*., 1975; Shannon, 1977; Jenkins and Chen, 1981; McCormick *et al*., 1984). Furthermore, McCormick and co-workers (1984), reported considerable lowering of reverse transcriptase activity in the presence of the drug.

The structure of ribavirin, as noted by single crystal X-ray studies of Prusiner and Sundaralingam (1973), is strikingly similar to guanosine (Fig 4a), with the carbonyl

oxygen and the amide nitrogen occupying stereochemically similar positions to the carbonyl oxygen and the amide ring in guanosine. The antiviral effect of ribavirin against measles in Vero cells is partially reversed by guanosine (Streeter et al., 1973). Guanosine also reverses the inhibition of haemagglutinin produced by influenza A virus in MDCK cells. Ribavirin 5' monophosphate is 1000 times more potent than GMP acting as a feedback inhibitor of IMP dehydrogenase (Streeter et al., 1973) ($Ki=2.5 \times 10^{-7}$ compared to $Ki=2.2 \times 10^{-4}$ for GMP). This potent inhibition prevents the formation of XMP and therefore GMP in the cell. In this sense there is a remarkable self-potentiation effect of ribavirin, since the natural GTP pool is considerably lowered which diminishes the reversing effect of GTP, since GTP competes with ribavirin 5' triphosphate for the GTP sites on viral RNA polymerase.

The 5' triphosphate form of ribavirin has also been shown to affect capping of mRNA. Goswami and co-workers (1979) showed that ribavirin 5' triphosphate exhibits a potent inhibitory effect on the 5' terminal guanylation of *in vitro* synthesised uncapped viral mRNA of vaccinia virus. It has not been established whether ribavirin as the 5' triphosphate is actually incorporated into the cap in place of GTP. Further studies have shown that 5' triphosphate is an effective inhibitor of vaccinia viral mRNA guanine-N^7-methyltransferase (Goswami et al., 1981). Ribavirin 5' triphosphate at 25uM gives a 70% inhibition of this viral enzyme. Studies on the mechanism of action of ribavirin against VSV in CHO cells, indicated no effect on cellular protein synthesis, but while VSV mRNA was synthesised, it was not functional and could not be translated. Such studies support the concept of interferences with viral 5' cap formation (Toltzis and Huang, 1986).

Despite its' broad antiviral spectrum, ribavirin has been licensed in the U.S.A. and Canada, only for the treatment of respiratory syncytial virus (RSV) infections in infants. This is partly because of unpersuasive evidence of antiviral activity in man and partly because of indications of toxicity. More recently however, two areas have shown particular promise. Firstly in laboratory animal models ,

ribavirin has been shown to have significant inhibitory effects against potentially dangerous pathogenic viruses such as Rift Valley fever virus, Punta Toro, Hantaan, Pichinde, Machupo and Lassa fever virus (Canonico et al., 1984). Lassa fever virus-infected cynomologus monkeys die with a mean time of 15 days. All monkeys treated with ribavirin on days 0-4 survived. When treatment was delayed until day 7, 4 out of 8 monkeys still survived. This remarkable protection with ribavirin, even late in infection, prompted a major clinical trial of ribavirin in Sierra Leone, West Africa against Lassa fever. McCormick et al. (1986), reported that ribavirin is effective in the treatment of Lassa fever either intravenously or orally and may be used at any point in the illness. In high risk patients, the fatality rate was reduced from 55% to 5% by ribavirin treatment.

Secondly, as stated previously, ribavirin displays activity against a range of retroviruses, and this prompted a study of the effects of this drug on HIV. McCormick and co-workers (1984), studied the effects of ribavirin against HIV in vitro, and reported viral suppression at 50ug/ml and considerable lowering of reverse transcriptase activity. A double-blind placebo controlled trial (Roberts et al., 1986) seemed to show that the drug delayed the progression of lymphadenopathy syndrome (LAS) to developing full blown AIDS. However, the Food and Drug Administration, citing irregularities of the protocol, rejected the manufacturers new drug application. Despite this, NIH has not totally given up on ribavirin.

AZIDOTHYMIDINE (AZT, 3'-AZIDO-3'-DEOXYTHYMIDINE,Fig 4b)

Azidothymidine (AZT), represents a 3'-modified derivative,first synthesised by Horwitz et al., (1964). It can inhibit a range of retroviruses, including the aetological agent of acquired immune deficiency syndrome (AIDS), human immunodeficiency virus (HIV), and enjoys the distinction of being the first compound, licensed by the Food and Drug administration for the treatment of AIDS (1987). This discussion will be confined to the effects of AZT on HIV, in vitro and in vivo. It was first shown to exhibit anti-HIV activity in vitro in 1985 (Mitsuya et al., 1985) inhibiting HIV replication at concentrations in the range 50-500nM. Furthermore, this inhibition was shown to be selective for HIV as the growth of uninfected human fibroblasts and lymophocytes was observed only at concentrations of AZT above 1uM.

AZT is converted to the 5' mono, di and triphosphate forms in both uninfected and HIV-infected cells, phosphorylation being performed by cellular kinases (Furman et al., 1986). Conversion to the monophosphate form is performed by cellular thymidine kinase, while conversion to the di and triphosphate forms is performed by cellular thymidylate kinase. Kinetics of this process have been investigated and reveal that the phosphorylation process is highly dependent on the cell species i.e. in murine cells AZT is readily converted to the triphosphate form whereas in human and caprine cells, AZT accumulates as the 5' monophosphate form, with relatively low levels of the triphosphate form (De Clercq, 1988b).

At the triphosphate level, AZT is a very potent inhibitor of the HIV-encoded reverse transcriptase being a 100 fold (Furman et al., 1986), or 1000 fold (Vrang et al., 1987) more potent than the inhibition of DNA polymerase . Cheng et al., (1987), found AZT triphosphate to be 100 fold more inhibitory to HIV reverse transcriptase than to cellular DNA polymerase, the IC_{50} (concentration of inhibitor that inhibits enzyme activity 50%), being 0.04uM and 39uM respectively. Therefore, although AZT is non-selectively phosphorylated, the triphosphate derivative efficiently and selectively binds to HIV reverse transcriptase. It is not clear whether AZT is incorporated

into the synthesised DNA, but if it were then chain termination should ensue since AZT has no 3' hydroxyl (Furman et al., 1986; Ostertag et al., 1974). Furthermore AZT severely reduces intracellular dTTP due to inhibition of dTMP kinase by the monophosphate form of the drug (Furman et al., 1986), thereby facilitating interaction of the triphosphate form of the drug with the reverse transcriptase by reducing levels of the competing substrate.

As stated previously, AZT was licensed for use against AIDS in 1987, only two years after it was first shown to be active against HIV in vitro. In July 1985, the first patient was entered into a clinical study. A multicentre placebo controlled trial was initiated in early 1986, with 282 AIDS patients with pneumocystis carinii pneumonia or other AIDS-related complex (ARC). Sixteen weeks into the study an independent review committee noticed significant differences in the mortality rates. Sixteen of the 137 patients on the placebo had died, whereas none on AZT died. Patients taking AZT had fewer opportunistic infections, improved immune function with increased circulating T cells, weight gain and a general sense of well being. For these reasons the independent review board, prematurely terminated the study, and those on placebo were given AZT. This drug was therefore licensed in 1987, with many questions on long term benefits and effects left unanswered.

Until recently, AZT was recommended for the approximately 40,000 people with full blown AIDS or advanced "AIDS-related complex". However, clinicians and researchers hoped that if AZT could help these very sick individuals, it might be even more beneficial for infected people who were still healthy.

Recent clinical studies have confirmed these hopes (Marx, 1989). NIAID released the results of a study showing that AZT could slow the progression of AIDS in people with very early symptoms. These potential new users are infected with the AIDS virus but remain asymptomatic even though their counts of T4 immune cells have dropped below 500. (The normal count is in the range of 600 to 1200).

Another study included 3200 people, 1300 of whom had T4 counts less than 500. These individuals were divided into three equal groups; one of which received a placebo, the

second a low dose of AZT (500mg/day), and the third a high AZT dose (1500mg/day). The end point for the study was progression to AIDS or severe AIDS related complex. It was shown that a person was twice as likely to progress on placebo as he was on AZT. The low dose worked as well as the high dose. In the placebo group, 38 people got worse, compared to only 17 in the low dose group and 19 in the high dose category. Many patients with advanced AIDS cannot take AZT because of the severe side effects it causes them. This may not be a problem for people who begin taking the drug before symptoms begin. The side effects were mild in asymptomatic patients, limited to nausea, in 3% of those who took the lower dose. On the basis of these results, NIAID officials immediately stopped the portion of the trial that included people with T4 counts below 500, so that those in the placebo group could begin taking AZT. Meanwhile the study continues for those with T4 counts greater than 500, as there is not yet enough data to test whether the drug slows AIDS progression in these people as well.

Larder et al. (1989), recently reported that isolates of HIV from patients receiving long term AZT therapy may have reduced sensitivity to the drug. Although no one knows just how long AZT will remain effective in asymptomatic patients, it may at the very least buy time for them until more effective and less toxic AIDS drugs can be developed.

DIDEOXYCYTIDINE (ddCyd) (Fig 4b)

Dideoxycytidine (ddCyd) and other 2',3'-dideoxynucleosides including ddAdo, ddGuo, and ddThd, are all potent and selective inhibitors of HIV replication, with ddCyd being the most potent in the series (Mitsuya and Broder, 1986). At 0.5uM, it confers complete protection of ATH8 cells against the cytopathogenicity of HIV, while not being toxic for the host cells at concentrations up to 25uM. The mode of action of ddCyd is thought to be similar to that of AZT, with the triphosphate form interacting with HIV reverse transcriptase, ~~and~~ being incorporat~~ion~~ed into the DNA chain, leading to termination of elongation (Cooney et al., 1986). Unlike AZT, however ddCyd does not reduce the intracellular pyrimidine pools and therefore may not result in the bone marrow suppression that occurs with AZT. In addition, pre-clinical studies have found good oral bioavailablity, direct excretion by the kidney and relative lack of cytotoxicity in animals (Mitsuya and Broder, 1986). Comparative studies of AZT and ddCyd revealed that ddCyd was less potent than AZT, with an ED_{50} of 0.01uM compared to 0.001uM for AZT (De Clercq, 1988). (ED_{50}: 50% effective dose required to achieve 50% protection of ATH8 or MT4 cells against the cytopathic effects of HIV). However, the selectivity of these compounds was the same, with a selectivity index of 500 (ratio of the 50% cytotoxic dose [required to reduce the viability of the uninfected host cells by 50%] to the ED_{50}) (De Clercq, 1988b).

The potency and selectivity of this compound and its' relative lack of toxicity (at least in animals), has led to the setting up of clinical trials, which are currently being conducted.

OTHER INHIBITORS OF REVERSE TRANSCRIPTASE

PYROPHOSPHATE ANALOGUES: PHOSPHONOPHORMIC ACID (PFA) AND CARBONYLBIPHOSPHONIC ACID (CPFA) (Fig 4c)

The pyrophosphate analogue PFA, is best known as an anti-herpetic compound. It has been shown to have selective activity against viral DNA polymerase, binding directly to the pyrophosphate binding site (Helgstrand et al., 1980). More recently PFA and another pyrophosphate analogue carbonylbiphosphonic acid, have been shown to inhibit reverse transcriptase (Vrang and Oberg, 1986; Sarin et al., 1985; Sanstorm et al., 1985). PFA has recently undergone clinical evaluation in man for the treatment of AIDS, although preliminary results have been disappointing.

SURAMIN, EVANS BLUE AND AURINTICARBOXYLIC ACID (Fig 4d)

All of these compounds have activity against HIV. However, their mode of action is not entirely clear. It is thought that they owe their selectivity to

1.) greater affinity for HIV reverse transcriptase than for cellular DNA polymerase and/or

2.) easier accessibility to HIV reverse transcriptase (located in the cytosol) than to cellular DNA polymerase (located in the nucleus and mitochondria).

All three compounds probably exert their effect against HIV by reducing the affinity of the key reverse transcriptase enzyme for the template/primer molecule (De Clercq, 1986).

Although suramin exhibited *in vitro* activity, and *in vivo* viral inhibition was observed, no significant clinical benefit or immunological improvement was found. This compound does not cross the blood/brain barrier, and has various toxic side effects, including lymphotoxicity and renal and adrenal toxicity. Not surprisingly therefore, suramin has not been selected for large scale clinical trials. The clinical efficacy of evans blue and aurinticarboxylic acid remains to be determined.

OLIGONUCLEOTIDES

Modified oligonucleotides (triphosphates), have been made which are stable but are still internalised by the target cell (Gallo and Reitz, 1988). These compounds seem to inhibit the reverse transcriptase of genomic RNA early in

infection with HIV 1. They are thought to act by binding to the genomic RNA and blocking elongation of DNA. The *in vivo* efficacy and clinical application of these compounds remains to be determined.

INHIBITORS OF CAPPING mRNA

NEPLANOCIN A AND RELATED COMPOUNDS: DHPA, AHPA, C-cADO, DHCA[1], AND DHCA[2] (Fig 4e)

All of these compounds are adenosine analogues which inhibit a range of RNA and DNA viruses *in vitro* (De Clercq, 1988a) (Table 8). These compounds are all inhibitors of the enzyme s-adenosylhomocysteine hydrolase (AdoHcy), which plays a regulatory role in the transmethylation reaction required for capping of mRNA. Since capping and methylation of mRNAs were first identified (Reddy *et al.*, 1974; Perry and Kelly, 1974), it has become apparent that cap structures are present on virtually all eukaryotic mRNAs. Similarly, mRNAs from most plant and animal viruses have been shown to contain caps (Banerjee, 1980). Only picornaviruses (e.g. polio), and the plant viruses, cowpea mosaic virus, satellite tobacco necrosis virus and its' helper virus have been found to lack cap structures. However, in place of a cap, the first two of this group (polio and cowpea mosaic virus), have a protein covalently linked to the 5' end of their RNA genome.

Much has been learned about the structure, mechanism of synthesis and the functional role of caps (Banerjee, 1980). Capping has been shown to protect mRNA against nuclease digestion, from the 5' end of the molecule, thereby enhancing its' stability in the cytoplasm (Furuichi, Finandra and Shatkin, 1977). In addition, methylation of the blocking nucleosides increases the affinity for ribosome binding to the 5' end of the mRNA during formation of the translational initiation complex (Both *et al.*, 1975). As a result, translational efficiency of capped mRNAs is significantly increased over that of uncapped mRNAs (Both, Banerjee and Shatkin, 1975). Because capping is observed to occur during the early steps of precursor heterogeneous nuclear RNA (hnRNA) synthesis, it has also been proposed that these structures are intimately involved in, and perhaps required for biosynthesis of complete precursor transcripts (Darwell, 1979). In several studies of viral transcriptional systems, there appears to be a direct relationship between the presence of 5' cap structures and completed viral mRNA transcripts and thus, viral replication

(Banerjee, Abraham and Colonno, 1977).

The general structure of the 5' cap (Fig 5a), includes a blocking guanosine nucleoside bound to the penultimate residue of the mRNA through a unique 5'-5' inverted linkage. While the capping moiety is, with few exceptions, 7-methylguanosine, the penultimate (N1) and adjacent nucleoside (N2) can be either purine or pyrimidine bases. Moreover, the methylation of these latter two nucleosides can vary giving rise to three distinct cap structures: ^{7}mG(5')ppp(5')N1pN2p...(cap 0); ^{7}mG(5')ppp(5')N1^{m}pN2p...(cap 1), and ^{7}mG(5')ppp(5')N1^{m}pN2^{m}p...(cap 2). At least three specific enzymes are responsible for the biosynthesis of capped and fully methylated mRNAs (Fig 5b). mRNA guanyltransferase, in the presence of GTP, catalyzes the first step in these reactions in which the capping nucleoside is attached to a 5' nucleotide chain (primer), through the 5'-5' inverted linkage (Fig 5b). Subsequent methylation of this capped structure is accompanied by two specific AdoMet-dependent methyltranferases; guanine-7 methyl transferase which methylates the 7 position of the guanosine residue and nucleoside-2' methyl transferase which methylates the 2' O-ribose moieties in nucleotides N1 and N2. All AdoMet-dependent methyltranferases follow a reaction scheme (Fig 5c), which results in the formation of the product S-adenosylhomocysteine (AdoHcy).As AdoHcy is a potent competitive inhibitor of these AdoMet-dependent methyltranferases, the rate of methylation is regulated by the existing intracellular ratio of AdoHcy/AdoMet (Cantoni and Chiang, 1980; Chiang and Cantoni, 1979). Consequently, AdoHcy must be continuously degraded or eliminated in order to maintain some potential for methylation to proceed.

In eukaryotic cells, the only route for metabolism of AdoHcy is via hydrolysis by AdoHcy hydrolase to adenosine and homocysteine (de la Haba and Cantoni, 1959) (Fig 5c). In vivo, the rapid metabolic elimination of both adenosine and homocysteine drive the reaction in the hydrolytic direction, maintaining relatively low intracellular levels of AdoHcy and permitting methylation to continue at the required rate.

In the light of the evidence that capped and methylated viral mRNAs, are required for the efficient translation into proteins which are essential for viral

replication, these post-transcriptional modifications have become an attractive target for the design of antiviral agents. The most promising approach has been to design inhibitors of AdoHcy hydrolase, the key enzyme responsible for AdoHcy metabolism (Fig 5c); inhibition of the hydrolase will result in a direct increase in intracellular levels of the endogenuous product inhibitor of AdoMet-dependentmethyl transferase reactions.

On the basis of earlier reports that 5'-deoxy-5'-(isobutylthio)-adenosine apparently elicited antiviral effects (Raies et al., 1976), through the inhibition of viral RNA methylation (Jacquemont and Huppert, 1977), Cantoni and his colleagues established AdoHcy hydrolase as an antiviral target by showing that two inhibitors of this enzyme (3-deazaadenosine and 5'-deoxy-5'-(isobutylthio)-3-deazaadenosine) could prevent the replication of Rous sarcoma and other viruses as well as the viral transformation of chick embryo cells (Bader et al., 1978; Chiang et al., 1978; Bodner, Cantoni and Chiang, 1981). As a result of the increased understanding of the biochemical properties and mechanism of catalysis of AdoHcy hydrolase obtained since these initial studies, a wide range of adenosine analogues have been synthesised and evaluated as hydrolase inhibitors with antiviral activity: these include: Dihydroxypropyladenine (DHPA) (Vortuba and Holy, 1980; De Clercq et al., 1978b; De Clercq and Holy, 1979); Adeninylhydroxypropanoic acid (AHPA) (De Clercq and Holy, 1985); Carboxylic deazaadenosine (C-cAdo) (Montgomery et al.,1982) and neplanocin A (Borchardt, Keller and Patel-Thombre, 1984; De Clercq, 1985) (Fig 4e).The antiviral spectrum of these compounds is similar, and their antiviral potency increases in the order DHPA < AHPA < C-cAda < neplanocin A, as does their inhibitory potency for AdoHcy hydrolase. In fact a close correlation has been established between the antiviral potency of these compounds (i.e. against vesicular stomatitis virus) and their Ki/Km values for bovine liver AdoHcy hydrolase activity (measured in the direction of AdoHcy synthesis. (De Clercq and Cools, 1985).

Current studies have focused on the most potent of these compounds, neplanocin A. The catalytic mechanism of AdoHcy hydrolase, as described by Palmer and Abeles (1976,

1979), involves an NAD+-dependent oxidation of the 3' hydroxyl group of AdoHcy, resulting in the generation of a series of 3'-keto intermediates prior to the release of adenosine and homocysteine. Inhibition,of AdoHcy hydrolase by neplanocin A is thought to be the consequence of the reduction of NAD^+ to NADH. This is supported by the observation that *in vitro* enzymatic activity can be recovered by incubation with excess NAD+ (Matuszewska and Borchardt, 1987).

Neplanocin A, has been shown to inhibit AdoHcy hydrolase in a variety of cultured cell lines (Borchardt, Keller and Patel-Thombre, 1984; Aarbakke *et al*., 1985; Keller and Borchardt, 1986; Whaun *et al*., 1986), resulting in an accumulation of AdoHcy and a dramatic increase (10-12 fold), in the intracellular ratio of AdoHcy/AdoMet. These changes however, are subsequently reflected in a decrease in AdoMet-dependent methylation in the treated cells. Neplanocin A however, exhibits multifunctional activity (Fig 6a), since it is also a substrate for adenosine deaminase (Tsujino *et al*., 1980), which rapidly converts it to the biologically inactive neplanocin B, and for adenosine kinase leading to the formation of the triphosphate derivative which AdoMet synthetase converts to the corresponding AdoMet derivative S-neplanocymethionine (NpcMet) (Glazer and Knode, 1984; Keller *et al*., 1985; Saunders, Tan and Robins, 1985; Inaba *et al*., 1986). Although deamination appears to be of little significance in studies with cultured cells (i.e. inhibitors of adenosine deaminase do not enhance the activity of neplanocin A), the latter metabolic route has been proposed to be responsible for the observed suppression of cellular RNA synthesis and the significant cytotoxic action of neplanocin A. After inoculation intranasally with VSV, young mice treated with neplanocin A showed partial reduction in the mortality rate. However, toxicity of the compound was a problem e.g. 20ug of neplanocin A per mouse was found lethal to newborn mice if infected intraperitoneally (De Clercq *et al*., 1985b). This cytotoxicity of neplanocin A demonstrable both *in vivo* and *in vitro* is the major factor limiting its' use as an antiviral.

In an attempt to design more specific monofunctional

antiviral agents (i.e.AdoHcy hydrolase inhibitors), with minimal other side effects (i.e. adenosine deaminase, adenosine kinase), two analogues 9-(trans-2'trans-3'-dihydroxycyclopent-4'enyl)-adenine [DHCAl] and 3-deazaadenine [DHCDA 2], have been synthesised (Borcherding, 1987), which lack the 4' hydroxymethyl group of the corresponding parent molecule (Fig 4e). These analogues, like neplanocin A, produce time and concentration dependent inhibition of AdoHcy hydrolase (Borcherding, 1987), while lacking substrate activity with either deaminase or the kinase, confirming their monofunctional design (Fig 6b). In mouse L 929 cells, the concentrations required to give 95% inhibition of AdoHcy hydrolase activity were 0.2uM, 0.5uM and 0.5uM for neplanocin A, DHCA 1 and DHCA 2 respectively (Hasobe et al., 1988). Moreover, these analogues exhibited inhibition of cellular AdoHcy hydrolase, which persisted throughout 72h whereas that of neplanocin A began to recover after 48h. Similarly, the elevation of intracellular levels of AdoHcy by DHCA 1 and DHCA 2 was maintained throughout 72h in comparison to that of neplanocin A treated cells which had decreased to near control levels by this time. These compounds had no effect on either cellular RNA or DNA synthesis at concentrations up to 10uM.

Both compounds had potent antiviral activity against vaccinia virus-infected L 929 cells, exhibiting IC_{50} at 0.28uM and 0.95uM, respectively as compared with 0.08uM for neplanocin A (Hasobe et al., 1988). More striking however. was the reduced cytotoxicity observed with these analogues: ID_{50} for neplanocin A, DHCA 1 and DHCA 2 was 0.5uM, 17uM and 56uM respectively. As a consequence of the reduced cytotoxicity of compounds DHCA 1 and DHCA 2, their antiviral effectiveness (ID_{50}/IC_{50}), 61 and 59 respectively was found to be significantly higher than that of neplanocin A (i.e. 6). These studies also indicated that the intracellular ratio of AdoHcy/AdoMet required for antiviral activity is significantly lower than that which elicits cytotoxic affects, suggesting that the virus specified methyltransferases may be inherently more sensitive to changes in this ratio than cellular counterparts, conferring some selectivity. It should be noted however, that the

studies described have all been performed *in vitro*, and that the *in vivo* efficacy of these compounds needs further study.

OTHER INHIBITORS OF CAPPING mRNA

ADOMET AND ADOHCY ANALOGUES (Fig 4f,g and h)

ADOMET ANALOGUES

Another approach to blocking the capping process, has been the design of inhibitors against the various AdoMet-dependent methyltransferases themselves. Numerous structural analogues of both AdoMet and AdoHcy have been prepared and examined with the aim of increasing their specificity and eliminatimng potential metabolic side effects (Zappia, Zydek-Cwick and Schlenk, 1969; Schlenk and Dainko, 1974; Borchardt et al., 1976; Schlenk, Hannum and Ferro, 1978). In general, these analogues have exhibited poor substrate and/or inhibitor properties, reflecting the high degree of structural specificity required by the methyltransferase enzymes. Two AdoMet analogues however, Sinefungin and A9145C (Fig 4f), which are artificial antibiotics were found to elicit potent inhibitory activity towards a variety of methyltransferases (Vedel et al., 1978; Fuller and Nagarajan, 1978; Borchardt et al., 1979), including the virion mRNA (guanine-7)-methyltransferase from both vaccinia and Newcastle disease viruses, and the mRNA (nucleoside-2')-methyltransferase from vaccinia virus (Pugh, Borchardt and Stone, 1978). These compounds were also observed to inhibit vaccinia virus plaque formation in monolayer cultures of mouse L 929 cells, but only in relatively high concentrations (Borchardt and Pugh, 1979), reflecting their low cellular permeability. This appears to be a major problem limiting the in vivo efficacy of AdoMet analogues which exhibit good in vitro activity.

ADOHCY ANALOGUES (Fig 4g and h)

Systematic analysis of base, amino acid or sugar modified analogues of AdoHcy was attempted to determine the structure-activity relationships for binding of specific inhibitors to particular methyltransferases. Using purified viral mRNA methyltransfersases, this approach has delineated significant differences in the structural requirements not only between Newcastle disease virus (NDV) and vaccinia virus mRNA (guanine-7)-methyltransferase, but also between this enzyme and the accompanying mRNA (nucleoside-2')-methyltransferase within the vaccinia virion

itself (Borchardt and Pugh, 1979), suggesting that it is possible to design site specific agents. For example, the base modified analogues, S-tubercidinyl-L-homocysteine (Tub H) and 8-aza-AdoHcy were found to have potent inhibitory activity toward NDV mRNA (guanine-7-) methyltransferase but were essentially inactive toward the vaccinia virion enzyme, although Tub H did exhibit potent activity toward the vaccinia mRNA (nucleoside-2') methyltransferase. 3-deaza-AdoHcy however, was more selective for both the vaccinia enzymes as compared to that from NDV. In contrast to Tub H which like AdoHcy has been observed to be a more general inhibitor of AdoHcy-dependent methylation, other AdoHcy analogues such as AdoHcysulfone, AdoHcy sulfoxide and N^6-methyl AdoHcy are reported to be more selective inhibitors for virion mRNA (guanine-7) methyltransferases (Pugh, Borchardt and Stone, 1977). Despite the promising activity that some of these analogues have exhibited *in vitro*, few have been found to elicit significant *in vivo* activity. Those with most notable activity are Tub H, which has been shown to inhibit internal N^6-adenosine methylation in mRNA (as high as 80% at 500uM) in phytohaemagglutin stimulated rat lymphocytes, Novikoff heptoma cells and Hela cells (Chang and Coward, 1975; Kaehler, Coward and Rottman, 1979; Camper *et al.*, 1984) and 5'-deoxy-5'-(isobutylthio) adenosine (SIBA) which has been observed to inhibit the replication of several viruses and virus-induced transformation of chick embryo cells at relatively high (500uM) concentrations (Raies *et al.*, 1976; Jacquemont and Huppert., 1977). Thus, like AdoMet analogues, the low cellular permeability of AdoHcy analogues appears to be a major factor limiting their *in vivo* effectiveness.

INTERFERON

Interferon (IFN), (Isaacs and Lindemann, 1957) is produced by virus-infected cells and protects uninfected cells from viral infection, inducing in them an antiviral state against essentially all viruses. Investigation of IFNs' mode of action has revealed that it has multiple biological effects (Gresser *et al.*, 1979). Several stages in the virus life cycle, including DNA, RNA and protein synthesis are all affected (Stewart, 1979). Protein synthesis is inhibited by interferon in several ways

including the inhibition of methylation of viral mRNA . Sen et al. (1977), first discovered that a labile inhibition of reovirus mRNA is induced in interferon treated cells. However, as only the 2-O-methylation of the ribose moieties is diminished the mRNA is translated satisfactorily. Subsequently, it was demonstrated that VSV mRNA is not translated in interferon treated cells because in this case the 5'-terminal guanosine cap is unmethylated, apparently as a result of interferon-induced changes in the levels of AdoMet and AdoHcy (de Ferra and Baglioni, 1981, 1983).

COMPOUND	ANTIVIRAL SPECTRUM	TARGET/S	PROPOSED MODES OF ACTION
RIBAVIRIN	RNA AND DNA VIRUSES (SEE TABLE 7)	RNA POLYMERASE (TRANSCRIPTION) REVERSE TRANSCRIPTASE mRNA GUANYL TRANSFERASE (CAPPING) IMP DEHYDROGENASE (POOLS OF GTP)	RIBAVIRIN-TRIPHOSPHATE COMPETES FOR THE SUBSTRATE SITE/S RIBAVIRIN-MONOPHOSPHATE COMPETES FOR THE SUBSTRATE SITE
AZIDOTHYMIDINE (AZT)	RETROVIRUSES INCLUDING HIV	REVERSE TRANSCRIPTASE dTMP KINASE (POOLS OF dTTP)	AZT-TRIPHOSPHATE COMPETES FOR SUBSTRATE SITE INCORPORATION INTO DNA; TERMINATION OF ELONGATION AZT-MONOPHOSPHATE COMPETES FOR SUBSTRATE SITE
DIDEOXYCYTIDINE (ddCyd)	HIV	REVERSE TRANSCRIPTASE	SAME AS AZT
PHOSPHONOFORMIC ACID (PFA) CARBONYL-BIPHOSPHONIC ACID (CBPFA)	HIV	REVERSE TRANSCRIPTASE	BINDS TO PYROPHOSPHATE BINDING SITE
SURAMIN, EVANS BLUE, AURINTRICARBOXYLIC ACID	HIV	REVERSE TRANSCRIPTASE	NOT CLEAR; PROBABLY REDUCES THE AFFINITY OF RT FOR THE TEMPLATE
OLIGONUCLEOTIDES (THIOPHOSPHATES)	HIV	REVERSE TRANSCRIPTASE	PROBABLY BY BINDING TO GENOMIC RNA AND BLOCKING ELONGATION
NEPLANOCIN A AND RELATED COMPOUNDS	A RANGE OF CAPPED RNA AND DNA VIRUSES (SEE TABLE 8)	SAH HYDROLASE (CAPPING OF mRNA)	REDUCES NAD (NAD---NADH) REQUIRED FOR ACTIVITY OF SAH HYDROLASE
OLIGONUCLEOTIDES (THIOPHOSPHATES)	HIV	REVERSE TRANSCRIPTASE	PROBABLY BY BINDING TO GENOMIC RNA AND BLOCKING ELONGATION
NEPLANOCIN A AND RELATED COMPOUNDS	A RANGE OF CAPPED RNA AND DNA VIRUSES (SEE TABLE 8)	SAH HYDROLASE (CAPPING OF mRNA)	REDUCES NAD (NAD---NADH) REQUIRED FOR ACTIVITY OF SAH HYDROLASE; RESULTS IN ACCUMULATION OF SAH WHICH FEEDS BACK AND BLOCKS METHYL TRANSFER
SAM, SAH ANALOGUES	SOME CAPPED RNA AND DNA VIRUSES VACCINIA, NDV	SAM DEPENDENT METHYLTRANSFERASES (CAPPING OF mRNA)	COMPETES FOR SUBSTRATE SITE
INTERFERON	VSV	SAM AND SAH (CAPPING OF mRNA)	UPSETS THE RATIO OF SAM:SAH, THEREBY INHIBITING METHYLATION

Table 6

Compounds which affect transcription and/or processing of mRNA: their antiviral spectrum and proposed mode of action.

※Antiviral spectrum: viruses affected by the action of the compound against the particular target shown.

○VSV is given as a specific example where capping of mRNA has been shown to be affected, contributing to the antiviral effect of interferon.

References: Ribavirin (Streeter et al., 1973; Sidwell et al., 1975; Shannon, 1977; Jenkins and Chen, 1981; McCormick et al., 1984); AZT (Furman et al., 1986; Vrang et al., 1987; Cheng et al., 1987; Ostertag et al., 1974); ddCyd (Mitsuya and Broder, 1986; Cooney et al., 1986; De Clercq, 1988); PFA/CBFA (Vrang and Oberg, 1986; Sarin et al., 1985; Sanstrom et al., 1985); Sumarin/Evans Blue/Aurintricarboxylic acid (De Clercq, 1986); Oligonucleotides (Gallo and Reitz, 1988); Neplanocin A etc. (Borchardt, Keller and Patel-Thombre, 1984; Matuszewska and Borchardt, 1987; Borcherding, 1987); SAM (AdoMet) and SAH (AdoHcy) analogues (Jacquemont and Huppert, 1977; Pugh, Borchardt and Stone, 1978; Camper et al., 1984); Interferon (de Ferra and Baglioni, 1981, 1983).

DNA VIRUSES	RNA VIRUSES (EXCLUDING RETROVIRUSES)	RETROVIRUSES
Herpes simplex virus I	Parainfluenza I/3	Friend Leukaemia
Herpes simplex virus 2	Influenza A/B	Gross murine leukaemia
Pseudorabies	Rhinovirus	Rauscher murine leukaemia
Murine Cytomegalovirus	Coxsackie B	Rous sarcoma virus
Vaccinia	Vesicular Stomatitis virus	Human Immunodeficiency virus
Myxoma	Semliki Forest virus	
Adenovirus-3		

MODERATELY TO HIGHLY SUSCEPTIBLE

Rhabdoviruses (vesicular stomatitis virus)
Poxviruses (vaccinia virus)
Iridoviruses (African swine fever virus)
Paramyxoviruses (parainfluenza virus, measles virus)
Reovirus (reovirus, rotavirus)

NOT OR LESS SUSCEPTIBLE

Adenovirus
Herpesviruses (HSV-I, HSV-2, VZV, ...)
Picornaviruses (polio virus, coxsackie B virus, rhinovirus)
Togaviruses (sindbis virus, semliki forest virus)

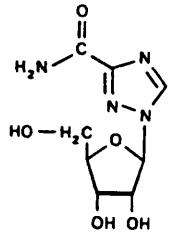
Table 7

Antiviral activity spectrum of Ribavirin (Witowski et al., 1972; Sidwell et al., 1972).

Table 8

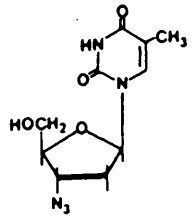
Antiviral activity of neplanocin A and related compounds (De Clercq, 1988).

a

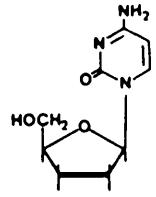


Ribavirin

b



AZT



2', 3'-Dideoxy-cytidine

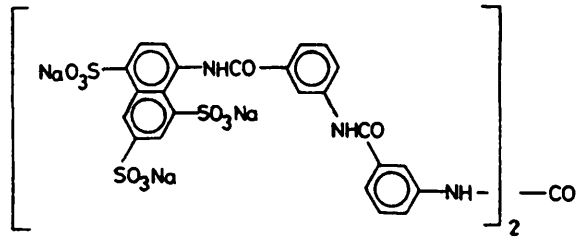
c $(HO)_2P(O)CO_2H$

Phosphonoformic acid (PFA)

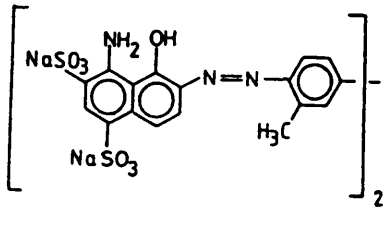
$(HO)_2P(O)CH_2CO_2H$

Phosphonoacetic acid (PAA)

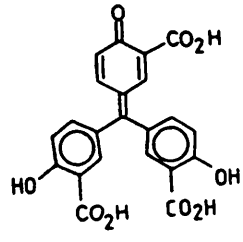
d



Suramin



Evans Blue

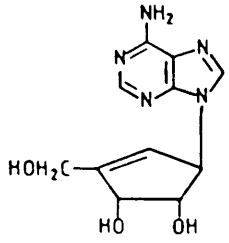


Aurintri-carboxylic acid

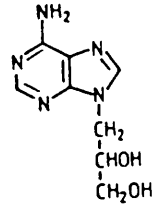
Figure 4

Structure of compounds which affect transcription and/or processing of mRNA (a-d).

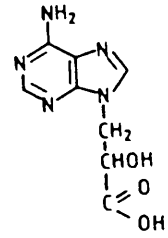
e



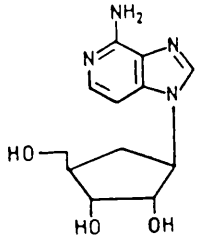
Neplanocin A



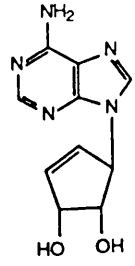
(S) - DHPA



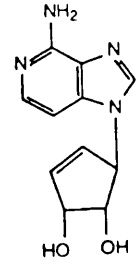
(RS) - AHPA



C - c³ Ado

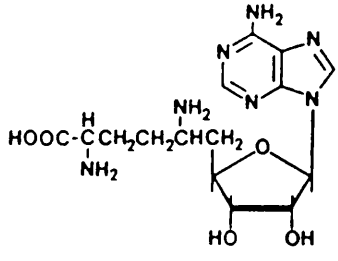


DHCA
[1]



DHCDA
[2]

f



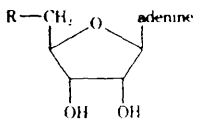
Sinefungin

Figure 4

Structure of compounds which affect transcription and/or processing of mRNA (e,f).

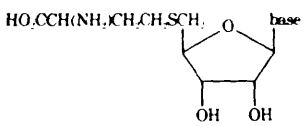
Structural Analogues of AdoHcy.

Amino Acid Modifications



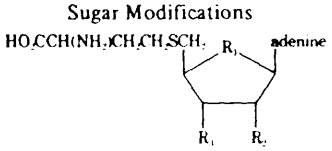
Compound	R
AdoHcy	$-SCH_2CH_2CH(NH_2)CO_2H(L)$
D-AdoHcy	$-SCH_2CH_2CH(NH_2)CO_2H(D)$
AdoHcy sulfone	$-S(\rightarrow O)CH_2CH_2CH(NH_2)CO_2H(L)$
AdoHcy sulfoxide	$-S(\overset{O}{\diagup\diagdown}\overset{O}{})CH_2CH_2CH(NH_2)CO_2H(L)$
Ado-*N*-AcHcy	$-SCH_2CH_2CH(NHAc)CO_2H(L)$
AdoTba	$-SCH_2CH_2CH_2CO_2H$
AdoTbMe ester	$-SCH_2CH_2CH_2CO_2CH_3$
AdoTpa	$-SCH_2CH_2CH_2NH_2$
Ado-*N*-AcTpa	$-SCH_2CH_2CH_2NHAc$
AdoCy	$-SCH_2CH(NH_2)CO_2H(L)$

Base Modifications



Compound	Base
TubHcy	7-Deazaadenine
N^6-Methyl-AdoHcy	N^6-Methyladenine
8-Aza-AdoHcy	8-Azaadenine
2-Aza-AdoHcy	2-Azaadenine
3-Deaza-AdoHcy	3-Deazaadenine
N^6-Methyl-3-deaza-AdoHcy	N^6-Methyl-3-deazaadenine
N^6-Dimethyl-3-deaza-AdoHcy	N^6-Dimethyl-3-deazaadenine
InoHcy	Hypoxanthine
GuaHcy	Guanine
UriHcy	Uracil
CytHcy	Cytosine

Sugar Modifications

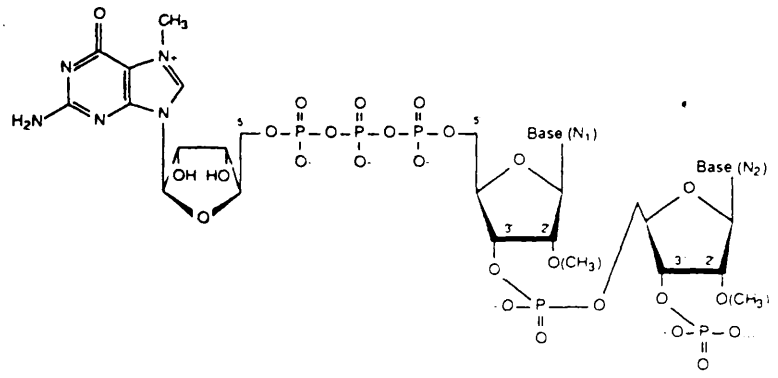


Compound	R_1	R_2	R_3
AriHcy	CH_2	OH	OH
2′-Deoxy-AdoHcy	O	H	OH
3′-Deoxy-AdoHcy	O	OH	H

Figure 4

Structure of compounds which affect transcription and/or processing of mRNA (g).(from Pugh,Borchardt and Stone, 1977).

a



b

1. RNA Polymerase:
   ATP + CTP + GTP + UTP ⟶ pppN- + PPi
2. guanylyltransferase:
   pppG + pppN- ⟶ GpppN- + Pi + PPi
3. guanine-7 methyltransferase:
   GpppN- + AdoMet ⟶ m^7GpppN- + AdoHcy
4. nucleoside-2' methyltransferase:
   m^7GpppN- + AdoMet ⟶ m^7GpppNm- + AdoHcy

c

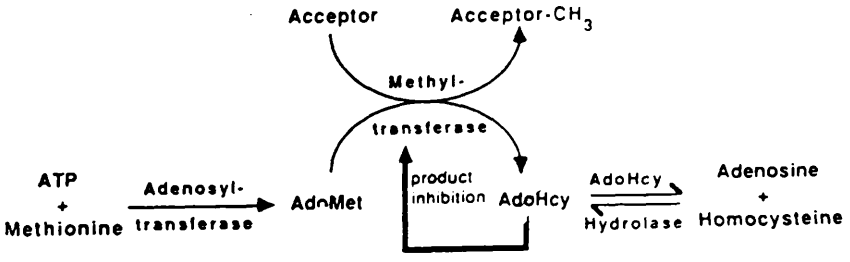
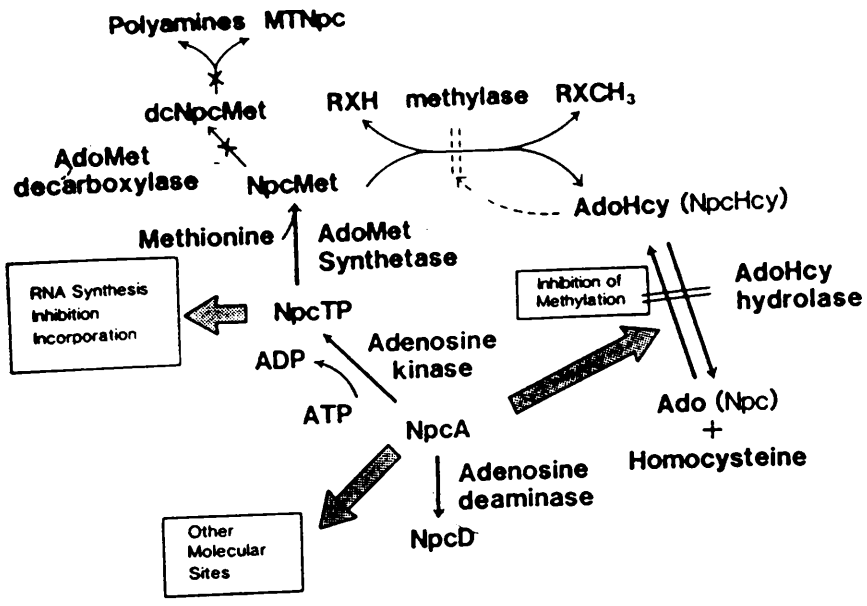


Figure 5a: Structure of mRNA 5'-terminal CAP.

Figure 5b: Enzymes involved in the biosynthesis of mRNA CAP structures.

Figure 5c: General reaction scheme for AdoMet dependent transmethylation.

a


Polyamines
MTNpc
dcNpcMet
AdoMet
decarboxylase
NpcMet
RXH
methylase
RXCH3
AdoHcy (NpcHcy)
Methionine
AdoMet
Synthetase
RNA Synthesis
Inhibition
Incorporation
NpcTP
Inhibition of
Methylation
AdoHcy
hydrolase
ADP
Adenosine
kinase
ATP
NpcA
Ado (Npc)
+
Homocysteine
Adenosine
deaminase
NpcD
Other
Molecular
Sites

b

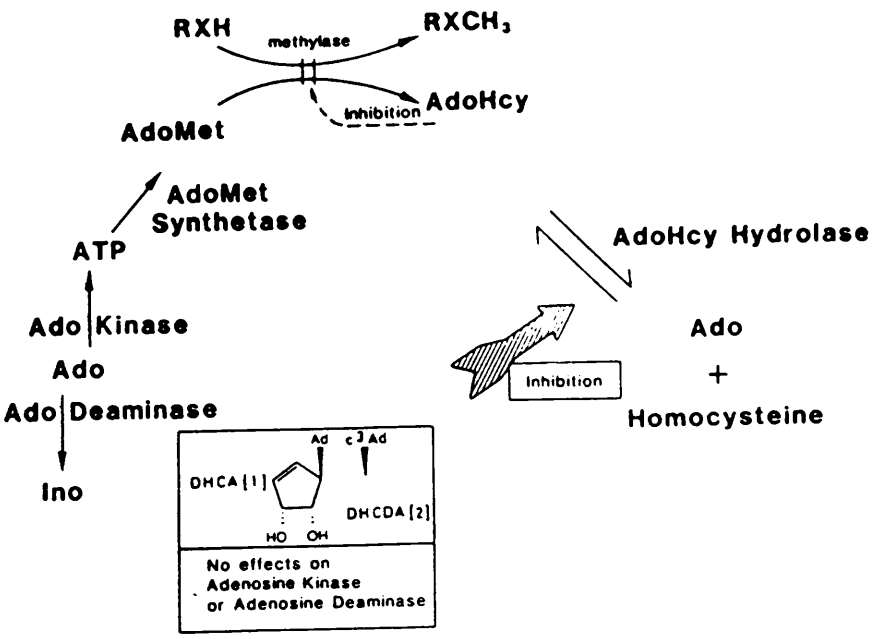

RXH
methylase
RXCH3
AdoHcy
Inhibition
AdoMet
AdoMet
Synthetase
ATP
AdoHcy Hydrolase
Ado Kinase
Ado
Ado
+
Inhibition
Ado Deaminase
Homocysteine
Ino
Ad
c Ad
DHCA [1]
DHCDA [2]
HO
OH
No effects on
Adenosine Kinase
or Adenosine Deaminase

Figure 6a

Multifunctional activity of neplanocin A.

Npc, neplanocin A; Npc D, Neplanocin D; NpcTP, neplanocin A triphosphate; NpcMet, S-neplanocylmethionine; dcNpMet, decarboxylated neplanocylmethionine; MTNpc, methylthioneplanocin; NpcHcy, neplanocylhomocysteine; ADO, adenosine; RXH, methyl acceptor molecule; $RXCH_3$, methylated acceptor molecule.

Figure 6b

Multifunctional activity of DHCA [1] and DHCA [2].

ADO, adenosine; INO, inosine; RXH, methyl acceptor molecule; RXCH3, methylated acceptor molecule.

1.1.3. COMPOUNDS WHICH INHIBIT PROTEIN SYNTHESIS AND/OR POST-TRANSLATIONAL MODIFICATIONS: GLYCOSYLATION, PHOSPHORYLATION

1.1.3.1. COMPOUNDS WHICH AFFECT PROTEIN SYNTHESIS

INTERFERON

The cause of tissue immunity was discovered in 1957 by Issacs and Lindemann, when they were studying the interfering capacity of inactivated influenza virus. They incubated the inactivated virus with fragments of chick chorio-allantoic membranes and expected that, because the virus was absorbed by the chick cells, the interfering capacity of the preparation would decrease. To their surprise, the reverse happened, and they were able to show that interfering activity was caused by a non-viral agent. They named the agent interferon. Interferon appeared to be produced by quite a variety of species including fish and reptiles after induction by a variety of viruses, both alive and inactivated, and also an impressive number of viruses appeared to be sensitive to interferon (Stewart, 1979)

The antiviral activity of interferon has been a main theme of research for years. The paramount question is: how is viral replication inhibited in interferon treated cells and at what stage ? The initial lack of pure interferon preparations resulted in many different answers to this question. The mode of action of interferon is certainly complex and virtually all stages of virus replication have been reported to be affected by interferon (Stewart, 1979). Here, I will not attempt to provide an in-depth review of interferon, but illustrate very simply how interferon is thought to affect protein synthesis.

Protein synthesis in cell-free extracts from interferon treated cells is extremely sensitive to inhibition by double stranded RNA (ds RNA)(Kerr, Brown and Ball, 1974). This appears to be due to the activity of 2 interferon induced, dsRNA-activated enzymes; a cAMP independent protein kinase and the 2-5 A synthetase (Baglioni, 1979; Revel, 1979; Lengyel, 1981, 1982) (Fig 7).

PROTEIN KINASE

A dsRNA dependent protein kinase was found independently by Lebleu et al. (1976) and Roberts et al. (1976) to phosphorylate at least one protein besides itself

(autophosphorylation) in extracts of interferon treated cells, to which dsRNA had been added. This particular protein kinase, which is induced by interferon, was first purified by Sen et al. in 1977. The protein phosphorylated by the activated kinase was shown to be the alpha subunit of the eukaryotic peptide chain initiation factor, eIF-2 (Farrell et al., 1978). The phosphorylated eIF-2 is inactive, hence can no longer catalyze the formation of the ternary complex between itself, met-tRNA and GTP.

2-5 A SYNTHETASE

The enzyme (2'-5')$(A)_n$ synthetase, induced in interferon treated cells, catalyzes the synthesis of 2-5A from ATP (Baglioni, 1983; Lengyel, 1982). It is active only when bound to dsRNA, and its' products are a family of oligoadenylates of a unique kind. The compounds designated (2'-5')oligo(A) or (2'-5')$(A)_n$, or more strictly, (2'-5')ppp$A(pA)_n$, were shown to be oligonucleotides consisting of short chains of adenosines linked uniquely, by 2'-5' phosphodiester bonds (rather than 3'-5' bonds found in all natural polynucleotides); the most abundant were di, tri and tetra-adenylates, but only trimers and longer oligomers were active (Kerr and Brown, 1978). Though 2-5A has a short half-life, it could be demonstrated in interferon treated, virus-infected cells. When introduced artificially into cells, 2-5A inhibited protein synthesis, cell division and viral multiplication (Cayley et al., 1981).

The mechanism of the antiviral action of 2-5A was unravelled as a result of the work of several independent groups. In the presence of dsRNA and ATP, both reovirus mRNA and cellular mRNA were degraded more rapidly in extracts of interferon-treated cells (Lengyel, 1982). Clemens and Williams (1978), showed that addition of 2-5A to an in vitro protein synthesising system reduced the translation of mRNA. This was shown to be attributable to an endoribonuclease that is activated by 2-5A, which destroys both viral and cellular mRNA, as well as rRNA and perhaps other cellular RNAs, by specific cleavage on the 3'-side of U_pN doublets (Baglioni, 1983; Lengyel, 1982).

1.1.3.2. COMPOUNDS WHICH AFFECT GLYCOSYLATION

Oligosaccharides can be added to proteins via the NH_2 group on the side chain of an asparagine residue (N-linked glycosylation), or less frequently, via the OH group on the side chain of serine, threonine or hydroxylysine residue (O-linked glycosylation). This discussion will be concerned only with N-linked glycosylation. The oligosaccharide to be transferred to an asparagine residue must be present on the luminal side of the ER in an activated form. Activation is achieved by linking the oligosaccharide to a donor molecule via a high energy bond. A major advance came in the early 1970s with the discovery that the activated donor is a special lipid molecule, dolichol, to which the oligosaccharide is linked via a pyrophosphate bridge.

The oligosaccharide is built up sugar by sugar on this membrane-bound lipid molecule. Sugars are first activated in the cytosol by the formation of nucleotide-sugar intermediates, which then donate their sugar (directly or indirectly) to the lipid in an orderly sequence (Fig 8a). Dolichol is very hydrophobic and quite long; with its' 22 five-carbon units, it can span the thickness of a lipid bilayer more than three times. Since it is firmly embedded in the membrane, the attached oligosaccharide is firmly anchored to the luminal surface of the ER, where glycosylation occurs.

Asparagine linked oligosaccharides are synthesised by the en-block transfer of the oligosaccharide (core sugar) $Glc_3Man_9GlcNac_2$, from the lipid carrier dolichol phosphate to the nascent polypeptide (Kornfeld and Kornfeld, 1985) (Fig 8b). Glucose and several mannose residues are successively removed in the ER. The modified glycoprotein is then transported in vesicles to the Golgi apparatus where subsequent processing occurs. The Golgi apparatus consists of three distinct stacks; cis, medial and trans, with specific enzymes located in each stack (Fig 8b). The progress of a glycoprotein through cellular membranes can be traced by the extent to which the oligosaccharides are processed. Thus the enzyme, N-acetylglucosaminyltransferase 1 is localized to the medial Golgi stack (Dunphy, Brands and Rothman, 1985), and a glycoprotein must reach that Golgi stack to obtain the peripheral GlcNAc residues. The

galactosyl and sialyltransferases reside in the Golgi stack, and the addition of galactose or sialic acid reside in the trans Golgi stack, and the addition of galactose or sialic acid to an asparagine-linked oligosaccharide indicates that the particular glycoprotein has reached the trans Golgi membranes (Griffiths et al., 1983; Griffiths, Quinn and Warren,, 1983; Rothman, Miller and Urbani, 1984). The fully processed glycoprotein is then transported in vesicles to the plasma membrane and may be secreted from the cell.

Inhibitors of glycosylation were for a long time ignored as antiviral agents because cellular as well as viral glycoproteins are affected. This was despite the fact that these compounds could be safely appied topically to epithelial surfaces such as skin, eye or genital tract. However, more recently, a series of glucosidase inhibitors have been examined for their anti-HIV potential.The antiviral properties of a series of polyaroxyalkaloids against the growth of HIV in vitro have recently been described (Gruters et al., 1987; Tyms et al., 1987; Walker et al., 1987). These natural substances, which accumulate in plants and microorganisms, but can also be synthesised, bear a structural resemblance to monosaccharides. Three different structural types have been shown to be active, namely: 1.) polyhydroxy derivatives of octahydroindolizine (casternospermine; CAST); 2.) piperidine (deoxynorimycin; DNJ); 3.) pyrolidine (dihydroxymethyldihydroxypyrolidine; DMDP) (Fellows, 1986) (Fig 9a). These compounds are characterised by their ability to inhibit glucosidase activities involved in the trimming of the oligosaccharide chains of glycoproteins (Fig 8b). CAST and DMDP both inhibit glucosidase 1 (Elbein et al., 1984) (Fellows, 1986), while DNJ inhibits both glucosidase 1 and 11 (Furmann, Bause and Proegh, 1985).

The external glycoprotein of HIV (gp 120), is the major surface component by which the virion attches to CD4 (T4), cellular receptors on helper T cells (Dalgleish et al., 1984). The envelope gene of HIV-1 encodes a precursor glycoprotein, gp160 which is cleaved to yield gp120 and gp41, a transmembrane protein (Muesing et al., 1985; Ratner et al., 1985; Robey et al., 1985; Veronese et al., 1985). The gp120 is heavily glycosylated with up to 24 N-linked

glycosylation sites (Muesing et al., 1985; Ratner et al., 1985). The expression of this gp on the surface of the host cell after infection is responsible for cell fusion and synctium formation (Lifson et al., 1986), and this may contribute to the loss of CD4+ cells in AIDS (Barnes, 1986; Lifson et al., 1986).

Treatment of HIV-infected T cells in vitro with CAST, DNJ or DMDP at concentrations of 1uM or greater, had a profound effect on the production of synctia and virus production (Gruters et al., 1987; Tyms et al., 1987; Walker et al., 1987). The specificity of this effect was shown by using CD4+ cells, constitutively expressing the HIV tat gene and transfected with a plasmid containing the art and env genes of HIV-1 (Sodroski et al., 1986). The spontaneous production of large synctia in these cells displayed a dose dependent inhibition when exposed to CAST (Walker et al., 1987). In contrast, deoxymannojirimycin (DMJ) (Gruters et al., 1987), which blocks the cleavage of alpha 1,2 mannose residues by inhibiting mannosidase 1 (Fig 8b), or swainsonine (Montefion, Robinson and Mitchell, 1987), which prevents the removal of alpha 1,3 and alphal,6-linked mannose residues by alpha-mannosidase 11, were both ineffective against HIV.

As indicated by the lectin binding studies (Lifson et al., 1986), these results support the hypothesis that oligosaccharides of the high mannose type, present on gp120 are critical for correct interaction with the host cell. When measured by reverse transcriptase or p24 antigen assays the extracellular levels of HIV from CAST treated cells were equal to those of untreated controls, but infectivity was reduced (Walker et al., 1987). It appeared therefore, that retention of glucose residues on the surface glycans of HIV interfered with infectivity (Gruters et al., 1987; Walker et al., 1987). However, incorrect trimming of glucose residues is also known to affect the cleavage of precursors and intracellular transport of gp and this can alter surface expression.

Recent studies with CMV have also shown large increases in the particle/p.f.u. ratio in infected cells treated with CAST (>1mM), which clearly correlated with an altered expression of viral gp on the virion surface (Taylor

et al., 1988). In this case, evidence points to the non-cleavage of a precursor glycoprotein when the glucose residues are retained on the glycan moiety. Similarly a block in proteolytic treatment of pE2 (Sindbis virus) after treatment with methyl-DNJ accounted for the loss of extracellular virus (McDowell et al., 1987) and was consistent with earlier observations made with VSV (Schlensinger, Malfer and Schlesinger, 1984) and mouse hepatitis virus (Repp et al., 1985)

CAST, DNJ and DMDP are well tolerated in cell culture at concentrations with antiviral activity, but only limited studies have been performed on in vivo toxicity. The injection of young rats with CAST (up to 2000mg/ip), daily for three days induced diarrhoea suggesting an inhibitory effect against gut glucosidases (Saul et al., 1985). This was eliminated when they were fed on a simple diet based on glucose rather than complex carbohydrate, although the inhibitory activity of 500mg/kg on alpha glucosidases found in the liver and other organs was not reversed. Evidence for the non-toxic nature of polyhydroxyalkaloids in man was provided when derivatives of DNJ were used to control Type 11 diabetes (up to 200mg/day, orally for three days). The medication was well tolerated with no apparent side effects (Joubert, Bam and Manyane, 1986; Schnack et al., 1986). Further information on toxicity and antiviral activity of glucosidase inhibitors in vivo should come from the study of retroviral infections in animal models.

Enough is known about the antiviral activity of glucosidase inhibitors to warrant serious clinical studies. Although essential supplies of these natural compounds are scarce, synthetic substitutes could alleviate the problem. Derivatives of these compounds are currently being investigated with the aim of identifying more potent agents with greater specificity for viral glycoprotein processing. Indeed, minor changes in the chemical structure of polyhydroxyalkaloids has already been shown to be important in influencing the antiviral activity (Tyms et al., 1987)

Finally, if altering the oligosaccharide structure of virus glycoproteins proves to be an effective antiviral strategy against AIDS, then it might be useful to use these in combination with compounds which affect other targets

(e.g. AZT; reverse transcriptase).

1.1.3.3. COMPOUNDS WHICH AFFECT PHOSPHORYLATION: XANTHATES

Xanthates display antiviral activity against a range of unrelated viruses. Vaccinia, herpes, coxsackie and vesicular stomatitis (VSV) viruses and simian virus (SV 40), can be inhibited at xanthate concentrations which still permit the mitotic activity of uninfected cells (Sauer et al., 1984). The mode of action of ths xanthate compound, tricyclo-decane-9-yl-xanthogenate (D609) (Fig 9b), against VSV was recently investigated (Muller-Decker, Amtmann and Sauer, 1987). Virus production in D609 treated cells was inhibited by around 2 logs by 30ug/ml of D609 at pH7. In order to discern the inhibitory affect of D609, the various events in the VSV growth cycle were studied. VSV particles are taken up into the cell by receptor mediated endocytosis, uncoating being facilitated by the fusion of viral and cellular membranes. The first synthetic event in the life cycle of VSV, is the transcription of its' negative stranded RNA genome, to yield 5 monocistronic mRNAs. Transcription occurs in two stages termed primary and secondary transcription. Primary transcription is performed by the virion-associated RNA polymerase complex, thought to comprise proteins L, NS and possibly N (Emerson and Yu, 1975; Mellon and Emerson, 1978; Isaac and Keene, 1982). This step is therefore not dependent on new protein synthesis. Secondary transcription is performed by the viral polymerase consisting of proteins translated from the primary transcripts. This step therefore, is clearly dependent on protein synthesis following infection and can be blocked using inhibitors of protein synthesis, such as cycloheximide. The products of primary and secondary transcription are the same, consisting of 5 monocistronic RNAs. It was shown that while D609 did not affect primary transcription, secondary transcription and replication of viral RNA were markedly decreased in the presence of the compound (Muller-Decker, Amtmann and Sauer, 1987). Experiments in vitro have shown that NS protein is involved in regulation of the activity of the polymerase complex, as the highly phosphorylated form of NS protein stimulated viral RNA transcription more efficiently than did its' less phosphorylated form (Kingsford and Emerson, 1980; Hsu, Morgan and Kingsbury, 1982). The phosphorylated NS protein

binds L protein to the RNA template (Mellon and Emerson, 1978). It was found that D609 inhibited the phosphorylation of NS protein in the infected cell, thereby inhibiting both secondary transcription and replication of RNA. The unaltered primary transcription in the presence of D609 can be attributed to the phosphorylated NS protein contained in the infecting virus particles. Similarly, the degree of inhibition of late RNA synthesis (i.e. de novo mRNA and genomic RNA synthesis) was found to be multiplicity dependent. At a higher m.o.i. (e.g. 50 p.f.u./cell) D609 was less efficient. This can also be accounted for by larger amounts of previously phosphorylated NS protein L polymerase complexes from the infecting viruses. As they are unaffected by the inhibitory affect of D609, their biological activity is maintained. The phosphorylation of the bulk of cellular proteins was apparently not affected by D609, therefore inhibition of phosphorylation of the NS protein is a highly selective process. The basis for this selectivity is not clear. It was shown that a host-derived envelope associated kinase and purified L protein were both capable of phosphorylating NS protein in vitro (Imblum and Wagner, 1974; Sanchez, De and Banerjee, 1985), leading to the activation of the RNA polymerase complex. In vitro, recombined NS protein was phosphorylated by a cellular kinase in the absence of any other VSV-coded protein (Hudson et al., 1986). It would be of interest to determine if D609 has any differential effects on the phosphorylation of NS mediated by viral or cellular kinases.

As well as exhibiting antiviral activity, D609 also has the ability to cause the phenotype of transformed cells to revert to a normal morphology and growth pattern (Amtmann et al., 1985). In addition, the tumour promoter induced phosphorylation of a cellular protein (m.wt. 80K) in fibroblasts mediated by protein kinase C is abolished in the presence of D609 (Muller-Decker, Amtmann and Sauer, 1987). This suggets some role for the inhibition of phosphorylation in the reversion from transformed to normal phenotype, mediated by D609.

The effect of D609, on the replication of other viruses, has been investigated. It was shown that this compound suppresses DNA and RNA synthesis in herpesviruses,

SV 40 and episomal bovine papillomavirus infected cells. However, it remains to be determined if like VSV, this is a consequence of inhibition of phosphorylation, or if other as yet unknown factors play a more significant role. (Saver et al., 1984)

COMPOUND	ANTIVIRAL ACTIVITY	TARGET	PROPOSED MODE OF ACTION
INTERFERON	REOVIRUS EMC	mRNA	mRNA DEGRADATION BY ACTIVATION OF ENDORIBONUCLEASE
		eIF	INHIBITION OF eIF BY PHOSPHORYLATION
CASTANOSPERMINE DMDP	HIV/CMV	GLUCOSIDASE I	INHIBITS THE PROCESSING OF gp's NORMALLY OCCURING IN ER; BLOCKS REMOVAL OF GLUCOSE RESIDUES RESULTING IN THE PRODUCTION OF VIRUS WITH ALTERED EXPRESSION OF gp ON SURFACE (INCREASED PARTICLE/ P.F.U. RATIO)
DEOXYNOJIRIMYCIN (DNJ)	HIV	GLUCOSIDASE I & II	
XANTHATE D609	VSV	NS PROTEIN OF VSV	INHIBITS THE PHOSPHORYLATION OF VSV NS PROTEIN (BASIS FOR SELECTIVE INHIBITION OF VIRAL PROTEIN PHOSPHORYLATION NOT CLEAR) LEADS TO DECREASED VSV SECONDARY TRANSCRIPTION AND REPLICATION

Table 9

Compounds which affect protein synthesis and/or post-translational modifications: their antiviral spectrum and proposed mode of action.

※Antiviral spectrum: viruses affected by the action of the compound against the particular target shown.

○Reovirus and encephalomylocarditis (EMC) virus represent specific examples where inhibition of protein synthesis has been shown to play an important role in the antiviral action of interferon.

References: Interferon (Farrell et al., 1978; Cayley et al., 1987; Baglioni, 1983; Lengyel, 1982); Casternospermine (Gruters et al., 1987; Tyms et al., 1987; Walker et al., 1987); Xanthate D609 (Sauer et al., 1984; Muller-Decker, Amtmann and Sauer, 1987).

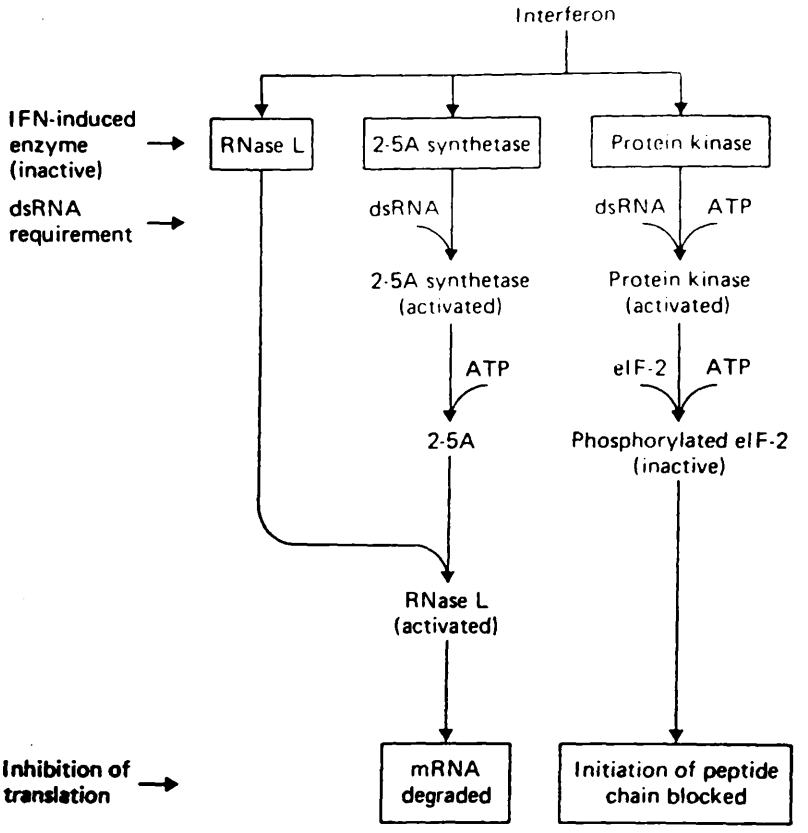

Interferon
IFN-induced enzyme (inactive)
dsRNA requirement
RNase L
2-5A synthetase
Protein kinase
dsRNA
dsRNA
ATP
2-5A synthetase (activated)
Protein kinase (activated)
ATP
eIF-2
ATP
2-5A
Phosphorylated eIF-2 (inactive)
RNase L (activated)
Inhibition of translation
mRNA degraded
Initiation of peptide chain blocked

Figure 8a

The synthesis of the lipid (dolichol)-linked oligosaccharide in the ER membrane.

(from Alberts et al., 1984)

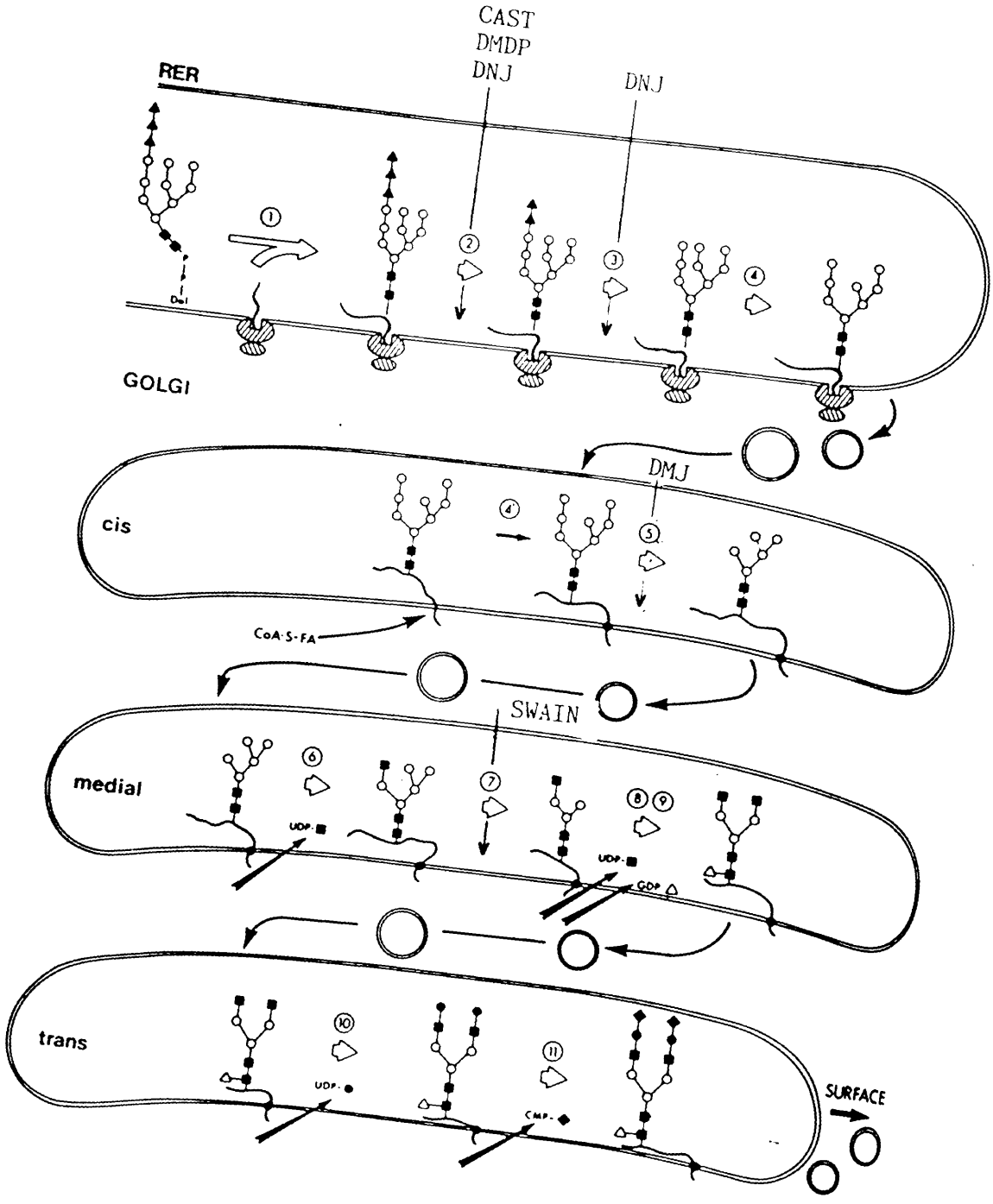
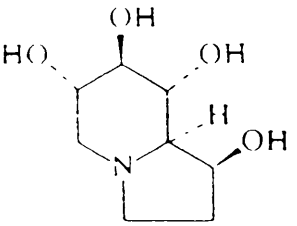
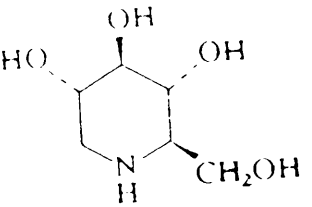

CAST
DMDP
DNJ
DNJ
RER
GOLGI
DMJ
cis
CoA-S-FA
SWAIN
medial
UDP-
UDP-
GDP
trans
UDP-
CMP-
SURFACE

Figure 8b

Schematic pathway of oligosaccharide processing and the site of action of glycosylation inhibitors: casternospermine (CAST),deoxynojirimycin (DNJ), dihydroxymethyldihydroxypyrolidine (DMDP), deoxymannojirimycin (DMJ), swainsonine (SWAIN). The reactions are catalyzed by the following enzymes: (1) oligosaccharyltransferase; (2) alpha-glucosidase 1; (3) alpha-glucosidase 11; (4) endoplasmic reticulum alpha 1,2-mannosidase; (5) fatty acyl CoA-protein transferase (postulated); (5) Golgi alpha-mannosidase 1; (6) N-acetylglucosaminyl transferase 1; (7) Golgi alpha-mannosidase 11; (8) N-acetylglucosaminyltransferase 11; (9) fucosyltransferase; (10) galactosyltransferase; (11) sialyltransferase. (RER) Rough endoplasmic reticulum. (■) N-acetylglucosamine; (○) mannose; (▲) glucose; (△) fucose; (●) galactose; (◆) sialic acid.
(from Kornfeld and Kornfeld, 1985).

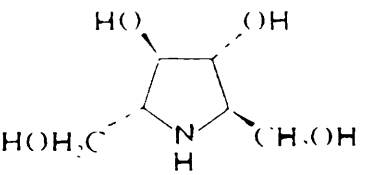


CAST



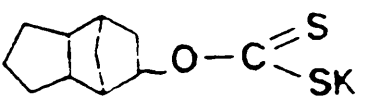
DNJ

a



DMDP

b



D609

Figure 9

Structure of compounds affecting post-translational modifications: a) glycosylation b) phosphorylation.

1.1.4. COMPOUNDS WHICH AFFECT DNA AND/OR RNA REPLICATION

NUCLEOSIDE ANALOGUES

Several nucleoside analogues have been shown to be potent inhibitors of DNA synthesis. These compounds rapidly cross the membrane of the cell by a facilitated transport mechanism. Most nucleoside derivatives are phosphorylated within the cell by a viral or cellular kinase to the 5' phosphate nucleoside analogue, which is then further converted to the 5' di and then triphosphate, the active form of the drug. Once the nucleoside is phosphorylated it does not readily leave the cell (except via gap junctions to neighbouring cells in cell-cell contact) and should stay within the cell long enough to sucessfully inhibit viral replication. This discussion of nucleoside analogues which affect DNA replication will be divided into three parts:

1.) Nucleoside analogues which affect DNA polymerase (although this may not be the only target for the compound). This group can be further divided into first-generation nucleosides, where phosphorylation of the compound is performed by cellular kinases and second-generation nucleosides, where the phosphorylation to the monophosphate form is performed by a virally encoded kinase, thus largely confining activity to virus-infected cells.

2.) Nucleoside analogues which affect thymidylate synthase (and are not dependent on virus-encoded thymidine kinase).

3.) A novel class of nucleoside analogues, phosphonylmethoxyalkylpurines and pyrimidines, whose mode of action is not yet clear.

NUCLEOSIDE ANALOGUES AFFECTING DNA POLYMERASE

FIRST GENERATION NUCLEOSIDES

IDOXURIDINE (IDU) (Fig 10a)

This pyrimidine nucleoside was first synthesised by Prusoff in 1959, and was shown to be active against a range of DNA viruses *in vitro* by Herrmann in 1961. It was successfully employed for the treatment of herpes simplex keratitis in rabbits and man in 1962 (Kaufman, 1962), and enjoys the distinction of being the first antiviral chemotherapeutic agent to be licensed for human use. However, it is too toxic to be used systemically and was applied topically as 0.1% eyedrops to HSV infections localized to the eye. It has now been overtaken by more specific nucleosides e.g. ACV and BVDU.

IDU utilises the same anabolic and catabolic pathways as thymidine (Fig 11a). The catabolic enzyme responsible for the cleavage of IDU to IU (Iodouracil) is thymidine phosphorylase and this activity did not vary much in cells post-infection. In order for IDU to be active it must be first phosphorylated to its' monophosphate form (IDUMP). Herpes simplex virus type 1 (HSV 1)., herpes simplex virus type 2 (HSV 2), varicella zoster virus (VZV), and Epstein-Barr virus (EBV)-infected cells, each induce a new species of virus specified thymidine kinase which in addition to the cellular thymidine kinase, is capable of phosphorylating IDU and other nucleosides with differing efficiency (Littler *et al.*, 1986; Turenne-Tessier *et al.*, 1986). The binding affinity of IDU to the virus-induced thymidine kinase is better than that of host thymidine kinase. Thus IDU phosphorylation takes place more efficiently in virus-infected than in host cells. Once it is converted to IDUMP, thymidylate synthetase could dehalogenate this nucleoside analogue to form the normal metabolite dUMP. However, IDUMP is primarily phosphorylated by TMP kinase to the diphosphate derivative, IDUDP, and then further to the triphosphate derivative IDUTP, by nucleoside diphosphate kinase.

IDU and its' various phosphorylated derivatives can competitively inhibit several enzymes concerned with the biosynthesis of DNA-thymine (thymidine kinase, thymidylate kinase and DNA polymerase), the triphosphate form can feed

back and inhibit several regulatory enzymes (thymidine kinase, deoxycytidylate deaminase and ribonucleotide diphosphate reductase), and gene expression can be altered by incorporation of IDU into DNA (Prusoff and Lin, 1988). Incorporation into DNA is believed to be responsible for toxicity as well as the antiviral activity of IDU. Buether and Werchau (1973) isolated infectious DNA from SV40 grown in the presence of IDU and showed a relationship between loss of infectivity and the extent of substitution of SV40 DNA-thymidine by IDU. A direct correlation between uptake of IDU into the DNA of HSV 1 and loss of infectivity was found by Fischer et al., 1980.

The incorporation of IDUMP into the DNA chain does not prevent further elongation, but the substituted DNA does not serve as a good template for either DNA relication or RNA transcription. Incorporation of IDU into HSV 1 DNA produced no apparent effect on the physical integrity of the substituted DNA molecules since Fischer, Chen and Prusoff, (1980), found no evidence of single strand or double strand breaks, when subjected to alkaline and sucrose density gradient centrifugation. Incorporation of IDU into the herpesvirus DNA produced pronounced effects on viral gene expression of those processes which rely on newly synthesised viral DNA, that is late RNA and protein synthesis. This treatment of HSV 1 infected Vero cells causes a 65% reduction in the amount of total viral RNA present at late times of infection. This decrease is apparent as early as 6h post infection, affecting the RNA viral cytoplasmic polyadenylated (poly A+)species to a greater extent than non-polyadenylated (poly A-) RNA. Cytoplasmic viral poly A+ RNA is present in treated cultures at a decreased amount relative to the control poly A+ levels, yet there is no evidence of a direct inhibition of RNA polyadenylation (Otto et al., 1984).

The RNA effects are reflected in the protein synthesised by HSV 1. Otto, Lee and Prusoff, (1982), found a reduction in the synthesis of late (Beta and Gamma), but not early (Alpha) proteins. There was a wide range of inhibition of Beta and Gamma proteins ranging from 46% to 100% of control levels. However, two proteins (ICP 35 and 39) were not affected and one (ICP 36) which migrated as

thymidine kinase was actually increased. Also, the progeny virions had altered protein patterns (Prusoff and Otto, 1983; Otto, Goz and Prusoff, 1984; Cheng and Prusoff, 1986). Therefore, although IDU acts at the level of DNA, DNA synthesis itself is not significantly inhibited, incorporation of IDU into DNA resulting in the inhibition of subsequent steps in the virus life cycle, such as RNA and protein synthesis.

ADENINE ARABINOSIDE (ARA-A) (Fig 10b)

Although IDU was the first antiviral agent to be licensed for human use, due to its' toxicity it could only be applied topically. Adenine arabinoside (Ara-A), was the first agent to be licensed for the treatment of systemic herpesvirus infections in man (Whitley and Alford, 1978, 1981; Muller, 1979; North and Cohen, 1979; Buchanan and Hess, 1980). It was first synthesised by Lee and co-workers at Stanford Research Institute in 1960. This compound can inhibit the replication of the human herpesviruses HSV 1 and HSV 2, varicella -zoster (VZV) and cytomegalovirus (CMV), in cultured cells with a considerably better therapeutic index than IDU.

The metabolic reactions to which Ara-A is subjected are shown in Fig 11b. Ara-A is rapidly deaminated by adenosine deaminase in vivo to give 9-B-D-arabinofuranosylhypoxanthine (Ara-HX), which is approximately 10 times less active than Ara-A in cell culture experiments. The in vivo efficacy and toxicity of Ara-A can therefore be augmented up to 20 fold by competitive inhibitors of adenosine deaminase such as erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (North and Cohen, 1979). Ara-A is relatively insoluble and so must be administered in a large volume of fluid by slow intravenous fusion. Ara-A monophosphate is much more soluble than Ara-A and can be administered intramuscularly or intravenously. It also has the added advantage that it is not subject to deamination by adenosine deaminase (North and Cohen, 1979). Ara-A is converted to its' monophosphate form by cellular adenosine and deoxycytidine kinase, the subsequent phosphorylation steps leading to the formation of the triphosphate form also being by cellular kinases; therefore, the formation of Ara ATP in HSV-infected cells is essentially the same as that in uninfected cells. There is direct correlation between levels of Ara ATP and inhibition of HSV replication. The selective action of Ara ATP against HSV, appears to be a consequence of the preferential action of Ara ATP against HSV 1 and HSV 2 polymerases. Ara ATP has been shown to be a competitive inhibitor of dATP for mammalian as well as viral DNA polymerases; however, HSV DNA polymerase is about 15 fold more sensitive with a Ki of

0.3uM versus 5uM for DNA polymerase (Ostrander and Cheng, 1980; Reink et al., 1978). Ara ATP is also a substrate for DNA polymerase and is incorporated in internucleotide linkage in both viral and cellular DNA. Pelling et al., 1981, demonstrated that Ara-A is incorporated uniformly tHroughout the HSV 1 genome, resulting in defective viral DNA. Slowing of DNA elongation has been reported and this could be a consequence of 3' exonuclease associated with HSV DNA polymerase. Thus by continuous incorporation and removal of Ara AMP from the terminal position , Ara ATP could act as a pseudo chain terminator. It is interesting in this regard, that Ara ATP has also been reported to inhibit terminal deoxynucleotidyltransferase. Muller et al. (1977), have also shown that Ara-A treatment of HSV-infected cells resulted in the accumulation of short DNA fragments. It should be noted that as well as affecting DNA polymerase in both infected and uninfected cells, Ara-A can also affect the procesing of mRNA, inhibiting both polyadenylation and capping. However, this is believed to play a major role in producing cytotoxicity rather than antiviral activity (North and Cohen, 1979; Drach, 1983; Prusoff and Otto, 1983; Shannon, 1984; Cheng and Prusoff, 1986). It is clear therefore, that there are multiple sites of inhibition for this compound, although it is still not clear which is responsible for the antiviral activity.

SECOND-GENERATION NUCLEOSIDES

ACYCLOVIR (9-(2-HYDROXYETHOXYMETHYL) GUANINE) (Fig 10c)

Elion et al. first described the antiviral activity of the guanosine analogue, acyclovir in 1977. Acyclovir is highly active in vitro against herpes simplex types 1 and 2 (HSV 1 and HSV 2, respectively), moderately active against varicella zoster virus (VZV) and Epstein-Barr virus (EBV), and relatively inactive against cytomegalovirus (CMV) (Fiddian, 1988). The full extent of the clinical value of acyclovir is still being defined, but we already know much about its efficacy and tolerance in a wide range of herpes virus infections. Briefly, intravenous, oral, dermal and ophthalmic formulations of acyclovir have variously been shown to be effective in systemic, mucocutanous and ocular manifestations of HSV infection. For example, acyclovir is the most potent agent available in the treatment of herpes simplex encephalitis (Whitley et al., 1986), is the only drug of proven clinical value in the treatment of both immunocompromised and normal patients with HSV infections of the skin and mucous membranes (Meyers, 1985),and constitutes first line treatment in HSV eye infections (Nicholson, 1984). In addition, there is extensive evidence to support the use of acyclovir for prophylaxis of HSV infections in normal patients (Fiddian, 1988).These data further attest to the excellent tolerance of acyclovir even over prolonged periods of time. Despite being less active against VZV, acyclovir has been shown to be effective on VZV infections, both in immunocompetent patients (Peterslund et al., 1981) and in immunocompromised patients (Balfour et al., 1983). Pagano, Sixbey and Lin (1983), reported that acyclovir reduced EBV-excretion in the throat but had minimal clinical benefit in patients with infectious mononucleosis. Andersson et al. (1986), confirmed these findings in a double-blind placebo controlled trial.

Studies of the mode of action of acyclovir (ACV), revealed the basis for the differential affect of the compond on herpesviruses. Elion et al. (1977), showed that in herpes-infected cells, ACV is converted to the monophosphate by a viral specific thymidine kinase (Fig 11c). In the uninfected host cell, phosphorylation occurs only to a very limited extent. Subsequently, ACV was found

to be phosphorylated 30-120 times faster with extracts from Vero cells infected with HSV 1 than with similar extracts of uninfected cells,and shown to be performed by viral thymidine kinase (Fyfe et al., 1978). HSV 1 and HSV 2 infected cells convert ACV to the 5' monophosphate, which is further phosphorylated by host cell GMP kinase to acyclovir diphosphate, which is turn further phosphorylated to acyclovir triphosphate by unidentified cellular kinases (Fig 11c). Obviously ACV can be expected to be active only against those viruses that encode a virus specific TK, as do HSV and VZV, and inactive against viruses which do not encode their own TK, such as CMV. It is noteworthy that EBV is quite sensitive to ACV, while it is still uncertain whether or not EBV encodes its' own kinase.

ACV triphosphate, has been found to be a competitive inhibitor of dGTP for HSV 1 and HSV 2 DNA polymerase (Larsson, Sanquist and Parnuud, 1986). In contrast, HeLa DNA polymerase is insensitive to inhibition by ACV triphosphate. Furthermore, ACV triphosphate was shown to be incorporated into thymus DNA primer template, this incorportion being much more rapid and extensive with HSV 1 DNA polymerase than with Vero cell DNA polymerase. This incorpoation of ACV ceased after 15 minutes since the template is chain terminated by acyclovir incorporation, due to the absence of a 3' hydroxyl group on which to continue elongation. The viral DNA polymerase was also inactivated by tight binding to the terminated template. McGuirt and co-workers (1984), have identified small DNA fragments synthesised in HSV 1 infected cells in the presence of ACV. These fragments were shown to be viral in origin by hybridization to purified HSV 1 DNA. ACV monophosphate terminated DNA thus was found to strongly inhibit HSV DNA polymerase catalyzed synthesis.

All this evidence indicates therefore, that ACV is selectively phosphorylated by herpesvirus TK to its' monophosphate form, then to its' triphosphate form by cellular kinases. The triphosphate form of ACV then acts as both a competitive inhibitor of dGTP for viral DNA polymerase and also as a substrate for viral DNA polymerase. Incorporation of ACV triphosphate into viral DNA follows, leading to chain termination, due to the absence of a 3' OH group.

ANALOGUES DHPG AND DHBG (Fig 10c)

DHPG

Variations in the carbohydrate portion of acyclovir, has resulted in a number of active analogues of acyclovir. The 2' deoxyguanosine analogue, DHPG, differs from ACV only by the addition of a 3' carbon and hydroxyl group attached to the acyclic chain. Orally administered DHPG is 50 fold more effacious than ACV in the treatment of systemic or local HSV 1 or HSV 2 intravaginal infections in mice (Smee et al., 1983). DHPG is reported to be a 30 fold more efficient substrate for HSV 1 TK than ACV and cellular GMP kinase phosphorylates DHPG monophosphate more readily than ACV monophosphate. These differences result in 7 times more DHPG triphosphate in HSV 1 infected rabbit kidney cells than ACV triphosphate 6h after infection. This analogue also has a greater spectrum of in vitro activity against herpes viruses than ACV. DHPG was reported by Lin, Smith and Pagano, (1984), to have a 6 fold greater activity aginst EBV than ACV as measured by its' ED_{50} 0.05uM versus 0.3uM for ACV. In addition, DHPG also displayed activity against CMV, a herpesvirus which is almost completely insensitive to ACV (ED_{50} values: 1.5uM (DHPG) versus 45uM (ACV)). Apparently DHPG is readily converted to its' active triphosphate form in the CMV-infected cells (Biron et al., 1986), providing some explanation for its' activity against this virus.

Like ACV triphosphate, DHPG triphosphate competitively inhibits incorporation of dGTP into DNA catalyzed by DNA polymerases, specified by both HSV 1 and HSV 2 (Robins and Revankar, 1988). DHPG also acts as an alternate substrate for dGTP for the viral specified DNA polymerase. However, unlike ACV, DHPG is incorporated into viral DNA in both terminal and internal linkages (Robins and Revankar, 1988), due to the presence of the additional hydroxyl group of DHPG. This incorporation results in the gradual slowing of viral DNA synthesis (like Ara-A in this regard).

Although the altered structure of this analogue, confers a greater spectrum of in vitro activity against herpes viruses, it also causes increased toxicity, particularly involving gonadal function, myelosuppression and mutagenicity. Nevertheless, the risk-benefit ratio may be sufficiently favourable in serious EBV infections and in

immunocompromised patients with severe and/or life-threatening CMV infections to warrant selected use in such target populations.

In particular, bone marrow transplant recipients and patients with AIDS are subject to high risk of morbidity and/or mortality from CMV diseases, including pneumonitis, retinitis and gastrointestinal disease. Several groups of such patients are being evaluated for acceptable tolerance and efficacy. Felsenstein et al. (1985) and Masur, Lane and Palestine, (1986), reported benifits in AIDS patients with CMV retinitis as evidenced by healing of retinal lesions and resolution of viraemia. However, relapse is common following cessation of treatment, so such patients may need to receive maintenance therapy for prolonged periods. Shepp et al., 1985, also demonstrated an antiviral effect in bone marrow transplant patients with CMV pneumonia, but survival was not influenced. This finding suggests that even a potent agent such as DHPG, may have to be employed prior to the development of pneumonia if the outcome is to be favourably modified.

DHBG (Fig 10c)

Another ACV analogue, DHBG (Fig 10c), has been reported by Larsson et al. (1986). DHBG is similar to ACV in its' activity against HSV 1 keratitis in rabbits, and somewhat superior to ACV regarding HSV 2 infections in mice. However, it is less effective than ACV against HSV 1 induced encephalitis in mice and in mice infected intravaginally with HSV 2, DHBG systemically administered did not prevent the mortality and spread of the virus to the brain (Lundgren et al., 1986). Like ACV it is phosphorylated to its' monophosphate form by viral TK. In Vero cells, DHBG proved to be a good substrate for HSV 1 and HSV 2 TK but not for cellular TK (Stenberg, Larsson and Datema, 1986). The monophosphate form of the compound is then further phosphorylated to its' triphosphate form by cellular guanylate kinase. The 5' triphosphate form is a selective and competitive inhibitor to dGTP as a substrate of the purified HSV 1 and HSV 2 DNA polymerases. However, unlike ACV, data suggests DHBG triphosphate inhibition of these polymerases occurs without incorporation into viral DNA (Stenberg, Larsson and Datema, 1986) (This may contribute to

its' reduced efficacy, compared with ACV against herpes encephalitis).

Given the variable *in vivo* results obtained with this compound, its' potential is unclear. A series of analogues of DHBG have been synthesised (Larsson *et al.*, 1986), although none of these has exhibited considerably better antiviral activity either *in vitro* or *in vivo*.

PRO-DRUGS OF ACV (Fig 10c)

The demonstration by McKendrick (1984, 1985), that higher doses of oral ACV (4 times), are required for herpes zoster than are recommended for HSV infections lends greater support for the development of prodrugs of ACV that might deliver higher plasma levels of active drug following oral administration. An additional benefit that might be achieved by such therapies is the convenience of less frequent dosing. The first pro-drug to be seriously considered was BW A134U (Fig 10c). Despite increased adsoroption compared with oral ACV, and subsequent conversion by the enzyme adenosine deaminase (Fig 11c), the consequent plasma levels of ACV in humans are disappointing. This finding as well as concerns about its' potential safety in humans (King and Fiddian, 1986), have diverted attention to another pro-drug candidate.

BW A515U (Fig 10c), is converted to ACV by xanthine oxidase (Krenitsy *et al.*, 1984) (Fig 11c), an enzyme that is abundant in humans. Phase 1 studies have been reported (Selby *et al.*, 1984). Absorption and subsequent conversion to ACV are almost complete, making this pro-drug an ideal candidate for further development. Phase 11 trials are currently in progress, but results will not be available for some time.

BVDU AND RELATED COMPOUNDS

BVDU (BROMOVINYLDEOXYURIDINE) (Fig 10d)

BVDU was synthesised by Jones, Verhelst and Walker in 1979 and was originally described by De Clercq *et al.* (1979), as a potent and selective inhibitor of HSV 1. The *in vivo* and *in vitro* efficacy of BVDU has been demonstrated in various experimental model systems (De Clercq and Walker, 1984), and its' antiviral spectrum encompasses quite a wide variety of herpes viruses including EBV and several herpes viruses of veterinary importance (Table 11). A number of clinical studies point to its' therapeutic value in the topical treatment of herpetic eye infections (Maudgal *et al.*, 1984, 85) and systemic (peroral) treatment of HSV 1 and VZV infections in immunocompromised patients (Maudgal *et al.*, 1985; De Clercq *et al.*, 1985b; Tricot *et al.*, 1986). Since the spectrum of BVDU extends to EBV, SHV 1, BHV 1 and bovine ulcerative mammilitis virus, BVDU should be further explored for its' potential in the treatment of EBV associated diseases (i.e. infectious mononucleosis, Burkitt lymphoma, nasopharyngeal carcinoma and certain lymphosarcommas in immunosuppressed patients), SHV 1 infections (pseudorabies), in pigs, and infectious rhinotrachatis and ulcerative mammalitis in cows.

Like ACV, BVDU is phosphorylated to its' monophosphate form by viral TK (De Clercq, 1982c), thereby confering selectivity to virus infected cells (Fig 11d). Unlike ACV, BVDU may be converted to its' 5' diphosphate form by the virus induced TK, or at least some virus induced TKs such as those specified by HSV 1, VZV, SHV 1 and BHV 1. These TKs therefore have dual enzyme activity. They successively act as both dThd kinase and dTMP kinase (Fig 11d). The thymidine kinases derived from HSV 2 and EHV 1 are able to phosphorylate BVDU to its' 5' monophosphate (BVDUMP) but not further onto its' 5' diphosphate (BVDUDP) (Ayisi *et al.*, 1984; Kit, Ichimura and De Clercq, 1987) (Fig 11d). This inefficient phosphorylation of BVUDP most probably explains why the replication of HSV 2 and EHV 1 is rather insensitive to BVDU. The diphosphate form of BVDU is then converted to the triphosphate by cellular kinases (Fig 11d). The interaction of BVDU triphosphate with DNA polymerase differs from that of ACV triphosphate in that it is incorporated

internally into the DNA strands, via an internucleotide linkage . The consequences of this incorporaton have been further studied with [^{125}I] IVDU and C-[^{125}] IVDU (Bernaurts, Verbuggen and De Clercq, 1987). At concentrations achieving an equivalent antiviral effect, [^{125}I] IVDU is incorporated into HSV 1 DNA to much greater extent than C-[^{125}] IVDU. Furthermore, the [^{125}I] substituted DNA becomes much more heterogeneous than the C-[^{125}I] IVDU labelled DNA. From these data, one may infer that the incorporation of [^{125}] IVDU but not C-[^{125}I] IVDU into DNA leads to an increased susceptibility to strand breakage. This together with the reduced functioning of the substituted DNAs as templates for DNA and RNA polymerases may account for the compounds ability to block virus replication.

ANALOGUES OF BVDU:

CARBOXYLIC BVDU (C-BVDU) (Fig 10d)

BVDU is an efficient substrate for pyrimidine nucleoside phosphorylases i.e. 2' deoythymidine (dThd) phosphorylase (Desgranges et al., 1983), which cleave the N-glycosidic linkage so as to release the free pyrimidine base BVU, which itself has no antiviral activity (De Clercq et al., 1986a). C-BVDU is completely resistant to degradation (De Clercq et al., 1985a), thus increasing its' bioavailbility and is as good if not better a substrate of the HSV 1 TK than BVDU (De Clercq et al., 1985a). Like BVDU, C-BVDU is incorporated into DNA strands (internally) via an internucleotide linkage. However, the extent of the incorporation of C-BVDU is low (3.6% at the most) and the exact mode of action of C-BVDU is currently being investigated.

Since it is not degraded to BVU, C-BVDU should have greater bioavailablity and perhaps greater in vivo efficacy than BVDU. Experiments with C-BVDU in vivo however, did not seem to indicate that it was more effective than BVDU in the systemic or topical treatment of HSV 1 infections in mice (Herdewijn et al., 1985).

BROMOVINYLURACIL ARABINOSIDE (BVaraU) (Fig 10d)

BVaraU, a closely related analogue of BVDU is like

BVDU, an effective anti-HSV and anti-VZV agent (Machida *et al.*, 1982). Its' *in vivo* efficacy has been demonstrated in some animal models i.e. HSV 1 encephalitis and simian varicella (De Clercq, 1988a). The anti- HSV 1 activity of BVaraU is more stringently dependent on the choice of the cell system than that of BVDU (De Clercq, 1982). Like C-BVDU, it is also not a substrate for dThd phosphorylase (De Clercq *et al.*, 1985c), again suggesting that it may have a longer bioavailability *in vivo* than BVDU. Whether this longer bioavailability would have any therapeutic implication and how BVaraU is eventually metabolized and eliminated by the organism are important issues that remain to be resolved.

OTHER 5' SUBSTITUTED 2' DEOXYURIDINES

ETHYLDEOXYURIDINE (EDU) (Fig 10e)

EDU has been known since 1967 (De Clercq and Shugar, 1975). Interest in the clinical potential of this drug has revived since Schinazi et al. (1985) and Spruance, Freeman and Smith, (1985), demonstrated its' effectiveness in the topical treatment of HSV 1 and HSV 2 infections in mice and guinea pigs. De Clercq and Bernaerts (1987), have studied the metabolism of this compound in uninfected and HSV-infected cells. EDU is phosphorylated in both mock and HSV-infected cells, albeit to a larger extent in the virus-infected than the mock-infected cells, and is incorporated internally into viral and cellular DNA strands via an internucleotide linkage, although again to a larger extent into DNA of virus infected cells. Concomitantly, with the incorporation of EDU into viral and cellular DNA of HSV-infected cells, there is a marked reduction in DNA synthesis and this reduction is more pronounced for viral DNA than for cellular DNA.

CHLOROETHYLDEOXYURIDINE (CEDU) (Fig 10e)

CEDU was synthesised by Griengl et al. in 1985. CEDU is about 10 times less potent than BVDU against HSV 1 in vitro and about equally active as BVDU in the topical treatment of cutaneous HSV 1 lesions in hairless mice; when given systemically however, CEDU appears to be effective at a 5 to 15 fold lower dose than BVDU or ACV (De Clercq and Rosenwith, 1985). CEDU also holds great promise for the topical treatment of both epithelial and stromal HSV 1 keratitis and HSV 1 uveritis readily penetrating the cornea (De Clercq, 1988). It is not clear why CEDU should be more effective than BVDU upon systemic (peroral or intraperitoneal) administration. This difference in efficacy does not seem to be related to differences in phosphorolytic cleavage by pyrimidine nucleoside phosphorylases (i.e. dThd phosphorylase), since CEDU is as good a substrate for this enzyme as other dThd analogues and it is as rapidly cleared from the bloodstream as is BVDU (Desgranges et al., 1986a).

NUCLEOSIDE ANALOGUES WHICH AFFECT THYMIDYLATE SYNTHETASE (Fig 10f)

FLUORODEOXYURIDINE (FDU), TRIFLUOROTHYMIDINE (TFT), NITRODEOXYURIDINE (NDU).

Some 5 substituted 2' deoxyuridines, that contain a small electronegative C-5 substituent such as fluorine (FDU, TFT) or nitro (NDU), exhibit antiviral activity against vaccinia virus, HSV 1, HSV 2 and VZV. These compounds are phosphorylated by cellular as well as viral TK. The initial phosphorylated product is the monophosphate, which interacts with and inhibits cellular thymidylate synthetase, the enzyme responsible for the de novo synthesis of thymidine monophosphate (dTMP), from dUMP (Fig 12).(the monophosphate form of IDU also interacts with thymidylate synthetase but unlike these compounds which inhibit this enzyme, IDUMP is dehalogenated to dUMP and then methylated to dTTP). That these compounds are inhibitors of thymidylate synthetase is based on a number of criteria such as:

1.) a much greater inhibitory effect on dUrd than dThd incorporation into DNA (Fig 12) (De Clercq et al., 1981)

2.) inability to sustain the growth of dTMP synthase-deficient murine mammary carcinoma (FM3A/TS-) cells (Balzirini et al., 1984a and b)

3.) inhibition of tritium release from [5-^{3}H]dCyd (Balzarini and De Clercq, 1984)

4.) demonstration of inhibitory action of the 5-substituted 2'-dUMPs on the isolated dTMP synthase (Balzarini et al., 1982).

FDU, TFT and NDU (Fig 10f), rank among the most potent inhibitors of dTMP synthase, with Ki/Km values (for their monophosphate) in the range of 0.001 to 0.01 (Balzarini et al., 1982).

The activity spectrum of the dTMP synthetase inhibitors extends to TK- virus strains i.e. those TK- HSV 1 mutants that may arise during therapy with ACV or any other viral TK dependent drug (De Clercq, Beres and Bentrude, 1987). Wild type TK+ HSV strains are less sensitive to the dTMP synthetase inhibitors than are their TK- counterparts. Apparently, TK- HSV mutants cannot rely on the dThd salvage pathway to ensure the necessary suply of dTMP and dTTP for their DNA synthesis (Fig 12), which makes them dependent on

the de novo biosynthetic pathway of dTTP starting from N-carbamoyl aspartate and going through the dTMP synthetase step. The latter can be considered as the bottleneck in the de novo biosynthesis of dTTP. TK- HSV mutants should be particularly vulnerable to any disturbance in the conversion of dUMP to dTMP as they cannot compensate for the loss in dTMP through salvaging Thd because they lack the necessary kinase for it.

Further evidence for the role of dTMP synthetase in the inhibitory effect of FDU on TK- HSV 1 replication stems from reversal experiments, which indicate when added exogenously, dThd completely reverses the antiviral activity of FDU at concentrations as low as 1ug/ml, whereas dUrd fails to show any reversing effect even at concentrations as high as 100ug/ml. According to the scheme shown in Fig 12, such a marked difference in the reversing effects of dUrd and dThd can only be accounted for by an inhibitory action of FDU at the dTMP synthetase level.

PHOSPHONYLMETHOXYALKYLPURINES AND PYRIMIDINES (Fig 10g)

(S)-HPMPA;
[(S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine], PMEA ;[9-(2-phosphonylmethoxyethyladenine] and their cogeners (Fig 10g), have been recognised as a novel class of potent and selective antiviral agents (De Clercq et al., 1986b). These compounds display antiviral activity against a range of DNA viruses: adenoviruses (Baba et al., 1987); herpesviruses including HSV 1, HSV 2, VZV, CMV, EBV, SHV 1, BHV 1, EHV 1, TK- HSV 1, TK- VZV as well as seal (phocid) herpesviruses (De Clercq et al., 1986b); iridoviruses (African swine fever virus) (Gil-Fernandez and De Clercq, 1987) and retroviruses including moloney murine sarcoma virus and HIV (De Clercq, 1988b) (Table 12).

(S)-HPMPA inhibits the replication of VZV in cell culture at a mean 50% inhibitory concentration of 1.8ng/ml (Baba et al., 1987), while not being toxic for the host cells at a concentration up to 52ug/ml, thus achieving a selectivity index of approximately 29,000. (S)-HPMPA is also active against TK- HSV 1 strains (De Clercq et al., 1986b), and this activity extends to the in vivo situation where (S)-HPMPA has been found to be effective against TK- HSV 1 keratitis in rabbits (Maudgal, De Clercq and Huyghe, 1987).

Among the different phosphonylmethoxyalkylpurines and pyrimidines, there exist rather striking differences in antiviral activity depending on the nature of the virus infection (De Clercq, 1988b)The pattern of activity observed with respect to adenovirus, vaccinia virus and varicella zoster virus (in order of decreasing activity) is: (S)-HPMPA > (S)-cHPMPA > (S)-HPMPDAP > (S)-HPMPC > PMEDAP > PMEA > PMEMAP. Towards HSV 1 and HSV 2 however, (S)-HPMPA, (S)-cHPMPA, (S)-HPMPC, PMEA and PMEDAP all appear about equally active, PMEMAP being definately less active. With a 50% inhibitory concentration less than 0.1ug/ml and a selectivity index > 1000, (S)-HPMPC is the most potent and selective inhibitor of CMV replication described so far, wheras in terms of anti-HIV activity, the order of (decreasing) activity is as follows: PMEDAP > PMEA > PMEMAP.

Using radiolabelled (S)-[U-^{14}C-adenine] HPMA, it has been demonstrated that the compound is as such taken up by the cells and subsequently converted to its' mono and

diphosphonyl derivatives. This phosphorylation must be achieved with cellular kinases as it occurs equally well in virus-infected and mock-infected cells (Vortuba et al., 1987). Thus, virus-specified phosphorylation, i.e. by the virus encoded dThd (dTMP) kinase, is not required for the antiviral action of (S)-HPMPA, a conclusion that could already be deduced from its' activity against TK- strains of HSV 1 and VZV.

Within the virus-infected cell, (S)-HPMPA selectively inhibits viral DNA synthesis without affecting cellular DNA synthesis. This has been clearly demonstrated in Vero cells infected with HSV 1. Only at a concentration of 500uM (150ug/ml), (S)-HPMPA effects a 50% reduction in cellular DNA synthesis (in both mock-infeted and virus-infected cells), while a 50% inhibition of viral DNA synthesis is achieved at a concentration of 5uM (1.5ug/ml). The differential effect of (S)-HPMPA on viral cellular DNA synthesis is even more pronounced in HEL cells infected with HSV 1, where (S)-HPMPA shuts off viral DNA synthesis at a concentration as low as 0.05uM (0.015ug/ml), while leaving cellular DNA synthesis unaffected at a concentration up to 50uM (15ug/ml) (Vortuba et al., 1987). The exact mechanism by which (S)-HPMPA interferes with viral DNA synthesis needs further investigation and hence, the exact target for its' action remains to be identified. Whatever target (S)-HPMPA is directed at, it is clear that its' selectivity as an antiviral agent results in a specific effect on viral DNA synthesis.

NON-NUCLEOSIDE INHIBITORS OF DNA AND/OR RNA REPLICATION

THIOSEMICARBAZONES

The antiviral activity of thiosemicarbazones was first reported in 1950 by Hamre, Bernstein and Donovick, who found that derivatives of benzaldehyde thiosemicarbazone, were active against neurovaccinial infection in mice when given orally. Sidwell and co-workers (1969), evaluated a series of purine analogues as antiviral agents and demonstrated that purine-6 carboxaldehyde thiosemicarbazone was effective in suppressing both the cytopathic effect and titres of human cytomegalovirus (HCMV). It was subsequently demonstrated that 2-acetylpyrimidine thiosemicarbazone derivatives inhibited the replication of HSV 1 and HSV 2 (Shipman Jr. et al., 1981). The mode of action of one of these compounds designated A723U (Fig 13a), against HSV 1 was examined (Spector et al., 1985). Ribonucleotide reductase induced by HSV 1 was studied for its susceptibility to inhibition by this compound. Ribonucleotide reductase, is a key enzyme in DNA synthesis and catalyzes the reduction of the 4 ribonucleosidediphosphates to the corresponding deoxyribonucleosidediphosphates. In this study, its' activity was measured by the conversion of CDP to dCDP. The mode of inhibition was unconventional as instead of remaining constant, the degree of inhibition increased as the reaction proceeded. To test whether this decelerating reaction rate was caused by inactivation of the HSV 1 ribonucleotide reductase, a small volume of fresh enzyme was added to the stagnant reaction mixture. This produced a second burst of CDP reduction that was similar to the first. Thus, it appeared that it was the enzyme that became defective during the reaction. The time dependence of this inactivation was investigated. No inactivation occurred when A723U was pre-incubated with HSV 1 ribonucleotide reductase prior to the initiation of the reaction with substrate. Inactivation only occurred when the enzyme was catalyzing the reduction of substrate. Thus the time dependence of the inactivation was not caused by the simple slow binding of the inhibitor to the resting enzyme. Studies with mammalian ribonucleotide reductase indicate that thiosemicarbazone inhibitors scavenge the free radical from the enzymes active site (Thelander and Graslund, 1983). It is possible that a

similar mechanism is involved in the inactivation of HSV 1 ribonucleotide reductase. However, because inactivation does not occur without catalysis, it is plausible that A723U inactivates by preventing the regeneration of the free radical.

A consequence of the inhibition of this enzyme is of course a reduction in the pool of dNTPs. In this same study (Spector et al., 1985), it was shown that A723U potentiated the action of ACV. As reported previously the triphosphate form of ACV competes with dGTP for DNA polymerase. A723U most likely potentiates the action of ACV by reducing dGTP pools and so reducing the amount of competitive inhibitor. If this is so, it would be of interest to determine if the action of compounds such as BVDU and AZT would also be enhanced by A723U.

PYROPHOSPHATE ANALOGUES

PHOSPHONOACETATE (PAA) AND PHOSPHONOFORMIC ACID (PFA) (Fig 13b)

Pyrophosphate analogues PAA and PFA exhibit in vitro activity against a range of herpesviruses (HSV 1 and HSV 2, CMV and EBV), and influenza virus. Helgstrand et al., 1978 demonstrated that antiviral activity of these compounds correlates well with their inhibitory effect on the polymerases of these viruses (DNA polymerase for herpesvirus and RNA polymerase for influenza viruses), and that inhibition is selective. These analogues inhibit DNA/RNA polymerase by interacting with the pyrophosphate binding site thereby being a competitive inhibitor of the dNTP pyrophosphate exchange reaction.

PAA is extremely irritating to the skin when applied topically and is retained by bone whatever the route of administration, hence it is unlikely to be licensed for human use. PFA when applied as a 3% cream is not an irritant and shortens the time mucocutaneous HSV 1 or HSV 2 lesions take to reach the stage of crusting and reduces the appearance of new vesicles (Helgstrand et al, 1980). PFA has also recently undergone clinical evaluation in man for the treatment of CMV infection of bone marrow and renal transplant patients (Helgstrand et al, 1980). Results have been encouraging although toxicity was observed (some 30% of systemically available

PFA is deposited in bone and cartilage). Given the toxicity problems associated with these compounds, the availablity and the effectiveness of ACV as well as attempts to broaden the antiviral range of ACV to include HCMV, by designing related analogues, the future of these compounds as anti-herpetic agents is not certain. These compounds and related analogues have been shown to have activity against HIV reverse transcriptase (Vrang and Oberg, 1986), and are inhibitory to HIV multiplication in cell cultures. However, preliminary clinical trials have not been encouraging.

GUANIDINE

Guanidine is an inhibitor of human enterovirus RNA replication (Crowther and Melnick, 1961). Many structural analogues of the guanidine derivative have been made; for example one phenyl ring can be replaced by a heterocyclic or certain acyclic ring systems. High *in vitro* activity was retained in a number of cases but none has shown significant activity in humans (Swallow and Kampfner, 1985).

Guanidine resistant variants of poliovirus were among the first drug resistant viruses to be isolated from tisue culture and experimentally infected monkeys (Melnic, Crowther and Barrero-Oro, 1961). Guanidine dependent viruses were also isolated (Loddo *et al.*, 1962). Early studies revealed that resistant mutants retained neurovirulence in monkeys (Carp, 1964), although the guanidine dependent mutants were apathogenic (Loddo *et al.*, 1962). These earlier workers were struck by the apparent ease with which resistance developed in their experimental systems and it was thought at that time to be a significant factor in the failure of chemotherapy in experimentally infected, guanidine treated monkeys (Barrera-Oro and Melnick, 1961)

Genetic recombination studies between guanidine resistant and sensitive strains identified the region encoding the coat protein as the important site for mutations leading to resistance (Cooper, 1968; Cooper,Wentwoth and MacMahon, 1970). Korant (1977), showed that resistant variants differed from the original sensitive strains in the electrophoretic and chomatographic behaviour of their structural proteins only. However, subsequent findings were not compatible with these early reports.

Recombination studies using a neutralizing monoclonal antibody resistant variant of the Mahoney strain indicated that guanidine resistance mapped to a region of the genome specifying the non-structural proteins (Emini et al., 1984). Anderson-Sillman et al., 1984 carried out peptide mapping and isoelectric focusing studies which demonstrated that 75% of guanidine resistant mutants had modifications in the 37kD protein known as 2C (formerly pX), which is a non-structural protein of unknown function. Sequence analysis of interstrain guanidine resistant and antibody resistant variant recombinants (Pincus and Wimmer, 1986), showed that 6 independently isolated guanidine resistant mutants each contained a mutation in 2C at amino acid 179 which resulted in a change of either ASP to ALA or ASP to GLY. Guanidine dependent mutants were also found to contain changes within 2C. Pincus and Wimmer (1986), produced resistant and dependent mutants from cloned cDNA demonstrating that mutations in polypeptide 2C are directly responsible for altered sensitivity to guanidine.

Similary, guanidine resistant mutations in foot and mouth disease virus have been mapped by isoelectric focusing , peptide mapping and recombination studies to p34, the counterpart of poliovirus 2C (Saunders and King, 1982; Saunders, Tan and Robins, 1985). A comparison of the amino acid sequence of the 2C equivalent protein in polio, foot and mouth, rhinovirus type 2 and 14 and encephalomyelocarditis virus reveals strong homology over a region of 115 residues in length and all the mutations associated with guanidine resistance appear to lie within this conserved region (Pincus and Wimmer, 1986). It may be that this region is involved in interaction with other viral proteins required for RNA replication. Membrane bound replication complexes contain 2C and guanidine could inhibit viral RNA replication by interfering with this interaction between 2C and replication proteins.

COMPOUND	ANTIVIRAL SPECTRUM	TARGET	PROPOSED MODE OF ACTION
IDU	HSV-I,HSV-2,VZV, EBV,CMV	DNA POLYMERASE	via IDUTP; 1.COMPETITIVE INHIBITOR OF dTTP 2. INCORPORATION INTO DNA : POOR TEMPLATE
ara A	HSV-I,HSV-2,VZV, CMV,EBV,VACCINIA	DNA POLYMERASE	via ara ATP; 1.COMPETITIVE INHIBITOR OF dATP 2. INCORPORATION INTO DNA: PSEUDO/CHAIN TERMINATION
ACYLOVIR (ACV)	HSV-I,HSV-2,VZV, EBV, (NOT CMV)	DNA POLYMERASE	via ACVTP; 1.COMPETITIVE INHIBITOR OF dGTP. 2. INCORPORATION INTO DNA: TERMINATION.
DHPG (ANALOGUE OF ACV)	AS ACV, PLUS CMV	DNA POLYMERASE	AS FOR ACV, EXCEPT INCORPORATED INTO BOTH TERMINAL AND INTERNAL POSITIONS : GRADUAL SLOWING OF DNA SYNTHESIS
DHBG (ANALOGUE OF ACV)	AS ACV	DNA POLYMERASE	AS FOR ACV, EXCEPT NOT INCORPORATED INTO DNA
BVDU	ANIMAL AND HERPES VIRUSES (SEE TABLE 12)	DNA POLYMERASE	via BVDUTP; 1. COMPETITIVE INHIBITOR OF dTTP 2. INCORPORATION INTO DNA: POOR TEMPLATE
C-BVDU (ANALOGUE OF BVDU)	AS BVDU	DNA POLYMERASE	AS FOR BVDU:GREATER BIOAVALIABILITY AS NOT A SUBSTRATE FOR dThd PHOSPHORYLASE
EDU, C-EDU	HSV-I, HSV-2	DNA POLYMERASE	PROBABLY SAME GENERAL MECHANISM AS BVDU. BASIS FOR DIFFERENTIAL EFFICACY NOT CLEAR
TFT,FDU,NDU	HSV-I,HSV-2, VZV,VACCINIA	THYMIDYLATE SYNTHASE	via MONOPHOSPHATE; COMPETITIVE INHIBITOR dUMP
(s)- HPMA, PMEA AND CONGENERS	A RANGE OF DNA AND RETROVIRUSES (SEE TABLE 13)	?	MODE OF ACTION UNCLEAR; SPECIFICALLY INHIBITS VIRAL DNA SYNTHESIS
THIOSEMI-CARBAZONES	HSV-I, HSV-2	RIBO-NUCLEOTIDE REDUCTASE	SCAVENGES THE FREE RADICAL FROM THE ENZYME`S ACTIVE SITE
PYROPHOSPHATE ANALOGUES; PFA AND PAA	HSV-I, HSV-2, CMV, EBV, VZV INFLUENZA	DNA POLYMERASE RNA POLYMERASE	BINDS TO PYROPHOSPHATE BINDING SITE
GUANIDINE	ENTEROVIRUSES	RNA POLYMERASE	IN POLIOVIRUS, RELATED TO PROTEIN 2C

Table 10

Compounds which affect DNA and /or RNA replication: their antiviral spectrum and proposed mode of action.

○phosphorylation performed by cellular and/or viral kinases.

●specific phosphorylation i.e. performed (at least to monophosphate) by viral kinase.

※Antiviral spectrum: viruses affected by the action of the compound against the particular target shown.

References: IDU (Hermann, 1961; Prusoff and Lin, 1988; Fischer et al., 1980); ara A (Pelling et al., 1981; Ostrander and Cheng, 1980; Reink et al., 1978); ACV (Elion et al., 1977; Larsson , Sanquist and Parnuud, 1986; McGuirt et al., 1984); DHPG (Smee et al., 1983; Robins and Revankar, 1988); DHBG (Larsson et al., 1986; Stenberg, Larsson and Datema, 1986); BVDU (De Clercq and Walker, 1984; De Clercq, 1982c; Bernaerts, Verbuggen and De Clercq, 1987); C-BVDU (De Clercq et al., 1985a); EDU/C-EDU (De Clercq and Bernaerts, 1987; Desgranges et al., 1986a); TFT/FDU/NU (Balzarini et al., 1982); (S)-HPMA/PMEA etc. (De Clercq et al., 1986b); Thiosemicarbazones (Spector et al., 1985); PFA/PAA (Helgstrand et al., 1978); Guanidine (Crowther and Melnick, 1961).

HIGHLY SUSCEPTIBLE

- Herpes simplex virus type 1 (HSV-I)
- Varicella zoster virus (VZV)
- Epstein Barr virus (EBV)
- Suid herpesvirus type 1 (SHV-I)
- Bovid herpesvirus type 1 (BHV-I)
- Simian varicella virus (SVV)

NOT (OR ONLY POORLY) SUSCEPTIBLE

- Herpes simplex virus type 2 (HSV-2)
- Cytomegalovirus (CMV)
- Equid herpesvirus type 1 (EHV-I)
- Adeno and poxviruses
- TK$^-$ variants of HSV-I and VZV
- RNA viruses

Moderately to highly susceptible

-Adenoviruses
-Herpesviruses (HSV-1, HSV-2, VZV, CMV, EBV, SHV-1, BHV-1, EHV-1, TK HSV-1, TK$^-$ VZV.....)
-Poxviruses
-Iridoviruses (African swine fever virus)
-Retroviruses (human immunodeficiency virus (HIV))

Not susceptible

-RNA viruses (except for retroviruses)

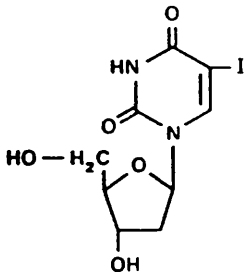
Table 11

Antiviral activity spectrum of BVDU and related compunds (De Clercq, 1988).

Table 12

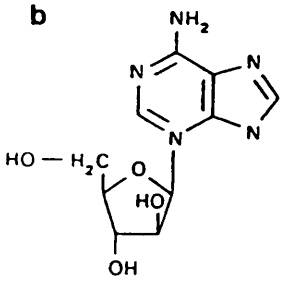
Antiviral activity spectrum of phosphonylmethoxyalkylpurine and purine derivatives (De Clercq, 1988).

a



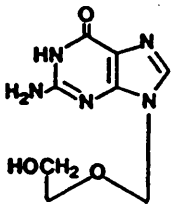
Idoxuridine (IDU)

b

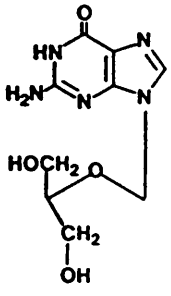


Adenine arabinoside (Ara-A)

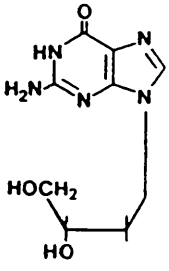
c



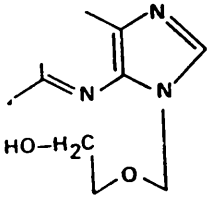
Acyclovir



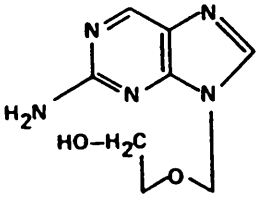
DHPG



DHBG

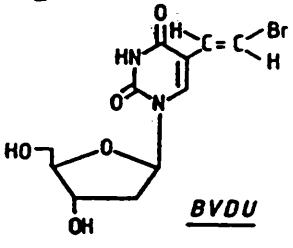


BW A134U

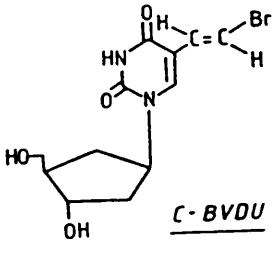


BW A515U

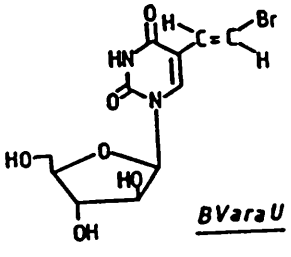
d



BVDU

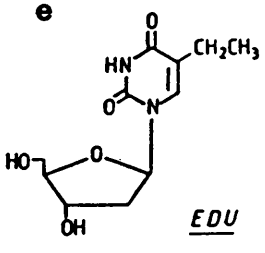


C-BVDU

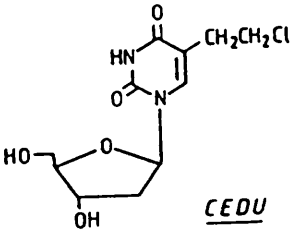


BVaraU

e



EDU

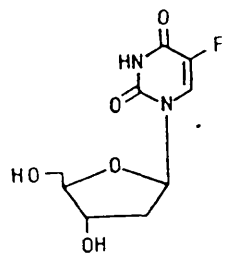


CEDU

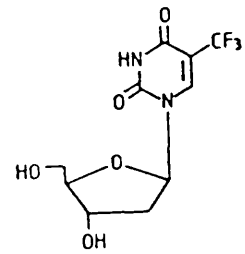
Figure 10

Structure of compounds which affect DNA and/or RNA replication (a-e).

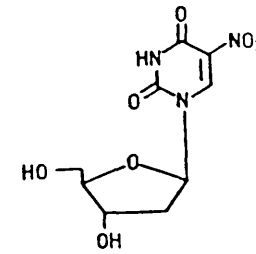
f



FDU

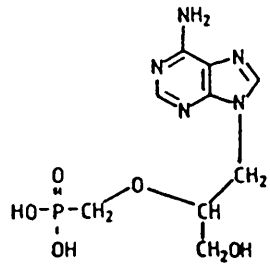


TFT

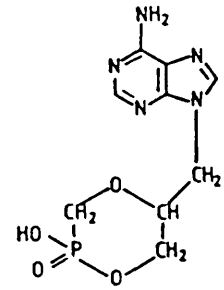


NDU

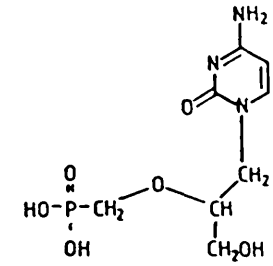
g



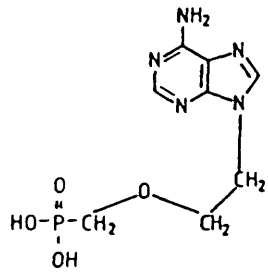
(S) - HPMPA



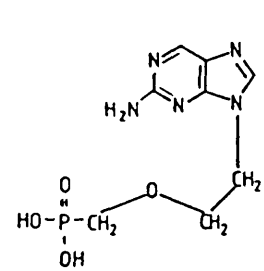
(S) - cHPMPA



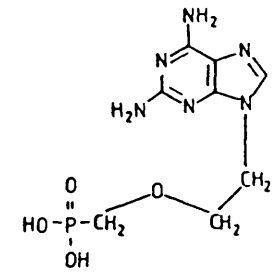
(S) - HPMPC



PMEA



PMEMAP



PMEDAP

Figure 10

Structure of compounds which affect DNA and/or RNA replication (f,g).

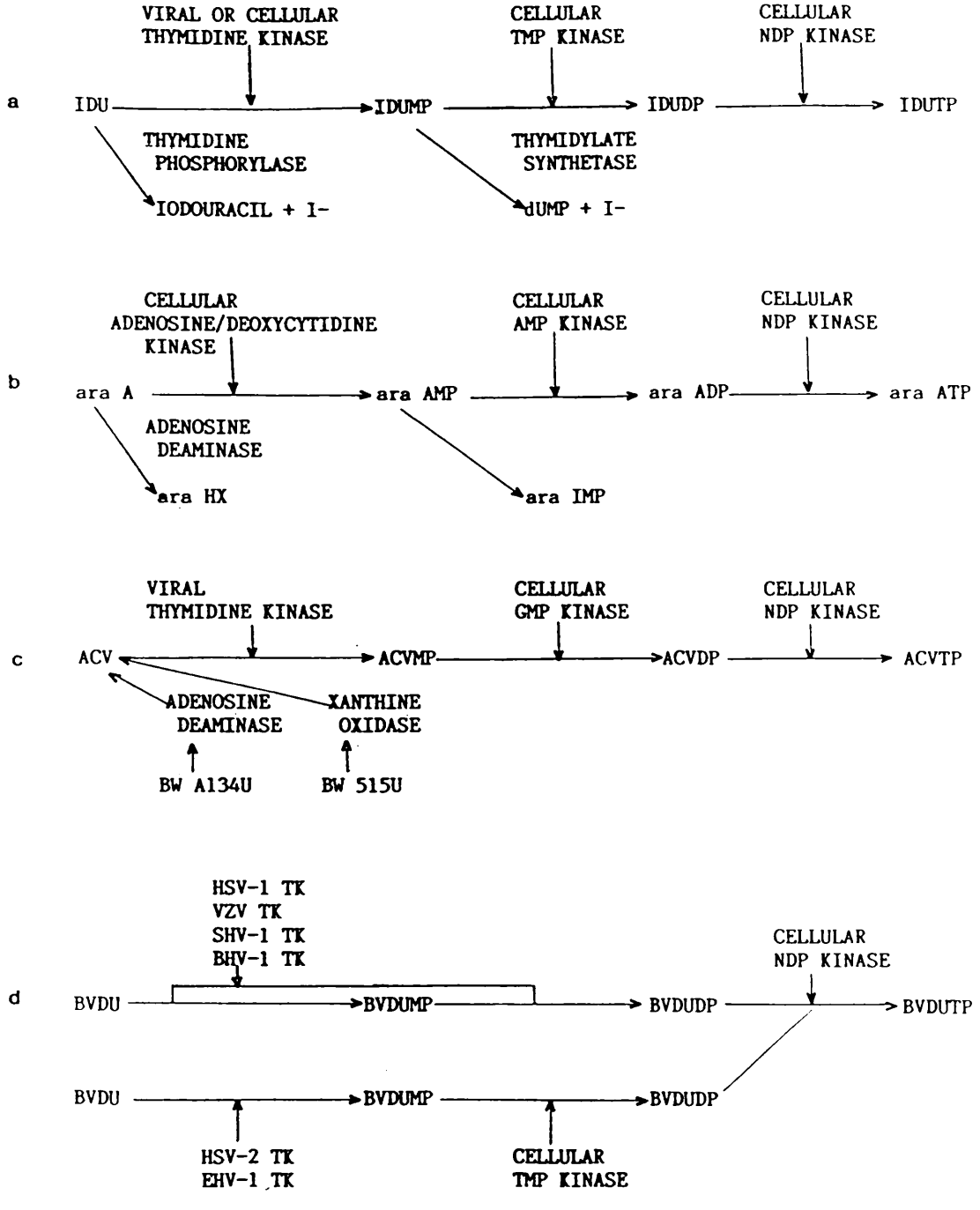

a
VIRAL OR CELLULAR
THYMIDINE KINASE
CELLULAR
TMP KINASE
CELLULAR
NDP KINASE
IDU
IDUMP
IDUDP
IDUTP
THYMIDINE
PHOSPHORYLASE
THYMIDYLATE
SYNTHETASE
IODOURACIL + I-
dUMP + I-
b
CELLULAR
ADENOSINE/DEOXYCYTIDINE
KINASE
CELLULAR
AMP KINASE
CELLULAR
NDP KINASE
ara A
ara AMP
ara ADP
ara ATP
ADENOSINE
DEAMINASE
ara HX
ara IMP
c
VIRAL
THYMIDINE KINASE
CELLULAR
GMP KINASE
CELLULAR
NDP KINASE
ACV
ACVMP
ACVDP
ACVTP
ADENOSINE
DEAMINASE
XANTHINE
OXIDASE
BW A134U
BW 515U
d
HSV-1 TK
VZV TK
SHV-1 TK
BHV-1 TK
CELLULAR
NDP KINASE
BVDU
BVDUMP
BVDUDP
BVDUTP
BVDU
BVDUMP
BVDUDP
HSV-2 TK
EHV-1 TK
CELLULAR
TMP KINASE

Figure 11

Metabolism of nucleoside analogues.

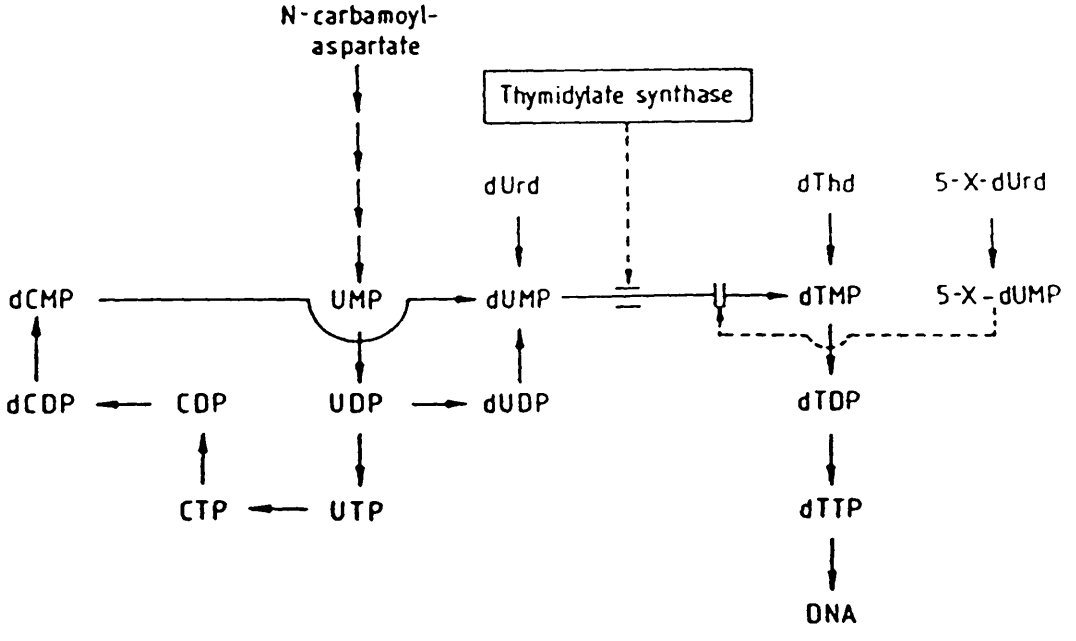
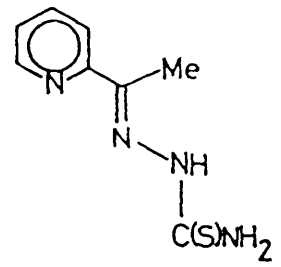

N-carbamoyl-
aspartate
Thymidylate synthase
dUrd
dThd
5-X-dUrd
dCMP
UMP
dUMP
dTMP
5-X-dUMP
dCDP
CDP
UDP
dUDP
dTDP
CTP
UTP
dTTP
DNA

Figure 12

Inhibitory action of 5-substituted 2'-deoxyuridines (5-X-Urd), such as FDU,TFT and NDU, on thymidylate synthase results in a marked reduction in the de novo biosynthesis of dTTP starting from N-carbamoylaspartate.

a 

2-acetylpyridine thiosemicarbazone

b $(HO)_2P(O)CO_2H$ $(HO)_2P(O)CH_2CO_2H$

Phosphonoformic acid (PFA) Phosphonoacetic acid (PAA)

c $(CN_3H_6^+)$
GUANIDINE

Figure 13

Non-nucleoside inhibitors of DNA and/or RNA replication.

1.1.5. CONCLUSION OF ANTIVIRAL REVIEW

Only few antiviral substances have been licensed and/or are widely available for medical use. These include at present, amantidine and rimantidine for the prophylaxis and early treatment of influenza A virus infections, idoxuridine, trifluridine, adenine arabinoside (vidaribine) and acyclovir for the topical treatment of herpetic keratitis, acyclovir for the systemic treatment (intravenous or peroral) treatment of HSV and VZV infections, adenine arabinoside for the systemic (intravenous) treatment of herpetic encephalitis (although ACV is superior in the treatment of herpetic encephalitis), ribavirin for the topical (aerosol) treatment of respiratory synctial virus infections in infants and AZT for the systemic (intravenous or peroral), treatment of AIDS and AIDS-related complex (ARC).

There are a number of other compounds which have been introduced into clinical practice albeit on a compassionate basis: phosphonoformate and dihydroxypropoxymethylguanine for the treatment of CMV infections. and BVDU for the treatment of HSV 1 and VZV infections, in immunocompromised patients. In addition ribavirin, has proven efficacious in the treatment of some haemmorrhagic fever virus infections (i.e. Lassa).

One of the major problems in the development of antiviral compounds is the relevance and use of testing and screening assays. Antiviral chemotherapy is aimed at preventing or curing a viral disease in a human patient; yet with the exception of anti-HIV compounds, that is the last thing which one is allowed to do. The close association of a virus with its' host, not only brings problems in the design of safe antivirals but it also makes the design of relevant test systems difficult. Because of cost and the quantity of material available one is usually initially restricted to *in vitro* assays in cell culture. Some viruses cannot be so cultured, other systems are so artificial that their relevance to the clinical disease is tenuous at best, and yet this is the sort of system which has to be used to find leads. Sometimes it is relatively easy to stop viral growth in infected cells, but it proves to be much more difficult when the compound is used *in vivo.* This can be because

either transport or cell targetting or uptake is very different or that the drug is metabolised before it reaches the target site. In vitro cell lines do not usually contain a full complement of functional enzymes and thus a compound like BVDU works even better in vitro than it does in vivo, where it is susceptible to nucleoside phosphorylase which is absent or present at only low levels in many cell lines. However,this does not have to be an all or none effect. Should a compound require activation by one or more enzymes before the active metabolite is produced, then small changes in concentrations of intermediates could mean that the Km for subsequent enzyme mediated transformations may not be achieved and so the active metabolite is not produced or some intermediate is side-tracked down another pathway.

Animal models often have their own problems. Usually one is trying to cure or prevent a human disease and the animal model, apart from being costly, may or may not be relevant. Uptake, distribution and metabolism of the drug may be very different, as is the nature of the viral infection and the immune system of the animal.

Toxicity assays also may not be relevant. For example, 1-(2'-deoxy-2'-fluoro-B-D-arabinofuranosyl)-5-iodocytosine (FIAC) (De Clercq et al., 1979), is poorly deaminated in dogs used in toxicity studies, whereas in humans it is rapidly deaminated to the corresponding uracil derivative FIAU. It is therefore a very naive assumption to make, that all metabolism of compounds in test animals and humans is likely to be the same or even similar, particularly when huge doses of compound are used.

The chance of getting anything clinically useful from an in vitro screen which will then show promise in animal models, pass the toxicity tests and yet still work against the clinical disease is very small.

It is also noteworthy that although the existence of specific viral targets for attack has been recognised for some time, with the exception of amantidine, all the licensed antivirals are nucleosides and are targeted against virally encoded enzymes. I would suggest the reason for this is a financial one, with researchers with limited funds following an already proven successful approach. The advent of AIDS however, has changed the situation with large

amounts of money being made available and multiple approaches adopted including random screening of compounds and design of specific antiviral agents.

Random screening of compounds and elucidation of the mode of action of compounds that show activity, play a valuable part in antiviral chemotherapy, providing an already active antiviral compound as well as information regarding susceptible targets in the virus life cycle. This information can then be used to improve the existing compound as well as to develop novel agents against the identified target.

1.2. CICLOXOLONE SODIUM

The subject of this thesis is the triterpenoid compound cicloxolone sodium (CCX), which is a synthetic analogue of glycchirizic acid (GA), which occurs naturally as a constituent of liquorice root (Fig 1). The parent compound GA, has been reported to have activity against a wide spectrum of unrelated RNA and DNA viruses *in vitro*: vaccinia, HSV 1, NDV, Influenza A and B and HIV (Pompei *et al.*, 1979, 1983; Ito *et al.* ,1987). Cicloxolone sodium (CCX) and the related compound carbenoxolone sodium (CBX), have been reported to have a beneficial effect in the treatment of HSV infections in man (Poswillo and Roberts, 1981; Csonka and Tyrrell, 1984; Partridge and Poswillo, 1984). More recently, Dargan and Subak-Sharpe have shown that both CCX and CBX inhibit the growth of HSV 1 and HSV 2 in Flow 2002 and BHK-21 cells. Both these compounds displayed greater antiviral activity than the parent compound GA. HSV 2 was consistently more sensitive than HSV 1, while CCX consistently exhibited greater antiviral activity than CBX . The mode of action of CCX and CBX *in vitro* in infected Flow 2002 (human) and BHK (hamster) cells has been investigated (Dargan and Subak-Sharpe, 1985; 1986a and b; 1988). The anti-HSV effect is characterised by the synthesis of poor quality progeny virus particles. This results in an elevated particle/p.f.u. ratio in the virus yield accompanied by a small reduction in the total number of virus particles made.

These authors demonstrated that both CCX and CBX affected cellular membranes. At least three drug effects on the PM were identified. First, adsorption of virus to cells pre-treated with CCX was inhibited, although not completely blocked (Dargan and Subak-Sharpe, 1985). Second, at concentrations greater than 50uM, both uninfected and HSV-infected cellular membranes became increasingly leaky and third, concentrations less than 10uM appeared to stabilise membranes (Dargan and Subak-Sharpe, 1986 a and b). Symons and Parke (1980), also reported that CBX had a concentration dependent biphasic effect on the stability of both isolated lysosomal membranes and artificial phosphatidylcholine/cholesterol liposomal membranes. Based on their analysis of protein synthesis in infected cells, Dargan and Subak-Sharpe have concluded that CCX and CBX also

disrupt the normal functioning of host cell membrane, affecting aspects of protein synthesis, transport and post-translational processing. It was shown that glycosylation and sulphation of both host and virus specific polypeptides are particularly sensitive to CCX inhibition, while phosphorylation is not greatly affected. This proposed antiviral action involving cell membranes, implies that the effect of CCX is of a more general nature and not just specifically directed against a particular virus protein, suggesting that the replication of several unrelated virus groups would be affected by the drug. In order to examine the effect of CCX on the replication of a range of viruses, cell lines permissive for virus growth and resistant to CCX treatment had to be identified to allow uncoupling of cytotoxic and antiviral effects. The aims of this thesis were three fold:

1.) to determine the tolerance of a range of cell lines to CCX

2.) to determine the antiviral activity spectrum of CCX (Table 13 shows the viruses examined and the families to which they belong).

3.) to more clearly elucidate the mode of action of CCX.

VIRUS	FAMILY	NUCLEIC ACID			ENVELOPED (E)/ NON-ENVELOPED (NE)
		RNA/DNA	SS/DS	S/NS	
HSV-I, HSV-2 (CONTROLS)	HERPESVIRUS	DNA	DS	NS	E
EHV-I,BHV-I, HCMV, VZV	HERPESVIRUS	DNA	DS	NS	E
VSV	RHABDOVIRUS	RNA	-SS	NS	E
SFV	TOGAVIRUS	RNA	+SS	NS	E
BUNYAMWERA, GERMISTON	BUNYAVIRUS	RNA	-SS	S(3)	E
POLIOVIRUS-I	PICORNAVIRUS	RNA	+SS	NS	NE
REOVIRUS-3	REOVIRUS	RNA	DS	S(10)	NE
ADENOVIRUS-5	ADENOVIRUS	DNA	DS	NS	NE
INFLUENZA	MYXOVIRUS	RNA	SS	S(8)	E

Table 13

Viruses screened for sensitivity to CCX.

+ positive strand viruses: genome is infectious i.e. can be immediately translated upon coating.

- negative strand viruses: genome is not infectious i.e. viral transcriptase must synthesise a complememtary + copy from the - genome, which can then be translated.

numbers in parentheses indicate the number of segments.

SS single-stranded

DS double-stranded

S segmented

NS non-segmented

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. STANDARD REAGENTS

Chemicals were obtained from BDH Chemicals Limited, Poole, Dorset. Acrylamide and N,N"-bisacrylamide were "Electran" grade, sodium dodecyl sulphate (SDS) was "Biochemical" grade, specially purified for electrophoresis. All other reagents were Anala R or the highest available grade. Analar ethanol was supplied by James Burroughs, London and dimethyl sulphoxide (DMSO) by Koch-Light Laboratories, Colnbrook, Berks. Bovine serum albumin, Ficoll 400 and Agarose (Type 1: Low EEO) were supplied by Sigma Chemical Company, Poole. Litex, Agarose Type HSA was purchased from Park Scientific Laboratories, Northhampton U.K. Ammonium persulphate and N,N,N",N"-tetramethyl-ethylenediamine (TEMED) were supplied by Bio-Rad Laboratories, Richmond, California.

All tissue culture reagents were supplied by Gibco-Biocult, Paisley, Scotland.

2.1.2. FINE CHEMICALS

Cicloxolone sodium, CCX (m.wt.668.8), carbenoxolone sodium (m.wt. 614.7), and diastereoisomers of CCX, BX 688A and BX 690A, were the gift of Biorex Laboratories Limited (London, U.K.) and kindly supplied by Miss L. Baxendale and Dr. P. Thornton.

The following fine chemicals were obtained from the Sigma Chemical Company, Poole: monensin, tunicamycin, furosemide, and disulfirum. Lyovac Cosmegen (actinomycin D) was obtained from Merck, Sharpe and Dohme, New Jersey, U.S.A.

2.1.3. RADIOCHEMICALS

[^{32}P]-inorganic orthophosphate (carrier-free) was supplied by the Western Infirmary, Glasgow. All other radiochemicals were obtained from Amersham International p.l.c., Amersham, Bucks :

[5-6 ^{3}H]-uridine (specific activity, 900Ci/mmol)

[^{35}S]-methionine (specific activity, 900Ci/mmol)

[^{35}S]-sulphate (specific activity,900Ci/mmol)

[^{14}C]-glucosamine hydrochloride (specific activity, 50-60mCi/mmol)

[^{14}C]-methylated protein mixture (catalogue no. CFA

626)
(Unlabelled protein markers were obtained from Sigma Chemical Company, Poole: catalogue no. SDS-6H and SDS-7B)

2.1.4. TISSUE CULTURE VESSELS

Roller bottles, flasks, petri dishes and 24-well Linbro trays were manufactured by Nunc, Denmark and were supplied by Gibco, Biocult Limited, Paisley.

2.2. METHODS

2.2.1. CELL CULTURE

2.2.1.1. MEDIA

Glasgow modification of Eagles medium supplemented with either 10% foetal calf serum (EFC10) or 10% newborn calf serum (EC10).

Eagles medium lacking methionine or phosphate was supplied by the Media Department, Institute of Virology, and was supplemented with either 2% foetal calf serum (EFC2) or 2% newborn calf serum (EC2).

L-15 medium (Leibovitz, 1963), supplied as a powder, was made up in 10l batches and supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 10% tryptose phosphate broth and 10% foetal calf serum. The medium was filter sterilised and stored at 4°C.

2.2.1.2. CELL LINES / CELLS

The cell lines used were as follows:

Human (Table 14); Rodent (Table 15); Miscellaneous (Table 16).

Primary Chick Embryo:

PCEF were prepared from 12 day old embryonated eggs of leukosis-free chickens. After washing the eggshell with ethanol, the area above the airspace was removed, allowing the chorioallantoic membrane to be removed. The embryo was gently lifted from the egg, decapitated and placed in a dish of sterile phosphate buffered saline (PBS) (Dulbecco and Vogt, 1954). Legs and wing buds were removed and discarded and then the embryos washed three times in PBS. The tissue was finely chopped, suspended in trypsin solution (0.25% Difco trypsin in 0.8% NaCl; 0.2% KCl; 0.01% Na_2HPO_4; 0.1% dextrose; 0.3% Tris HCl,pH7.7; 0.015% phenol red), allowing 5ml trypsin per embryo and stirred at 37°C for 30 minutes, when one tenth volume of serum was added. The supernatant was centrifuged at 8000g at 4°C for 10 minutes and the cell pellet washed twice with PBS. After resuspension in EC10 (1ml per embryo), the cells were filtered through a sterile gauze filter and seeded at 2 x 10^6 cells per 50mm petri dish. Cells were used the following day, after incubation overnight at 37°C, in an atmosphere of 5% CO_2. Fresh cells

CELL LINE	DERIVATION	REFERENCE
Chang Liver	a human liver (epithelial)	Nat. Cancer Inst. Monog (1962) 7,249
Detroit 562	b metastatic carcinomatous cells in pleural fluid from primary carcinoma of pharynx (epithelial)	Peterson,W.D., Stulberg,C.S. and Simpson,W.F. 1971
Fg 293	c human embryo kidney transformed with Adenovirus - 5 E1a and E1b (epithelial)	Graham,F.L.,Smiley,J., Russel,W.C. and Nairn,R., 1977
Flow 2002	b human embryonic lung (fibroblast)	originated by Flow Laboratories
Flow 4000	b human embryonic kidney (fibroblast)	originated by Flow Laboratories
HeLa	a cervical carcinoma, contains HPV genomes (epithelial)	Scherer,W.F.,Syverton,J.T., and Gey,G.O., 1953
HEp-2	b carcinoma of larynx (epithelial)	Toolan,H.W., 1954
MRC-5	b human embryonic lung (fibroblast)	Jacobs,J.P.,Jones,C.M., and Bailee,J.P., 1970

Table 14

Human cell lines.

a obtained from Institute cell bank and supplied by Mrs. MacNamara.

b obtained from Flow Laboratories.

c supplied by Dr. V Mautner.

CELL LINE	DERIVATION	REFERENCE
BHK-21	a baby hamster kidney (fibroblast) provided by the Cytology Unit, Institute of Virology	Macpherson,I.A., and Stoker,M.G.P., 1962
Hood	c rat embryo cells passaged continously (fibroblast)	-
Re	c rat embryo transformed with HSV-I strain HFEM (fibroblast)	-
Re 99	c rat embryo transformed with HSV-2 strain HG52 tsI (fibroblast)	-
RK-13	b rabbit kidney (epithelial)	Christofinns,G.J., and Beale,A.J., 1968
Re	d rabbit epithelial (epithelial) (rabbit corneal)	Cook,S.D., 1988
RKer	d rabbit keratocyte (differentiating epithelial) (rabbit corneal)	Cook,S.D., 1988

Table 15

Rodent cell lines.

a obtained from Institute cell bank and supplied by Mrs. MacNamara.

b obtained from Flow Laboratories.

c supplied by Dr. J. MacMab.

d supplied by Dr. S. Cooke.

CELL LINE	DERIVATION	REFERENCE
BS-C-I	a African Green Monkey kidney (epithelial)	Hopps,H.E., Bernheim,B., Nisalak,A.,Tjio,J.h., and Smadel,J.E.,1963
MDBK	b Madin-Darby Bovine Kidney (fibroblast)	-
MDCK	c Madin-Darby Canine kidney (epithelial) Cocker Spaniel Kidney	Rindler,M.J.,Chuman,L.M., Shaffer,W., and Saier,JR., M.H.,1979
XTC-2	d South African clawed toad Xenopus laevis (epithelial)	Pudney,M.,Varma,M.G.R., and Leake,C.J., 1973

Table 16

Miscellaneous cell lines.

a obtained from Flow laboratories.

b supplied by Dr. H. Field.

c supplied by Dr. U. Desselberger.

d supplied by Dr. R.M. Elliot.

were prepared as described for each experiment.

2.2.1.3. CELL CULTURE, STORAGE AND RECOVERY

CULTURE

Most cell lines were grown in EFC10, but BHK-21 cells and PCEF were grown in EC10, while XTC-2 cells were grown in L-15 medium (Section 2.2.1.1.). XTC-2 cells were passaged at 31°C in an ungassed incubator, while all other cells were passaged at 37°C in an atmosphere of 5% CO_2. Confluent monolayers (of all cell lines except XTC-2) were subcultured by washing the cell sheet twice with a solution of versene (0.6mm ethylenediaminetetra acetic acid) containing 0.05% trypsin. Detached cells were resuspended in an equal volume of medium and pipetted vigorously until a uniform suspension was obtained. Cells were then split and re-seeded as appropriate. XTC-2 cells were not trypsinised, but were sub-cultured by pipetting medium over the surface of the cell monolayer until all of the cells detached making a uniform suspension. XTC-2 and Fg 293 cells could only be propagated in tissue culture flasks.

STORAGE AND RECOVERY

Cells were stored in liquid nitrogen (-173°C), at a density of 2×10^6 cells per vial, in storage medium (40% normal growth medium, 40% foetal calf serum or newborn calf serum and 20% DMSO or glycerol). Prior to long term storage, the cells were frozen slowly by overnight incubation at -70°C in a polystyrene container. Cells were recovered by thawing and agitating in a 37°C water bath. Cells were then seeded in a $25cm^2$ tissue culture flask containing prewarmed medium. The medium was replaced after 24 hours and the cells cultured as normal.

2.1.1.4. DETECTION OF BACTERIAL AND MYCOPLASMA CONTAMINATION

Cells were routinely checked for both bacterial and mycoplasma contamination. Bacterial contamination was detected by streaking cell suspensions onto blood agar and sabourand agar plates, which were then incubated at 37°C for several days.

The genus Mycoplasma is a large group of prokaryotes which lack a cell wall. These organisms frequently

contaminate established cell lines. The fluorescent staining method of Chen (1977) was employed to facilitate detection of these contaminants.

2.2.2. VIRUS CULTURE.

2.2.2.1. VIRUSES

Vesicular Stomatitis virus, Indiana strain and Germiston virus were supplied by Dr. Joseph Szilagyi; Bunyamwera virus by Dr. Richard Elliot; Influenza A virus by Dr. Ulrich Desselberger; Semliki Forest virus and Reovirus type 3 by Professor Craig Pringle; Equine Herpes virus type 1 by Dr. Anne Cullinaine; Bovine Herpes virus type 1; strain 6660 by Dr. Hugh Field ; Human Cytomegalovirus and Varicella Zoster virus by Dr. Derrick Dargan and Adenovirus type 5 by Dr. Vivian Mautner. Poliovirus type 1 (Sabin vaccine strain) was purchased from Burroughs Wellcome Company, Greenville.

2.2.2.2. PREPARATION OF VIRUS STOCKS

Virus stocks were prepared by infecting confluent cell monolayers at low m.o.i. (0.001-0.01 p.f.u./cell), except Adenovirus-5 where the m.o.i. was 1-10 p.f.u./cell. Virus was added in medium containing 2% serum or in the case of Polio-1, in medium containing no serum. All virus stocks, except Adenovirus-5, were harvested by the same procedure, at the times indicated in Table 17. Growth media containing cell released viruses, was clarified by centrifugation at 8000g for 10 minutes, and aliquots of the supernatant stored at -70°C. Cell associated virus was harvested by resuspending the cell pellet in 2ml of growth medium and sonicating at least three times. Cell debris was pelleted after each sonication and the supernatants pooled and stored at -70°C.

The infectious yield of Adenoviruses is very cell associated, therefore the growth medium was discarded. The cell pellet was resuspended in 1ml of Tris Saline (0.8% NaCl; 0.038% KCl; 0.01% Na_2HPO_4; 0.1% Dextrose; 0.3% Sigma-Tris {hydroxymethyl} amino methane), freeze thawed three times, the cell debris pelleted and the supernatants stored at -20°C.

VIRUS	FAMILY	CELLS	GROWTH PERIOD (DAYS)	TEMPERATURE(°C)	TITRE TYPICAL P.F.U./CELL
VESICULAR STOMATITIS VIRUS, INDIANA STRAIN (VSV,IND)	RHABDOVIRUS	BHK-21	2	31	100 - 500
INFLUENZA TYPE A (INF. A)	MYXOVIRUS	MDCK	2/3	37	< 1
EQUINE HERPES VIRUS TYPE-I (EHV-I) BOVINE HERPES VIRUS TYPE-I (BHV-I)	ALPHAHERPES VIRUS	RK-13	2	37	10 - 50
SEMLIKI FOREST VIRUS (SFV)	TOGAVIRUS	BHK/ BS-C-I	2/3	31	50 - 100
REOVIRUS TYPE-3 (REO-3)	REOVIRUS	BS-C-I	2/3	37	10 - 50
POLIOVIRUS TYPE-I (POLIO-I)	PICORNAVIRUS	BS-C-I	2/3	34	10 - 50
BUNYAMWERA, GERMISTON	BUNYAVIRUS	BHK/ BS-C-I	4/5	31	50 - 100
ADENOVIRUS TYPE 5 (ADENO-5)	ADENOVIRUS	HeLa	3/4	37	1000

Table 17

Preparation of virus stocks

2.2.3. VIRUS PURIFICATION (VSV)

2.2.3.1. CONCENTRATION

Concentration of virus was by ultracentrifugation at 12,000 r.p.m. at 4°C for 2 hours in a Sorvall GSA rotor.

2.2.3.2 GRADIENT PURIFICATION

Concentrated virus, resuspended in Eagles medium without phenol red (EPr-), was layered onto Ficoll 400 gradients,(5-15%, made up in EPr-), 500ul per gradient. Centrifugation was at 15,000 r.p.m. for two hours at 4°C in the AH 650 rotor. Visible viral bands were removed by aspiration, made to 5ml with EPr- and pelleted by centrifugation at 26K for 90 minutes at 4°C, in the AH650 rotor. The pellet was resuspended in EPr- and stored at -70°C.

2.2.4. ASSAY OF VIRUS INFECTIVITY

One in ten serial dilutions of virus stocks were made in PBS containing 2% foetal calf or newborn calf serum. 100ul inoculum of each virus dilution was added to confluent monolayers of cells (4×10^6 cells/50mm petri dish) and allowed to absorb at the appropriate temperature for 60 mins. (90 mins. for Adenovirus-5), prior to adding overlay (Table 18). For all viruses, except Adenovirus-5, the overlay was made by mixing 370ml EPr-, with 125ml of molten agarose solution (2.4% in EPr-). Foetal calf or newborn calf serum was added to 3% final concentration and the overlay equilibrated to 42°C in a water bath, before 4ml was added to each petri dish. In the case of Influenza A, trypsin was added to the overlay, to a concentration of 4ug/ml.

The overlay for Adenovirus titrations was made by mixing 115ml of EPr- with 32ml of molten Noble agar (2.4% Noble agar in EPr-). Calf serum and 1.25M MgCl were added to a final concentration of 2% and 0.66% respectively and the overlay equilibrated to 42°C before adding 4ml to each petri dish.

Following incubation (as outlined in Table 18), the monolayers were fixed by flooding the plates with CIDEX/PBS (1:1). Cultures were allowed to fix for at least 4 hours, the overlay removed, the monolayers stained with Giemsa and

VIRUS	CELL	TIME OF INCUBATION (DAYS)	TEMPERATURE (°C) OF VIRUS ABSORPTION AND VIRUS GROWTH	MACROSCOPIC(MA) OR MICROSCOPIC(MI) COUNTING
VESICULAR STOMATITIS VIRUS, INDIANA STRAIN (VSV,IND)	BHK-21	2	37	MA
INFLUENZA TYPE A (INF. A)	MDCK	2	37	MA
EQUINE HERPES VIRUS TYPE-I (EHV-I) BOVINE HERPES VIRUS TYPE-I (BHV-I)	RK-13	2	37	MI MI
SEMLIKI FOREST VIRUS (SFV)	BHK	2	37	MA
REOVIRUS TYPE-3 (REO-3)	BS-C-I	5/6	37	MI
POLIOVIRUS TYPE-I (POLIO-I)	BS-C-I	2	34	MA
BUNYAMWERA, GERMISTON	BHK	5/6	31	MA
ADENOVIRUS TYPE 5 (ADENO-5)	HeLa	7	37	MA

Table 18

Assay of virus infectivity

the numbers of virus plaques determined and the virus titres calculated.

2.2.5. BIOLOGICAL ASSAYS

2.2.5.1. STOCK SOLUTIONS OF DRUGS

10mM preparations of cicloxolone sodium (CCX), and the diastereoisomers of CCX, 690 and 688 were made with sterile distilled water and then diluted 1:1 with sterile bovine serum albumin, which stabilises the drug in solution. Fresh stocks were prepared for each experiment and used immediately.

Monensin was prepared as a 10mM solution in ethanol and tunicamycin prepared at a concentration of 5mg per ml in DMSO.

Disulfirum stock solutions were made at a concentration of 30mM in DMSO.

All dilutions were prepared in EFC2 or EC2.

2.2.5.2. SCREENING OF CELLS FOR TOLERANCE TO DRUGS

DYE EXCLUSION

Cells were seeded at a low density (5 x 10^6 cells per 35mm petri dish or 10^5 cells per Linbro well). Following overnight incubation, cells were overlaid with growth medium supplemented with 2% serum containing increasing concentrations of drug. After one or two days treatment with the drug, cell cultures were treated as follows : the growth medium was decanted from each dish/well into a sterile container. The monolayer was washed twice with trypsin/versene (except for XTC-2 cells, where washes were in normal growth medium), and the washes added to the growth medium previously removed from the cells. Following centrifugation (at 8000g for 10 minutes), the supernatant was discarded, and the cell pellet resuspended in 1ml normal growth medium and returned to the trypsinised monolayer. A homogeneous suspension of the cells was obtained by vigorous pipetting. 100ul of the cell suspension was then mixed with 100ul of Trypan Blue dye (0.5% in PBS). After staining for 10 minutes, cells were counted in a Neubar Haemocytometer and two parameters measured : total cell number and the number of viable cells i.e. those excluding the dye.

2.2.5.3. DOSE-RESPONSE EXPERIMENTS

Cells were seeded on 35mm plastic tissue culture dishes (NUNC), at a density of 10^6 cells per dish or on 24 well Linbro trays at 5 x 10^5 cells per well, and incubated overnight at 37°C. Confluent monolayers of cells were infected with virus at a m.o.i. of 5 p.f.u./cell (10 p.f.u./cell for Adenovirus-5), and allowed to absorb for 1h (90 minutes for Adenovirus-5) at the appropriate temperature (Table 18). The monolayers were washed three times with PBS supplemented with 5% calf serum (4ml/plate or 1ml/well), then overlaid with EFC2 containing varying concentrations of drug.

Virus infected cells were incubated for 24h (or 48h in the case of Adenovirus-5) at the appropriate temperature (Table 18), after which the cells were harvested by scraping into the growth medium. Cell associated virus was released by ultrasonic disruption of the cells, and the yield of infectious virus determined by titration (Section 2.2.3.). To control for cytotoxicity, mock-infected cells (from the same batch as those used in the virus dose-response), were treated in parallel with drug. Cell viability was determined by Trypan Blue dye exclusion, after 24h (48h for Hela cells) drug treatment.

2.2.5.4. PLAQUE REDUCTION ASSAYS

VARICELLA ZOSTER VIRUS (VZV)

Monodispersed VZV infected Flow 2002 cells were mixed with uninfected Flow 2002 cells, then seeded into 50ml tissue culture flasks and incubated at 37°C overnight. Next day the medium was replaced with EFC2 containing either no drug or 12.5, 25, 50, 75 or 100uM CCX and the incubation resumed until 5 days post-infection, at which time the monolayers were fixed, stained and the number of virus plaques determined.

HUMAN CYTOMEGALOVIRUS (HCMV)

Monolayers of Flow 2002 cells were infected with 600 p.f.u. HCMV. Following absorption for 1h at 37°C, the monolayers were overlaid with EFC2 containing either no drug or 10, 20, 30, 40, 50, 60uM CCX, and incubated at 37°C for eight days (with a liquid medium change on the fourth day). The monolayers were then fixed, stained and the virus plaques counted.

These experiments were performed in duplicate and uninfected control monolayers checked microscopically for cytotoxicity : Flow 2002 cell monolayers are known to be resistant to CCX (Dargan and Subak-Sharpe, 1985).

2.2.5.5. ONE-STEP GROWTH CURVES

Cell monolayers (4×10^6 cells/50mm petri dish) were infected with virus at a m.o.i. of 5 p.f.u./cell. Following absorption (Table 18), the cells were washed three times with PBS and overlaid with EFC2 either drug-free or containing 300uM CCX. At the indicated times after absorption, the infectious virus yield from selected cultures were harvested, sonicated and titrated.

2.2.5.6. ABSORPTION CURVES

8×10^7 cells (Table 18), were suspended in 1ml PBS containing either no drug or 300, 600 or 900uM CCX. The cell suspension was incubated for 1 h at 37°C in a shaking water bath, to allow the drug to interact with the cells. The cells were then pelleted, the supernatant drawn off and the cell pellet washed with 10ml PBS to remove cell-free drug. The drug pre-treated cells were then resuspended in 1ml PBS containing 10^6 p.f.u. of virus and incubated in a shaking water bath at 37°C. At the indicated times post- infection, 100ul samples of the infected cell suspension, were withdrawn and mixed with 9.9ml of ice cold PBS. The cells were pelleted and the residual unbound virus titrated.

2.2.5.7. DIRECT INACTIVATION OF VIRUS PARTICLES

Virus suspensions were incubated at either 4°C or 37°C for 24h, with either 300uM CCX or an equal volume of BSA (mock drug). The virus suspensions were titrated before

addition of either 300uM CCX or BSA, immediately after addition and then 24h after treatment (48h in the case of Adenovirus-5).

2.2.5.8. THERMOSTABILITY OF VIRUS PARTICLES PRODUCED IN THE PRESENCE AND ABSENCE OF CCX

Infectious virus yields from CCX virus dose-response experiments were employed to investigate the thermolability of virus grown in the presence of CCX. The BSA content of each sample was standardised. Following standardisation, 1ml of virus grown in the absence or presence of 50, 150, 300uM CCX, was incubated at 42^{o}C in a shaking water bath. At 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes after incubation, 100ul aliquots were withdrawn from each sample and immediately titrated. The percentage of surviving infectious virus at each time was then calculated relative to that present at 0 time.

2.2.6. ELECTRON MICROSCOPY STUDIES

2.2.6.1.NEGATIVE STAIN

5ul of virus suspension was mixed with an equal volume of stain (uranyl acetate), containing a known number of latex beads (1.43 x 10^{10}/ml). After staining for two minutes the mixture was transferred to a plastic coated grid, and allowed to stand for a few minutes. Excess sample was removed by touching the meniscus of the sample with the edge of a filter paper. The virus remaining on the grid was examined under a Siemens 101 electron microscope. Particle counts were estimated by comparing the number of latex beads (Wildy et al, 1960).

2.2.6.2. EMBEDDING IN EPON / THIN SECTIONING

BS-C-1 cell monolayers (4 x 10^{6} cells/50 mm petri dish) were infected with SFV at m.o.i. of 5 p.f.u./cell, allowed to absorb for 1h at 37^{o}C, washed twice with PBS 5% FCS to remove unbound virus and overlaid with EFC2 without drug or containing 300 uM CCX or 10uM monensin. After 18 h incubation at 37^{o}C, selected cultures were harvested and either processed for electron microscopy or assayed for infectivity. The growth medium was removed and the monolayers fixed by adding 4ml of 2.5% gluteraldehyde (EM

grade) and incubating overnight at 4°C. The monolayers were then washed three times with PBS, and stained with 1% osmium tetraoxide in PBS for at least 2h. After further washing in PBS, the cells were scraped into PBS and pelleted by centrifugation at 8000g at 4°C for 10 minutes. The cell pellet was dehydrated by 15 minute washes in a series of increasing alcohol concentrations (30, 50, 70, 90 and 100%, made up in anala R H_2O).Cells were permeated with Epon : Ethanol (1:1) and allowed to stand overnight. The pellets were then embedded in fresh undiluted Epon which was polymerised by baking at 60°C, for 48h. Thin sections were cut with a diamond knife, stained with uranyl acetate, counter stained with lead citrate and examined in a Siemens 101 electron microscope operating at 60kV.

2.2.7. BIOCHEMICAL TECHNIQUES

2.2.7.1. RADIOLABELLING OF CELLULAR AND VIRUS-SPECIFIED POLYPEPTIDES

TREATMENT OF CELLS PRIOR TO VIRUS INFECTION

Cells were seeded in Linbro trays at 5 x 10^5 cells/well in normal growth medium or medium deficient in methionine (EFC2met- or EC2met-), or phosphate (EFC2phos- or EC2 phos-) as appropriate, and incubated at 37°C overnight. However in the case of Adenovirus-5, cells were seeded in normal growth medium, which was only replaced by the appropriate deficient medium 3h prior to the addition of label. Before infection with SFV, cells were treated with 5ug/ml actinomycin D for 3h and actinomycin D was maintained in the medium throughout infection.

VIRUS INFECTION AND ADDITION OF RADIOACTIVE LABELS

Virus infection and treatment with varying drug concentrations was as described in 2.2.5.3., except m.o.i. was 100 p.f.u./cell for Adenovirus-5 and 10 p.f.u./cell for all other viruses. Radioactive labels were added in the appropriate medium (^{35}S met : EFC2 / EC2 met- ; ^{32}P : EFC2 /EC2 phos- ; ^{14}Cglucosamine : EFC2 /EC2).The time of label addition varied and is indicated for each experiment.

At the time of harvest, the growth medium was removed and the monolayers washed twice with 1ml cold PBS. The washes and the growth medium for each sample were pooled and

centrifuged at 8000g at 4°C for 10 minutes to pellet any detached cells. Pelleted cells and cells remaining on the dish were lysed in 100ul protein dissociation mix, (0.125M Tris, HCl,pH6.8; 4% SDS; 10% 2-mercaptoethanol; 20% glycerol; 0.1% bromophenol blue) and pooled. Adenovirus-5 was lysed in a different dissociation mix (8mM Tris, HCl,pH6.8; 2% SDS; 10% glycerol; 2mM PMSF; 0.1M DTT; 0.2% bromophenol blue).

2.2.7.2. RADIOLABELLING OF PURIFIED VSV STRUCTURAL POLYPEPTIDES PRODUCED IN THE PRESENCE AND ABSENCE OF CCX AND MONENSIN

Sub-confluent roller bottles of BS-C-1 cells were incubated overnight at 37°C in EFC2 met-. The cells were then infected with VSV at m.o.i. 10 p.f.u./cell and the virus allowed to absorb for 1h at 37°C. Unabsorbed virus was then removed by washing the monolayers twice with EFC2 met-. Incubation was continued (for 24h) in EFC2 met- containing 25uCi /ml of ^{35}S met and either no drug or varying concentrations of CCX or monensin. After 24h, radiolabelled virions were purified as described in Section 2.2.3. Virus associated bands in Ficoll 400 gradients were resuspended in protein dissociation mix and analyzed by SDS-PAGE.

2.2.7.3. RADIOLABELLING OF CELLULAR AND VIRUS-SPECIFIED RNA SPECIES

VSV-INFECTED CELLS : LABELLING OF PRIMARY TRANSCRIPTS

For 2h prior to infection, monolayers of BS-C-1 cells (in 90mm petri dishes; 1 x 10^7 cells) were treated with 5ug per ml actinomycin D (Act. D) and 200ug per ml cycloheximide (CHX). Act D and CHX concentrations were maintained throughout the experiment. Cells were then infected in the usual way. Infected monolayers were overlaid with EFC2 containing 25uCi/ml 3H uridine and either no drug or various concentrations of CCX. RNA was extracted at 8h p.i. as shown in Figure 14.

VSV-INFECTED CELLS : LABELLING OF SECONDARY TRANSCRIPTS

BS-C-1 cells were seeded onto 90mm petri dishes in EFC2 phos- at a density of 1 x 10^7 cells per dish, and incubated overnight at 37°C. Two hours prior to infection

EXTRACTION OF CYTOPLASMIC RNA

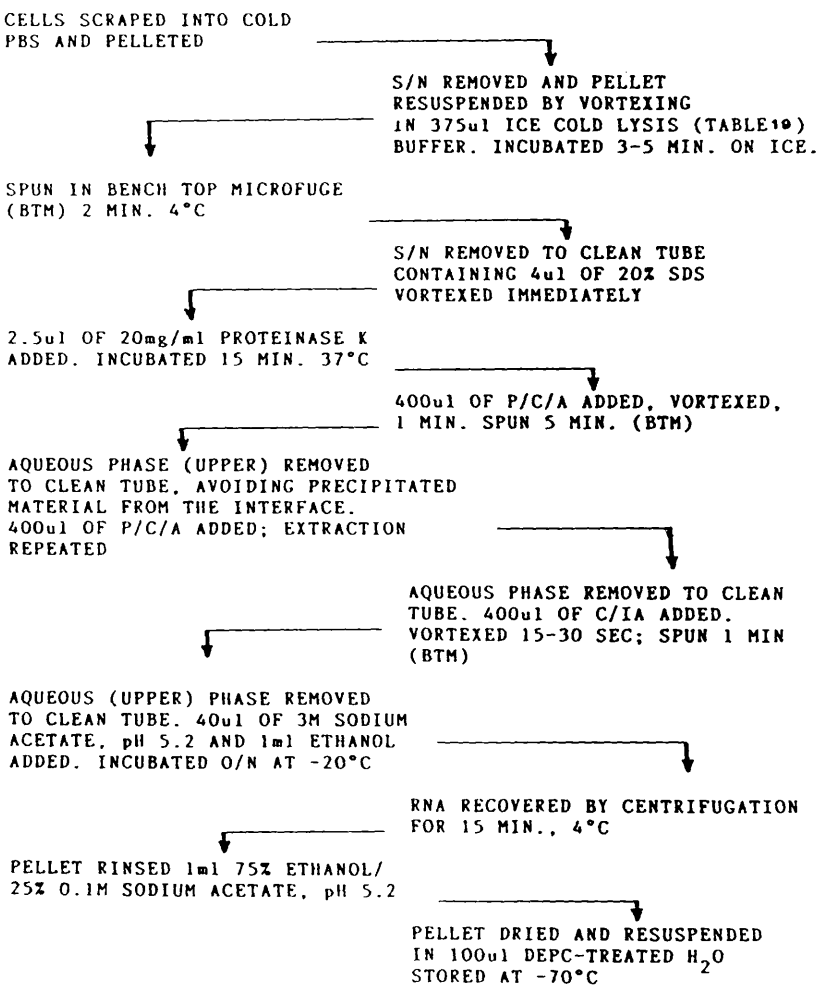


Figure 14

Extraction of cytoplasmic RNA

Footnotes:

1. All solutions were made up in Diethylpyrocarbonate (DEPC) treated water.
2. All volumes given are for $4x10^7$ cells.
3. Spins performed in bench top microfuge (BTM), at 13,000r.p.m.
4. P/C/A/ : 25:24:1 Phenol/Chloroform/Isoamylalcohol
   C/IA : 24:1 Chloroform/Isoamylalcohol
5. Lysis Buffer (see Table 19).

Sample Buffer

5ul 10 x MOPS running buffer
8.75ul 37% formaldehyde
25ul formamide

Formaldehyde Loading Buffer

1mM EDTA, pH8
0.25% Bromophenol Blue
0.25% Xylene Cyanol
50% glycerol

10 x MOPS [3-(N-morphilino)-propanesulphonic acid] running buffer

41.8g MOPS was added to 800ml DEPC-treated H_2O; pH adjusted to 7. To this was added 16.6ml of 3M DEPC-treated sodium acetate and 20ml 0.5M DEPC-treated EDTA, pH8, the final volume brought to 1l with DEPC-treated H_2O.
The buffer was filtered before use.

Cell Lysis Buffer

Chemicals	Volume (ml)
500mM Tris HCl, pH8	10
1mM NaCl	10
50mM $MgCl_2$	10
Nonidet P-40	0.5
H_2O	69.5

Table 19

Solutions and buffers used in agarose gel electrophoresis of RNA.

a

PERCENTAGE ACRYLAMIDE	30 % ACRYLAMIDE [29.25:0.75] (ml)	GEL BUFFER (ml)	DISTILLED H2O (ml)	10% AMMONIUM PERSULPHATE (ul)	TEMED (ul)
10	20	15	25	500	25
12.5	25	15	20	500	25

b

PERCENTAGE ACRYLAMIDE	30 % ACRYLAMIDE [28.5:1.5] (ml)	GEL BUFFER (ml)	DISTILLED H2O (ml)	GLYCEROL (ml)	10 % AMMONIUM PERSULPHATE (ul)	TEMED (ul)
5	5	7.5	17.5	-	187.5	12.5
8	8	7.5	8	6.5	120	12.5
12.5	12.5	7.5	5.5	4.5	81.25	12.5

Table 20

Single concentration (a) and gradient (b) polyacrylamide gels

and throughout the experiment cells were treated with 5ug per ml Act D. The cells were then infected with VSV at an m.o.i. of 50 p.f.u./cell, in the usual way, using EFC2 phosfor washes and overlays. Infected cells were then grown in the presence or absence of various concentrations of CCX. Secondary transcripts were labelled by adding ^{32}P-orthophosphate (25uC/ml) between 4 and 8 h p.i.RNA was extracted as shown in Figure 14.

LABELLING OF TOTAL RNA IN MOCK-INFECTED CELLS

Mock-infected BS-C-1 cells (90mm petri dishes; 1 x 10^7 cells/dish were labelled with ^{32}P-orthophosphate (25uCi/ml between 1 and 8h and 1 and 24h and RNA extracted as shown in Figure 14.

2.2.8. BIOPHYSICAL TECHNIQUES

2.2.8.1. POLYACRYLAMIDE GEL ELECTROPHORESIS OF POLYPEPTIDES (SDS PAGE)

Proteins were separated on polyacrylamide gels in the presence of 0.1% SDS using the discontinuous buffer system of Laemmli modified by Marsden. (Laemmli, 1970; Marsden, Crombie and Subak-Sharpe, 1976).The ratio of acrylamide to bisacrylamide was generally 39:1 for single concentration gels and 19:1 for gradient gels. Separating gels contained either 10 or 12.5% acrylamide and stacking gels contained 3% acrylamide (Table 20).The molecular weights of virus-specified proteins were estimated by reference to molecular weight protein standards (Section 2.1.).Electrophoresis of polypeptides was optimal when a constant current of 60mA was run through the gel.

2.2.8.2. PREPARATION OF POLYACRYLAMIDE GELS FOR ELECTROPHORESIS

Polyacrylamide gels containing electrophorised polypeptides were routinely soaked in fix / stain [0.2% Comassie Blue and methanol, H_2O, Glacial acetic acid (50:43:7)] for 1h. Bands were visualised by continuous shaking in destain [methanol, H_2O, glacial acetic acid (5:88:7)] overnight. When it was necessary to increase the sensitivity of detection of poorly labelled proteins, gels were first fixed (45% methanol, 5% acetic acid) for at least

45 minutes, then soaked in En^{3}Hance (New England Nuclear) for 1h and finally washed in water for 1h. Gels used to analyze ^{32}P-labelled proteins were treated according to the method of Petri et al (Petri, Patterson and Dimmock,1982). After electrophoresis gels were fixed in methanol and acetic acid as above, soaked for 1h at 90^{o}C in 5% TCA and washed twice in 5% TCA at room temperature for 1h. This treatment with hot and cold TCA removes the phosphate which is not covalently linked to protein (Bhorjee and Pederson, 1976) and so reduced background radiation in the resulting autoradiographs.

All gels were dried onto Whatman number 1 filter paper under vacuum (30 units) at 80^{o}C for 1.5h. Dried gels were placed against X-Omat film. Exposure of gels was at room temperature, unless they had been treated with En^{3}Hance when exposure was at -70^{o}C. Exposed autoradiograph films were developed and fixed automatically in an X-Omat film developer.

2.2.8.3. AGAROSE GEL ELECTROPHORESIS OF RNA

PREPARATION OF SAMPLES

The yield of RNA was first quantitated by measuring the O.D. (optical density) at 260nm with a Cecil CE 595 double beam digital spectrophotometer, assuming an O.D. of one at 260nm in a 1cm pathlength to be equivalent to 50ug RNA. Samples were prepared for electrophoresis by adding 38.75ul of sample buffer (Table 19) to 11.25ul of RNA, and then adding 10ul of formaldehyde loading buffer (Table 19).

ELECTROPHORESIS OF SAMPLES ON 1.2% AGAROSE / FORMALDEHYDE GELS

Samples were analyzed on a 1.2% agarose/formaldehyde gel containing 1% MOPS running buffer and 0.5% formamide. The gel was run at a constant voltage of 5V/cm until the bromophenol blue band had migrated halfway down the gel (-3h). The gel was then allowed to sit in excess water overnight. The next day the water was decanted and ethidium bromide added (25ul of 10mg/ml in 500ml H_2O). The gel was then allowed to stain for 45 minutes and then photographed under short wave U.V. illumination (260nm),thus allowing visualisation of total RNA. The gel was then dried and

placed against X-Omat S film. Exposed autoradiographs were developed and fixed automatically in X-Omat film developer, revealing radiolabelled RNA.

3. RESULTS

3.1. CELL SURVEY

3.1.1. CLASSIFICATION OF CELL LINES AS RESISTANT, INTERMEDIATE OR SENSITIVE TO CCX TREATMENT

Dargan and Subak-Sharpe (1985), reported that three cell lines differed in their sensitivity to treatment with up to 300uM CCX : Flow 2002 cells (human) were resistant, Vero cells (monkey) were sensitive, while BHK-21 cells (rodent) displayed intermediate sensitivity. The present study which has expanded the range of cell lines was performed with two objectives in mind. Firstly, to identify cell lines resistant to CCX treatment and permissive for virus growth to allow uncoupling of antiviral and cytotoxic effects. Secondly, to establish how a range of cell lines compare in their tolerance to CCX treatment and attempt to determine the basis for differential CCX sensitivity.

Cell monolayers overlaid with various concentrations of CCX were treated with trypan blue 24h and 48n after drug addition, and total cell number and the number of viable cells then determined (Materials and Methods, Section 2.2.5.2.). On the basis of cell viability in the presence of CCX, the cell lines fell into three groups (Resistant, Intermediate or Sensitive), the designation of individual cell lines to a particular group depending upon a consistent performance in three independent experiments. Curves showing cell viabilities and total cell numbers with increasing CCX concentrations are plotted in Figures 15-17.

RESISTANT

Nine cell lines fell into the Resistant class : Flow 2002, RK-13, Chang Liver, Detroit 532, HeLa, Hep2, MDBK, MDCK, BS-C-1 (Fig.15a-i). In general, for cell lines classified as Resistant the decrease in viability obtained with 300uM CCX was not greater than 20% at 24h, and 30% at 48h.

The decrease in cell viability was less than or equal to 10% after 24h treatment with CCX to 300uM, in all cases (Fig. 15a,b,c,d,e,g,h,i,), with the exception of Hep2 (Fig.15f), where the reduction was 19%. 48h treatment to 300uM CCX, resulted in the viability of Flow 2002, Chang Liver, Detroit 532, HeLa and BS-C-1 cells (Fig.15a,c,d,i) being reduced by less than or equal to 10%, with the reduction in Hep 2 cell viability (Fig.15f), being just slightly greater at 11%. RK-13, MDBK and MDCK (Fig.15b,g,h,) cell viability decreased by 28%, 15% and 24% respectively after 48h treatment with CCX to 300uM. The effect on cell viability increased with increasing CCX in the case of MDBK and MDCK (Fig.15g,h). In contrast, RK-13 cell viability (Fig. 15b) was unaffected by CCX to 200uM, with a sudden decrease achieved with 300uM CCX. This result was shown to be consistent in repeat experiments.

The total cell number typically rose 2 fold in a 24h period in the absence of CCX. 24h treatment at the highest concentration had some effect on the replication of cell lines in this class: cell numbers rose to between 68% (Hep2, Fig 15f) and 84% (Chang Liver, Fig 15c) of the drug free control. The effect on cell replication progressed with both increasing drug and time, cell numbers rising to between 52% (Flow 2002, Fig 15a) and 68% (Chang Liver and Detroit 532, Fig 15c and d) of the drug free control after 48h treatment with 300uM CCX. Therefore, while the viabilities of resistant cell lines was not significantly affected, their growth was impaired by CCX treatment. These cell lines were classified as suitable for use as hosts in virus dose-response experiments using concentrations to 300uM. As virus dose-response experiments were performed in confluent monolayers, the effect on cell number could not contribute to any effect on virus replication.

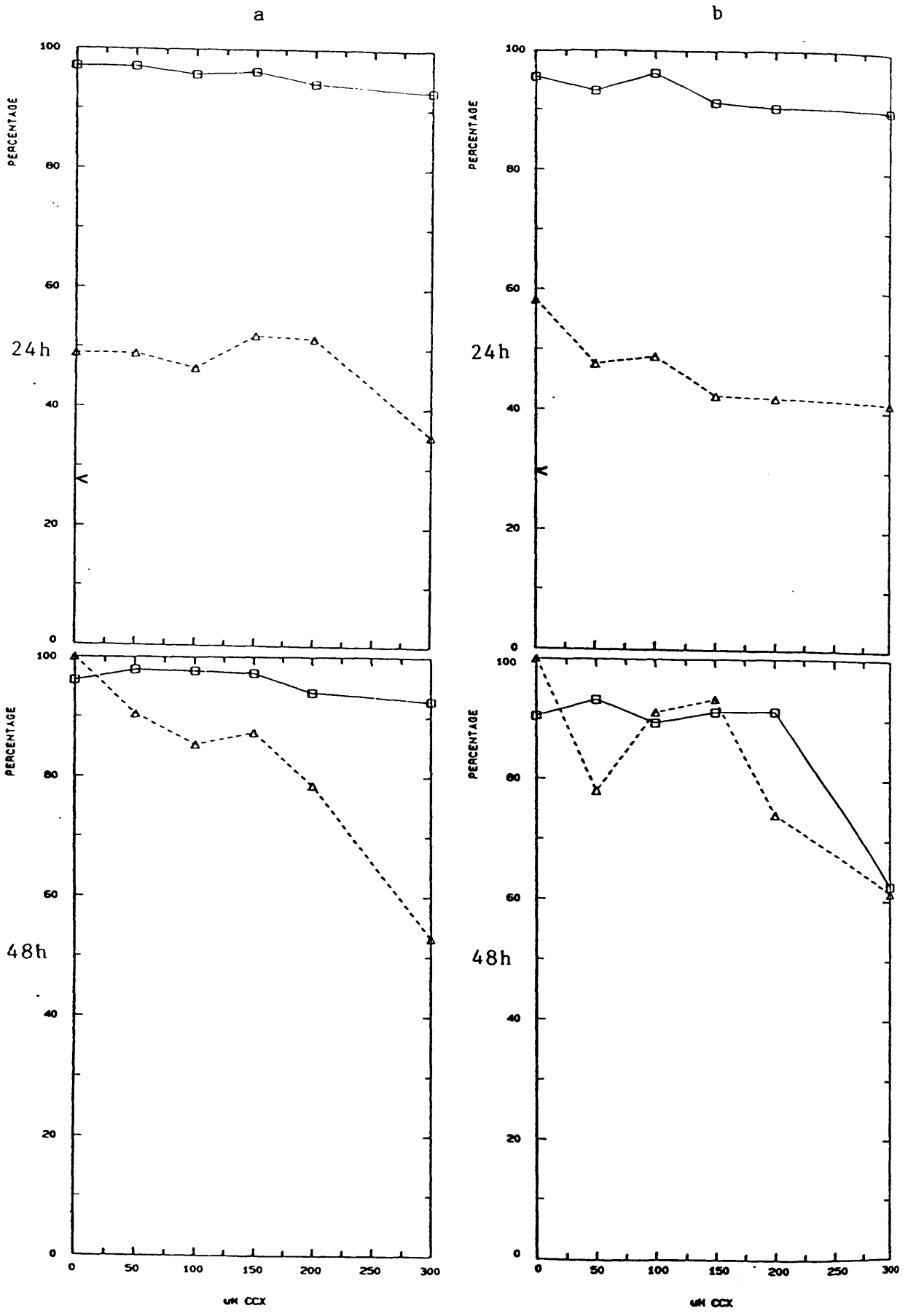

a
b
PERCENTAGE
24h
48h
uM CCX
0
20
40
60
80
100
50
150
200
250
300

Figure 15

CCX Resistant cell lines: a) Flow 2002, b) RK-13.

The effect of increasing CCX concentrations on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treatment. The total cell number at 0h is denoted by (<).

All total cell number percentages are relative to the drug free control after 48h, while cell viability percentages are relative to the total number of cells on each plate.

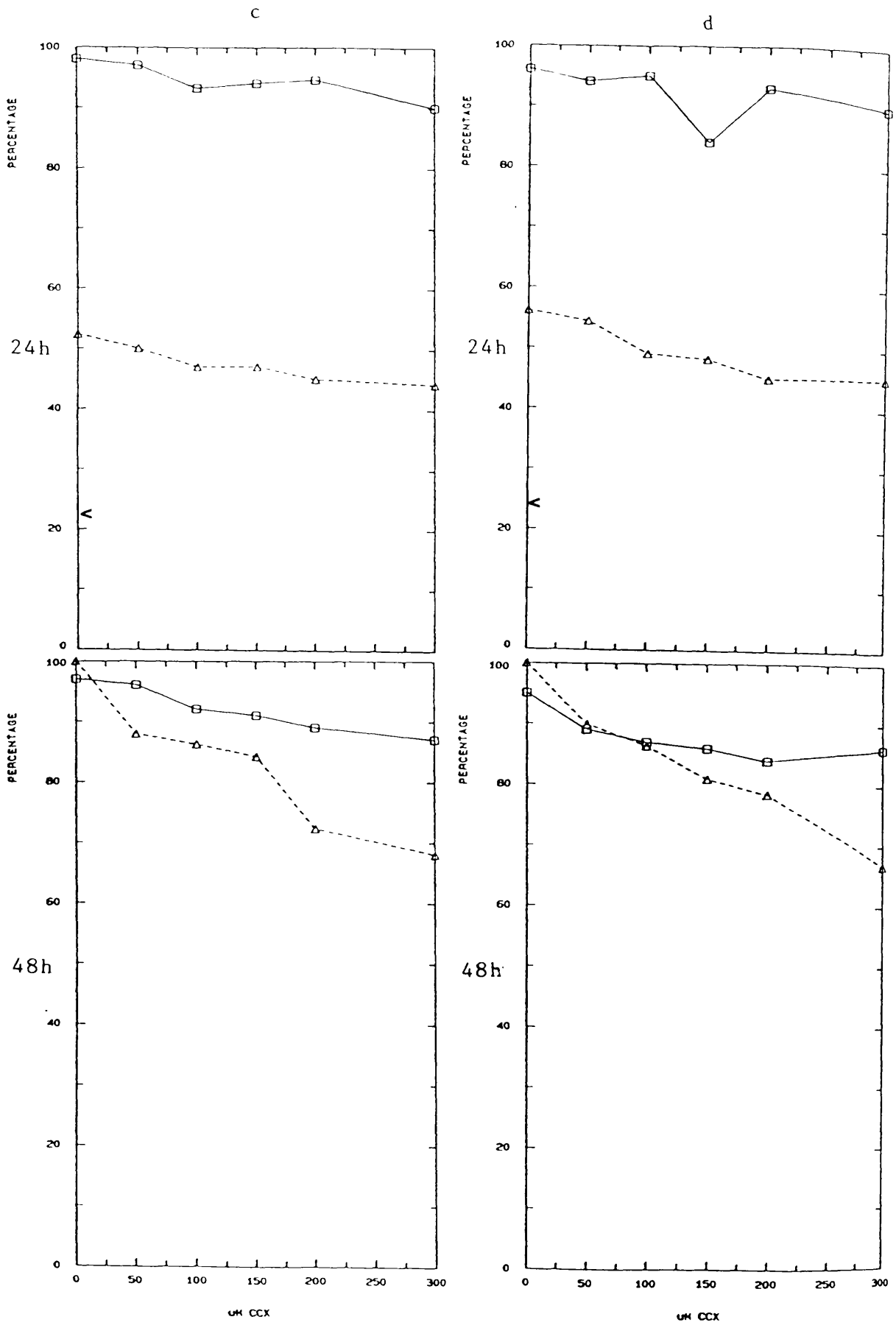
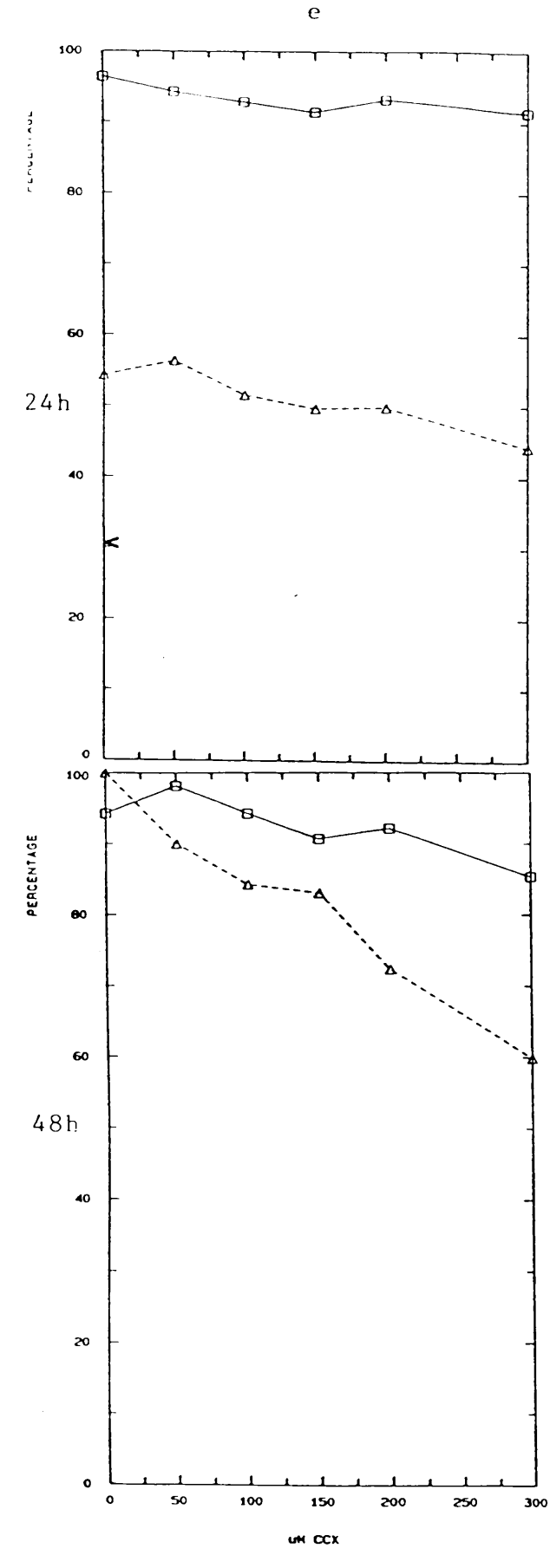

c
d
PERCENTAGE
24h
48h
uM CCX
0
20
40
60
80
100
50
150
200
250
300

Figure 15

CCX Resistant cell lines: c) Chang Liver, d) Detroit 532.

The effect of increasing CCX concentrations on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treament. The total cell number at 0h is denoted by (<).

All total cell number percentages are relative to the drug free control after 48h, while cell viabilty percentages are relative to the total number of cells on each plate.


e
PERCENTAGE
24h
48h
uM CCX

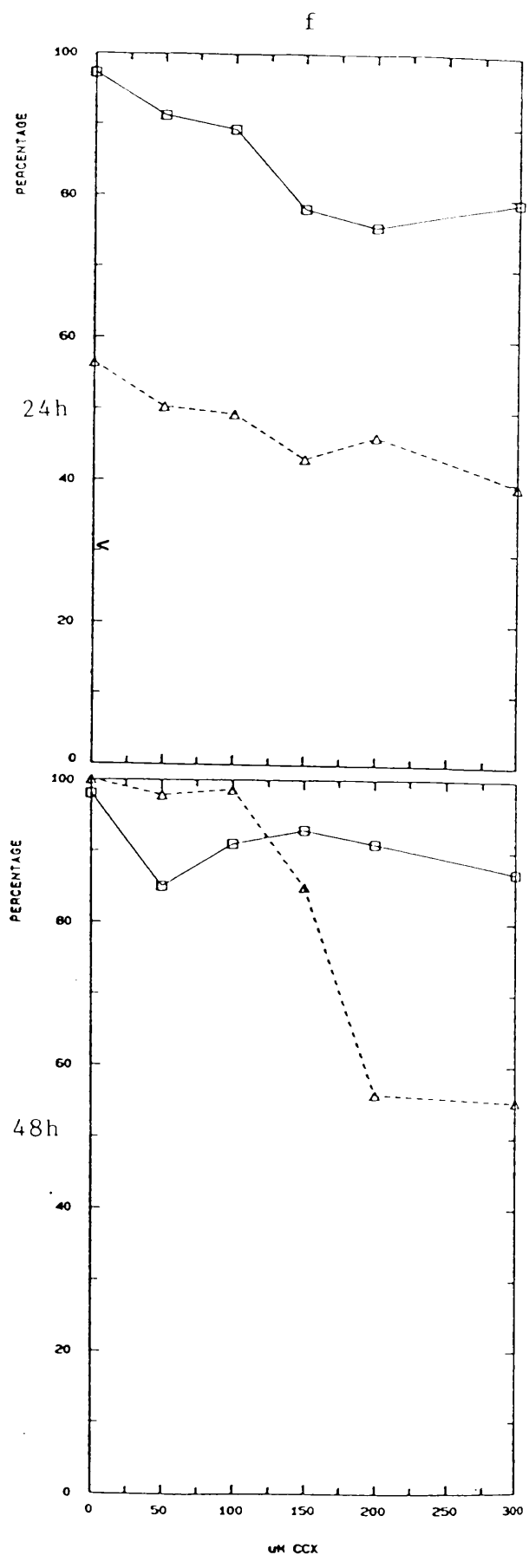

f
PERCENTAGE
24h
48h
uM CCX

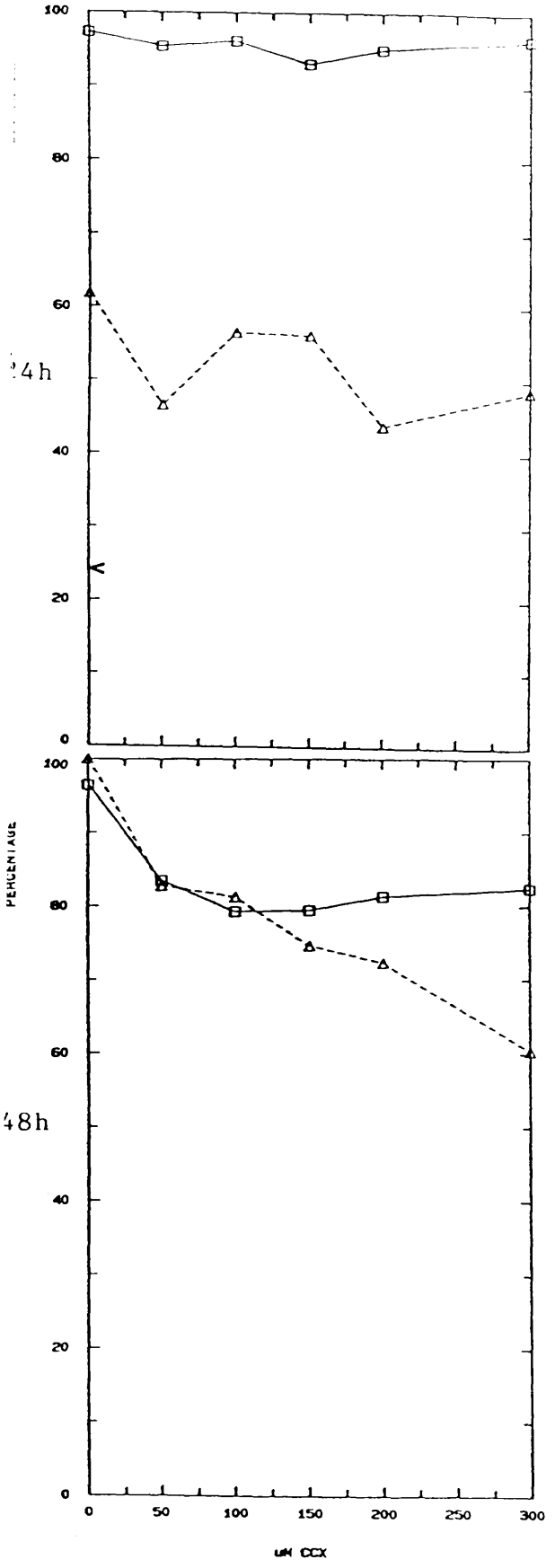
Figure 15

CCX Resistant cell lines: e) HeLa, f) HEp_2.

The effect of increasing CCX concentrations on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treatment. The total cell number at 0h is denoted by (<).

All total cell number percentages are relative to the drug free control after 48h, while cell viability percentages are relative to the total number of cells on each plate.

g


24h
48h
PERCENTAGE
100
80
60
40
20
0
0
50
100
150
200
250
300
uM CCX

h

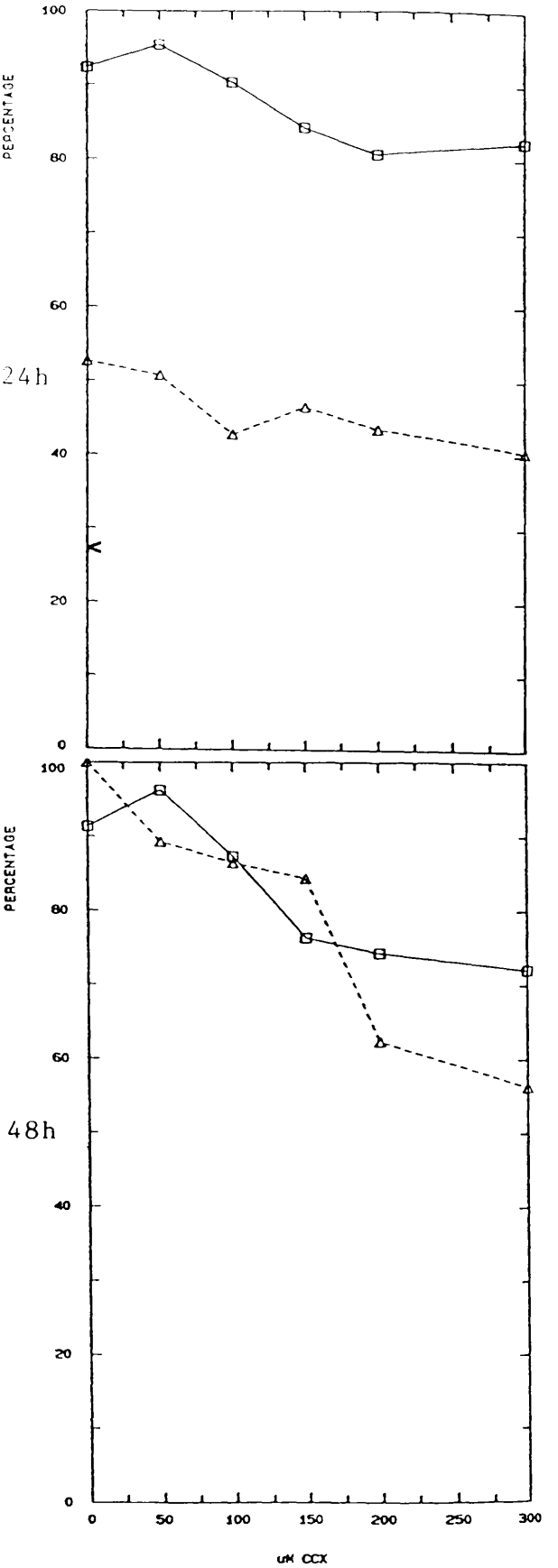

24h
48h
PERCENTAGE
100
80
60
40
20
0
0
50
100
150
200
250
300
uM CCX

Figure 15

CCX Resistant cell lines: g) MDBK, h) MDCK.

The effect of increasing CCX concentrations on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treatment. The total cell number at 0h is denoted by (<).

All total cell number percentages are relative to the drug free control after 48h, while cell viability percentages are relative to the total number of cells on each plate.

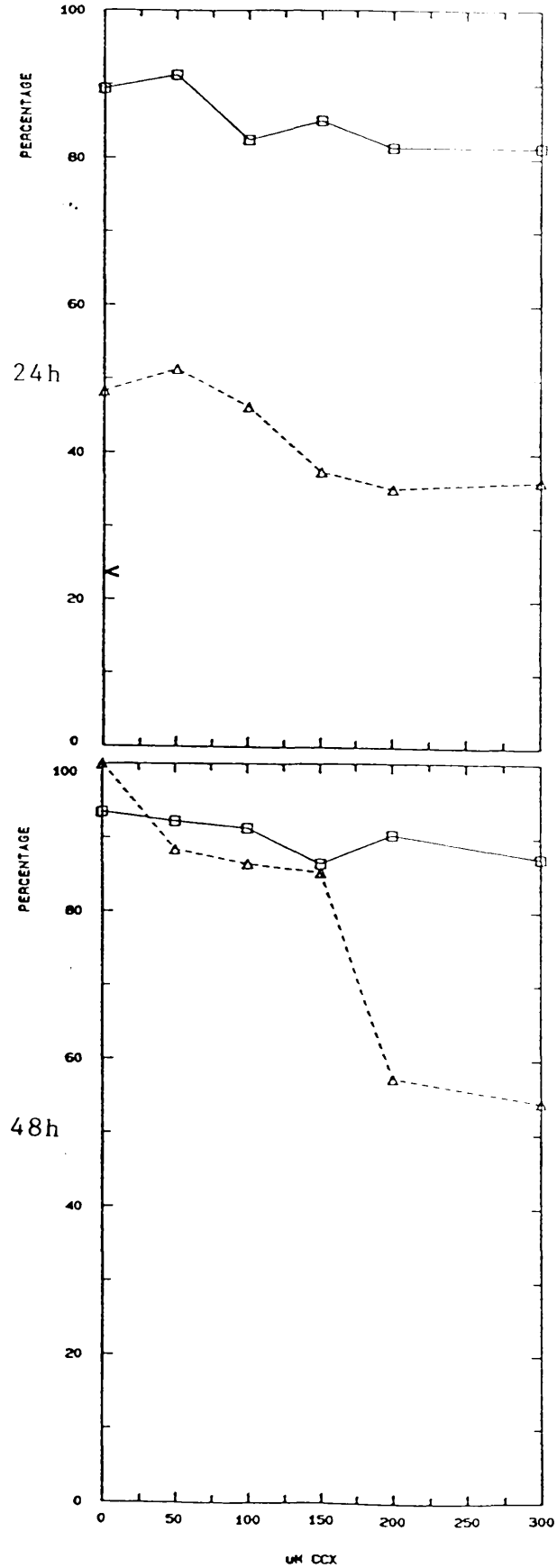

PERCENTAGE
100
80
60
40
20
0
24h
PERCENTAGE
100
80
60
40
20
0
48h
0
50
100
150
200
250
300
uM CCX

Figure 15

CCX Resistant cell lines: i) BS-C-1.

The effect of increasing CCX concentrations on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treatment. The total cell number at 0h is denoted by (<).

All total cell number percentages are relative to the drug free control after 48h, while cell viability percentages are relative to the total number of cells on each plate.

INTERMEDIATE

Five cell lines fell into the Intermediate class : BHK-21, Flow 4000, MRC-5, Rabbit Epithelium, Rabbit Keratocytes (Fig 16a-e). Viabilities of all cell lines belonging to this class were progressively reduced with both increasing drug and time.

After 24h, a significant reduction in cell viability was achieved with only 100uM CCX in all cases (BHK-21,15%; Flow 4004,21%; MRC-5,15%; Rabbit Epithelium,14%; Rabbit Keratocytes,15%) (Fig.16a-e). This reduction progressively continued as concentration of drug increased,so that BHK cell viability was reduced by 90%, MRC-5 by 80%, Rabbit Epithelium by 80% and Rabbit Keratocytes by 90% (Fig.16a,c,d,e). No viable MRC-5 cells could be detected at 300uM CCX (Fig.16c), with only 5%, 8% and 2% of the cells being viable in the case of BHK-21, Rabbit Epithelium and Rabbit Keratocytes (Fig.16a,d,e). Flow 4000 cell viability was not as dramatically affected by the drug, viability reduced by 66% with 300uM CCX, with 25% of the cells still viable (Fig.16b).

Treatment for 48h, with the lowest CCX concentration (50uM), had a significant effect on cell viability with BHK-21, Flow 4000, MRC-5, Rabbit Epithelium and Rabbit Keratocyte cell viability reduced by 24%, 23%, 11%, 13% and 14% respectively (Fig.16a-e). The effect was progressive with reductions in viability of between 45%-58% being achieved with 150uM CCX, and no viable cells detected with 300uM CCX in all cases, except Flow 4000, where only 3% of the cells were viable.

After only 24h, total cell number was decreased with increasing CCX concentration, resulting in almost complete inhibition of cell replication at 300uM in all cases (Fig.16a-e). After 48h, total cell number again decreased with increasing CCX concentration. However, some cell growth occurred at concentrations of CCX less than 300uM, with the exception of Rabbit Keratocytes where cell replication was completely inhibited with 200 and 300uM CCX (Fig.16e).

Replication of cell lines belonging to the Intermediate class was progressively reduced with increasing drug. However, no progression in the inhibition of cell replication was observed in the second 24h period.

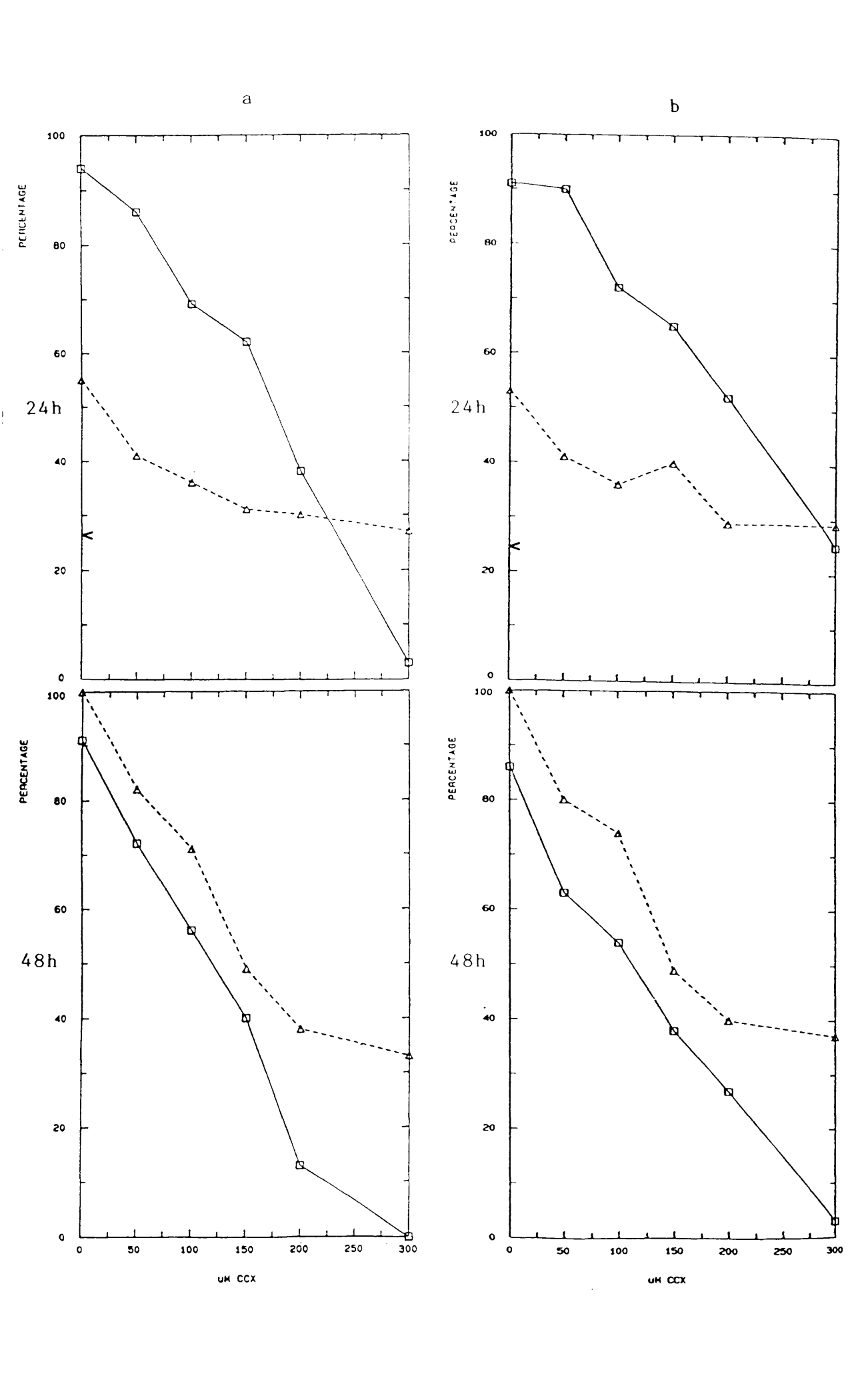

a
b
PERCENTAGE
24h
48h
uM CCX
0
50
100
150
200
250
300
20
40
60
80
100

Figure 16

CCX Intermediate cell lines: a) BHK-21, b) Flow 4004.
The effect of increasing CCX concentrations on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treatment. The total cell number at 0h is denoted by (<).
All total cell number percentages are relative to the drug free control after 48h, while cell viability percentages are relative to the total number of cells on each plate.

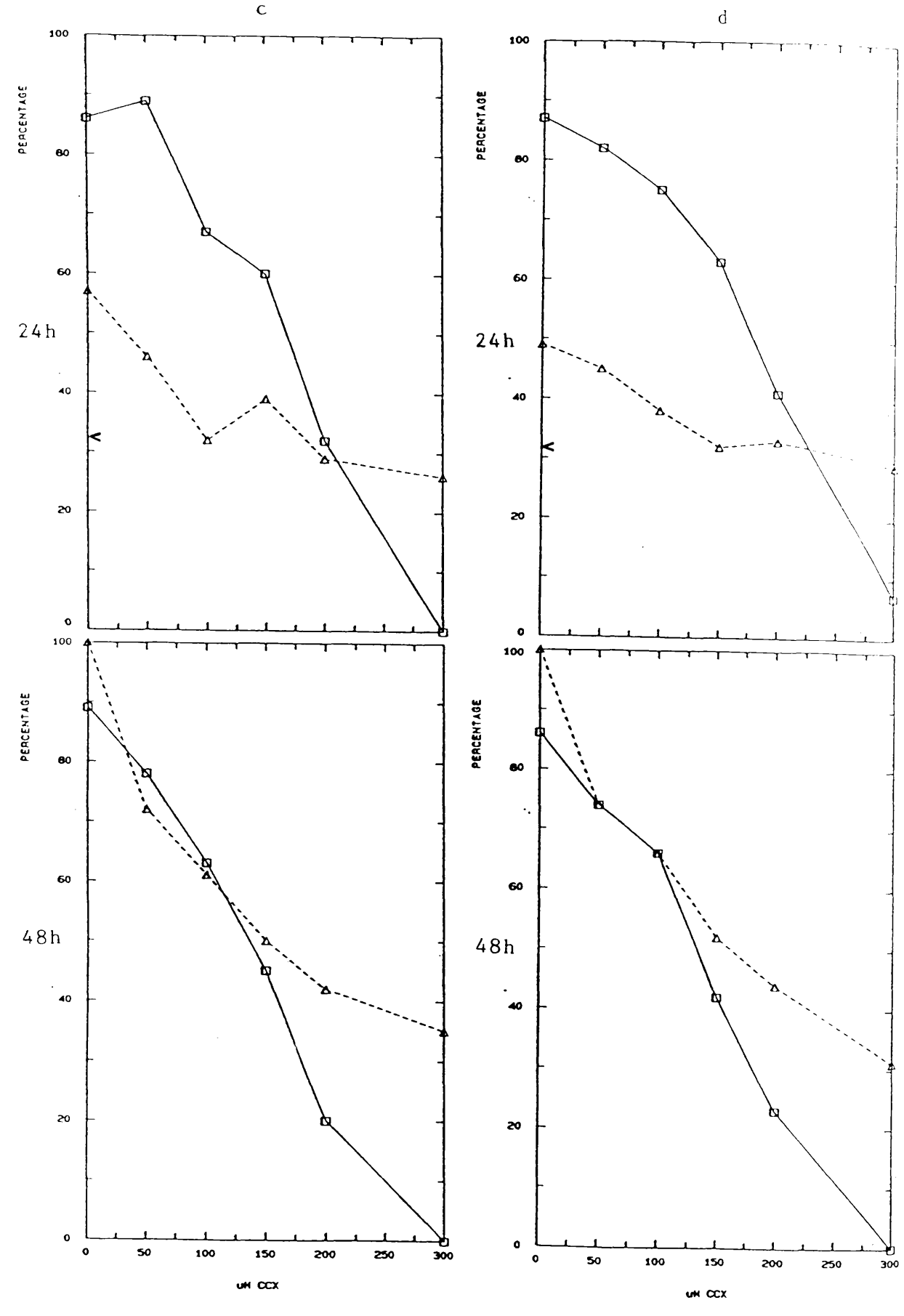

c
d
PERCENTAGE
24h
48h
uM CCX
0
50
100
150
200
250
300
20
40
60
80
100

Figure 16

CCX Intermediate cell lines: c) MRC-5, d) Rabbit Epithelium.

The effect of increasing CCX concentrations on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treatment. The total cell number at 0h is denoted by (<).

All total cell number percentages are relative to the drug free control after 48h, while cell viability percentages are relative to the total number of cells on each plate.

e

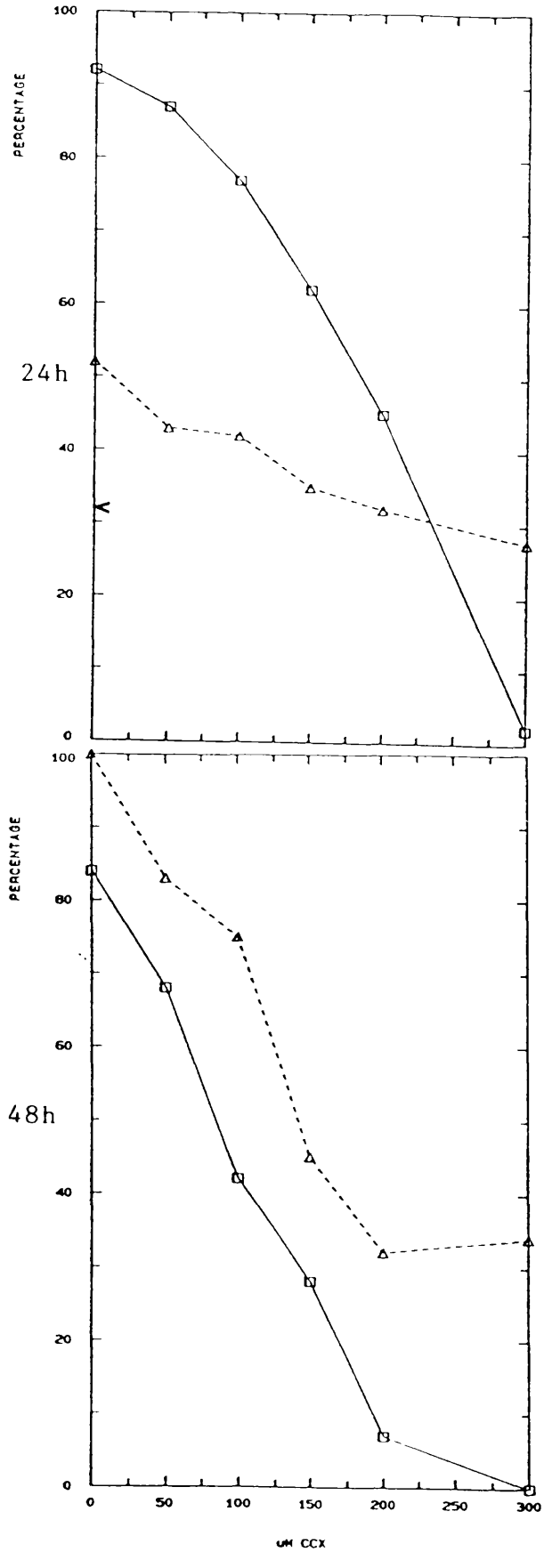

PERCENTAGE
100
80
60
24h
40
20
0
PERCENTAGE
100
80
60
48h
40
20
0
0
50
100
150
200
250
300
uM CCX

Figure 16

CCX Intermediate cell lines: e) Rabbit Keratocytes.

The effect of increasing CCX concentrations on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treatment. The total cell number at 0h is denoted by (<).

All total cell number percentages are relative to the drug free control after 48h, while cell viability percentages are relative to the total number of cells on each plate.

SENSITIVE

The Sensitive class consists of six cell lines : Fg 293, XTC-2, HOOD, Re-r, Re99 and PCEF (Fig 17a-f). The most sensitive cell line was Fg 293, the viabilty of these cells decreasing by 70% after 24h treatment with only 50uM CCX (Fig 17a). The same percentage decrease in cell viability after 24h, was achieved with Re-r cells at 100uM CCX and for all the rest of the cell lines in this group, except Re 99 at 150uM (Fig 17b,c,f). 48h treatment with only 50uM CCX almost completely abolished Fg 293 cell viability (Fig 17a). While XTC-2 and HOOD cell viabilities decreased by 71% and 73% respectively (Fig 17b,c), with Re-r and Re99 reduced by 50% and 30% respectively (Fig.17d,e), PCEF cells showed somewhat greater tolerance to treatment with CCX concentrations less than 150uM, over a 48h period (Fig.17f). At 50uM CCX, viability remained high and was similar to that observed with Resistant cell lines (Fig. 15a-i). However cell viability was progressively reduced culminating in total death of the cell culture with 200uM CCX, in common with other members of the group.

Little or no cell replication occurred even at low drug concentrations for all cell lines in this group, except PCEF, whose cell growth was ~~not~~ ~~marked~~less markedly affected, by 24h treatment with drug concentrations lower than 200uM CCX (Fig. 17f). Cell growth was affected by 48h treatment with all drug concentrations: there was a progressive reduction in total cell number, leading to almost total inhibition with 150uM CCX. Thus, for all cell lines in this Sensitive group the effect on total cell number parallels the effect on cell viability.

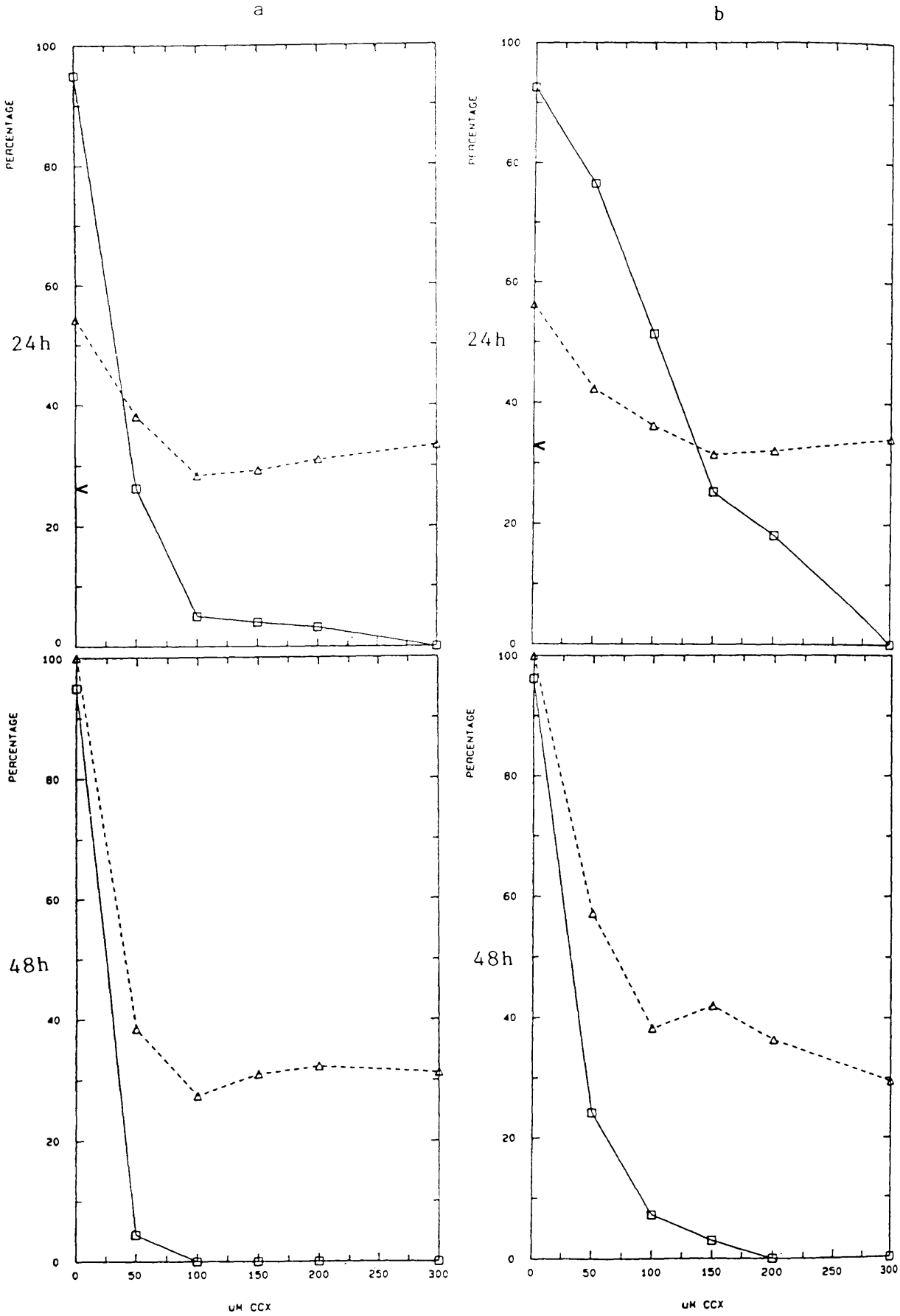

a
b
PERCENTAGE
24h
48h
uM CCX
0
20
40
60
80
100
50
150
200
250
300

Figure 17

CCX Sensitive cell lines: a) Fg 293, b) XTC-2.

The effect of increasing CCX concentrations on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treament.The total cell number at 0h is denoted by (<).

All total cell number percentages are relative to the drug free control after 48h, while cell viability percentages are relative to the total number of cells on each plate.

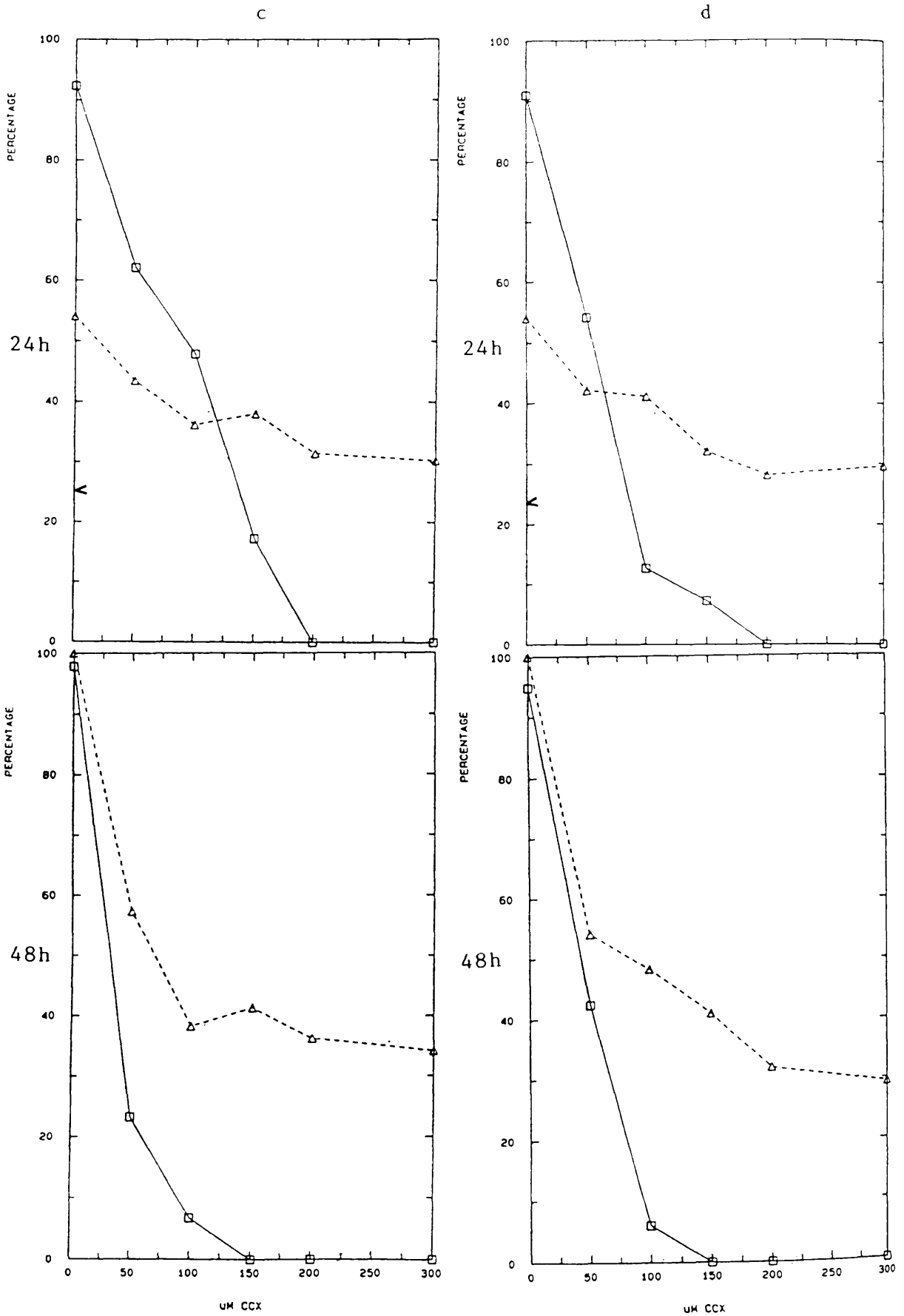

c
d
PERCENTAGE
24h
48h
UM CCX
0
20
40
60
80
100
50
150
200
250
300

Figure 17

CCX Sensitive cell lines: c) HOOD, d) Re-x .

The effect of increasing CCX concentrations on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treatment. The total cell number at 0h is denoted by (<).

All total cell number percentages are relative to the drug free control after 48h, while cell viability percentages are relative to the total number of cells on each plate.

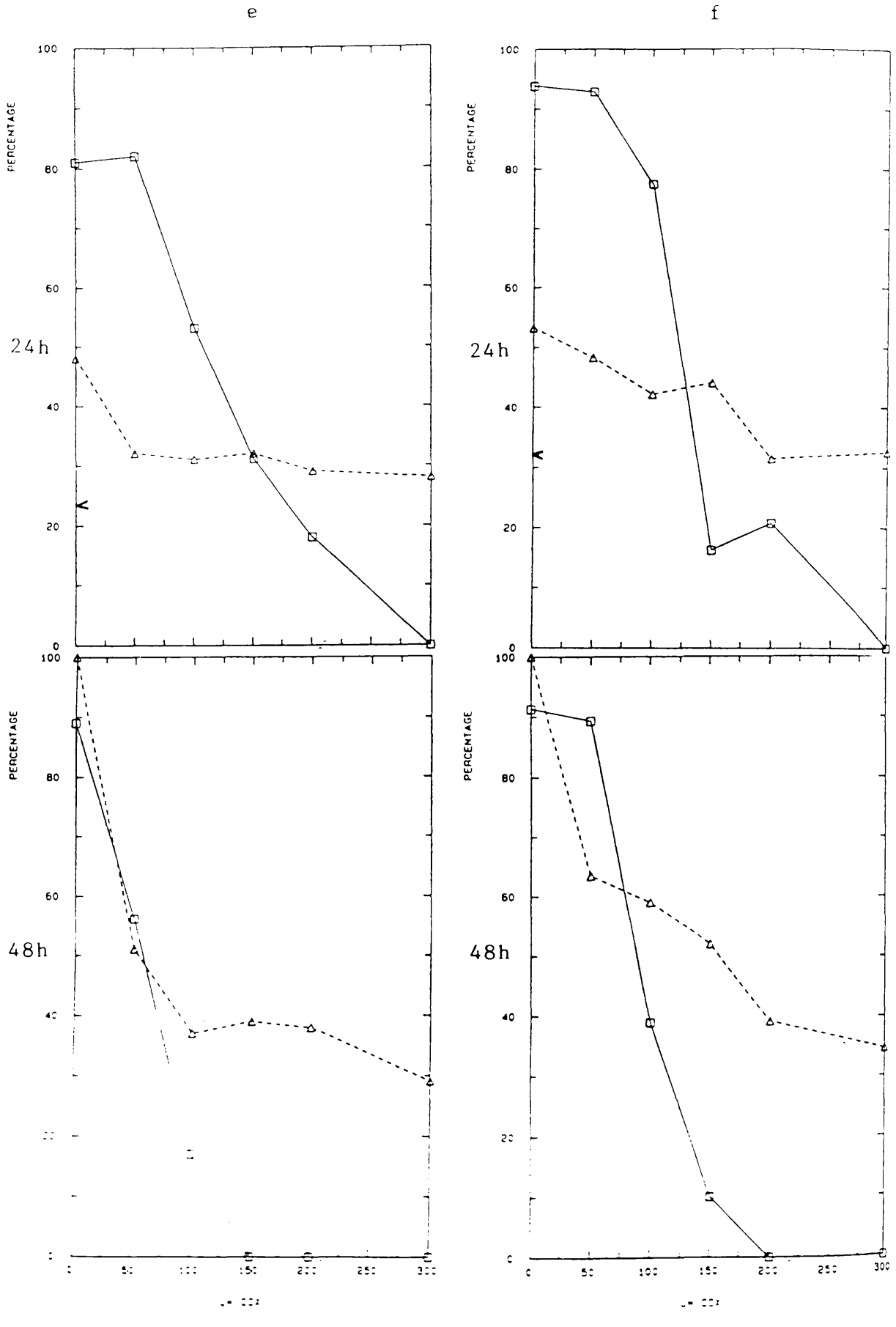

e
f
PERCENTAGE
24h
48h
100
80
60
40
20
0
50
150
200
250
300

Figure 17

CCX Sensitive cell lines: e) Re 99, f) PCEF.

The effect of increasing concentrations of CCX on cell viabilities (□) and total cell numbers (△), after 24h and 48h drug treatment. The total cell number at 0h is denoted by (<).

All total cell number percentages are relative to the drug free control after 48h, while cell viability percentages are relative to the total number of cells on each plate.

A summary of the cell lines examined and the classes into which they fall are shown in Table 21. Of the twenty cell lines in the survey, nine were found to be Resistant, five Intermediate, and six Sensitive to CCX treatment. The species may be important : of nine human cell lines, six proved Resistant, two Intermediate and only one Sensitive, while of eight rodent cell lines, only one was Resistant, three were Intermediate and three Sensitive. It should be noted that the only human cell line which is sensitive to CCX is the Adenovirus DNA transformed cell line Fg 293, which expresses the Adenovirus Ela and Elb genes. Several other transformed lines (HOOD, Re and Re99) also fall into this Sensitive group. Cell type per se may play some role in conferring tolerance to CCX: of the nine fibroblastic lines tested only 2 fell into the Resistant class, with 3 Intermediate and 4 falling into the Sensitive class.

Five of the nine cell lines identified as resistant to CCX were chosen because of their biological properties, as suitable hosts in which to perform virus dose-response experiments : Flow 2002, BS-C-1, HeLa, MDCK and RK-13. The most useful cell line identified was the BS-C-1 line as this is permissive for the growth of five viruses in the study.

RESISTANT	INTERMEDIATE	SENSITIVE
Flow 2002 [H]f	BHK-21 [R]f	Fg 293 [H]e
BS-C-I [S]e	FLOW 4000 [H]f	XTC-2 [A]e
CHANG LIVER [H]e	MRC-5 [H]f	HOOD [R]f
DETROIT 532 [H]e	RABBIT [R]e EPITHELIUM	Re∠ [R]f
HELA [H]e	RABBIT [R]e KERATOCYTES	Re 99 [R]f
HEP-2 [H]e		PCEF [Av]f
MDBK [B]f		
MDCK [C]e		
RK-13 [R]e		

Table 21

Classification of cell lines based upon their sensitivity to CCX.

[H] human
[R] rodent
[S] simian
[C] canine
[B] bovine
[A] amphibian
[Av] avian

Transformed Lines

Fg 293: transformed with Ela and Elb region of Adenovirus-5 DNA.

Re : transformed with HSV 1 (strain HFEM)

Re 99: transformed with ts 1 HSV 2 (strain HG 52)

HOOD: immortalised by serial passage

e: epithelial; f: fibroblastic

3.1.2. RECOVERY OF DRUG TREATED CELLS

For better evaluation of the cytotoxic effect of CCX on the cell lines employed in virus dose-response experiments, and to determine whether cell death is a factor in the antiviral effect, reversibility of the cytotoxic effect was examined. BS-C-1, HeLa, MDCK and RK-13 cells were treated with mock drug (the solution containing BSA in which CCX is made up), or various uM concentrations of CCX. After 24h or 48h as appropriate the CCX was removed from the cell cultures by three washes with $PBS_{5\%}$ FCS and then overlaid with drug-free medium and incubated at 37°C for a further five days. The total number of cells and the percentage of viable cells was then determined for each cell line in the usual way (Table 22). As previously noted with Flow 2002 cells, presence of increasing concentrations of CCX in all cases slows down cell replication; RK-13 < HeLa < BS-C-1 < MDCK. Cell viability is least affected in BS-C-1 cells suggesting they represent very favourable hosts for investigating the antiviral effects of CCX treatment. In every case, the effect on cell replication was reversible as evidenced by increasing total cell numbers and constantly high cell viabilities. The reversible effect of CCX on Flow 2002 (and BHK-21) cells was previously demonstrated by Dargan and Subak-Sharpe, 1985.

CELL LINE	CCX Conc. uM	TIME (h)				
		0	24		48	
		a	a	b	a	b
BSC-I	0	36 (89)	52.2 (96)	6.2 (89)	100 (94)	3.5 (91)
	50		47.2 (97)	7.5 (91)	83.3 (93)	4 (92)
	100		44.4 (91)	7.6 (87)	75.0 (85)	4.7 (84)
	200		41.6 (89)	8.7 (86)	55.5 (84)	6.1 (83)
	300		38.8 (87)	8.4 (89)	50.5 (83)	6.6 (81)
HeLa	0	29.4 (87)	55.8 (89)	8.2 (91)	100 (88)	4.8 (93)
	50		52.9 (91)	8.9 (87)	82.3 (87)	5.9 (94)
	100		47.0 (82)	10.11(86)	82.3 (76)	5.3 (87)
	200		45.3 (78)	10.3 (92)	58.8 (76)	7.9 (86)
	300		41.1 (79)	11.3 (89)	55.5 (75)	8.6 (85
MDCK	0	26.3 (88)	52.6 (96)	8.1 (91)	100 (94)	4.1 (91)
	50		47.3 (95.2)	8.8 (89)	89.4 (91)	4.6 (94)
	100		43.4 (88.1)	10.0 (89)	73.7 (81)	5.9 (89)
	200		36.8 (75.6)	11.4 (91)	49.7 (76)	8.2 (88)
	300		37.3 (76)	11.1 (93)	58.2 (74)	8.2 (87)
RK-13	0	29.4 (91)	52.9 (89)	4.7 (90)	100 (91)	3.1 (91)
	50		50.0 (84)	6.0 (89)	90 (87)	3.2 (94)
	100		49.4 (81)	4.5 (81)	82.3 (81)	3.4 (81)
	200		44.1 (80)	5.5 (84)	70.5 (75)	4.2 (83)
	300		44.1 (81)	5.7 (83)	58.8 (63)	4.9 (86)

Table 22

Recovery of drug-treated cells.
Cells were treated with increasing concentrations of CCX and after 24h or 48h drug addition cells were either harvested (column a), or washed and overlaid with drug-free medium, and incubation continued for a further 5 days before harvesting (column b).
Harvested cells were then subjected to dye exclusion (trypan blue) and both total cell number and the number of viable cells determined.

In both column a and b, numbers in brackets indicate percentages of viable cells.

Column a: numbers are percentages of the total cell count relative to the 48h drug-free control.
Column b: the given numbers are multipliers indicating the increase during the 5 day recovery period in total cell number, relative to the numbers observed in the individual cultures after 24h or 48h CCX treatment (column a).

3.1.3. STRESS PROTEINS INDUCED IN CCX TREATED CELLS

Dargan and Subak-Sharpe (1986a), previously reported the appearance of novel protein bands in CCX treated Flow 2002 and BHK-21 cell extracts, subjected to SDS PAGE. They proposed these bands represented proteins which might play a role in determining whether cells are resistant or sensitive to CCX treatment. A detailed study was undertaken. Firstly, to establish whether the CCX induced polypeptides were stress proteins and secondly to determine whether different classes of cells (i.e. Resistant, Intermediate and Sensitive), would induce different novel bands when treated with CCX.

Mammalian cells induce different sets of stress proteins in response to different enviromental insults. For example, they respond to heat shock and other enviromental insults by inducing the synthesis of a small set of heat shock proteins (hsp) and to glucose starvation and glycosylation inhibitors by inducing the synthesis of another set of proteins designated glucose-related proteins (grp) (Shiu et al., 1977). Although the grps are related to the hsps, they are not induced by heat shock (Sorger and Pelham, 1987; Munro and Pelham, 1986). The two major hsps, have m.wts. of 90K and 70K, while the two major grps are 94K and 78K.

To help identification of the novel bands induced by CCX in different cell lines, compounds known to induce different sets of stress proteins were employed in a comparative study with CCX : disulfirum (hsp control; Notarianni and Preston, 1982) or tunicamycin (grp control; Welch et al., 1983; Pouysseyur and Yamada, 1978). Figure 18 shows the SDS PAGE profile of extracts from CCX, tunicamycin and disulfirum treated cells. The cells used represent the three classes : Resistant - HeLa (1-4), Flow 2002 (9-12), BS-C-1 (13-16); Intermediate - BHK-21 (17-20) and Sensitive - Fg 293 (5-8).

In all CCX treated extracts, novel bands were induced. However, the number, molecular weight and intensity of these bands differs between cell lines. Although the bands corresponding to grp 78 and hsp 90 appear in all CCX treated extracts, Fg 293 cell extracts differ from other cells in the relative abundance of these bands; with the grp 78 band

present in moderate amounts, while the hsp 90 band is very marked. In all other cell lines, grp 78 is induced more strongly (particularly in HeLa and Flow 2002), than hsp 90. The amounts of grp 94 in all CCX treated cell extracts was very small (undetectable in BHK-21 cells). In contrast, hsp 70 was induced very strongly in all cells (particularly in Flow 2002 cells), treated with CCX, the exception being BS-C-1 cells where this band was virtually absent.

Additional novel bands not identified as stress proteins were also induced in CCX treated human cell lines, Fg 293, Flow 2002 and HeLa cells (Fig. 18, lanes 7,11 and 3).CCX treatment resulted in a reduction of ^{35}S methionine incorporation into the actin band with most cell lines, and was particularly notable in the case of Flow 2002 and BHK-21 cells.

The SDS PAGE gel (Fig 18), shows that CCX treatment of three CCX Resistant, one Intermediate and one Sensitive cell line (from three different species) resulted in the synthesis of a set of polypeptides some of which co-migrate with recognised major stress proteins, both of the grp and hsp types. The fact that similar polypeptides are made in cells of diverse origin, make it most likely that these are indeed cellular stress proteins. It is not yet clear how CCX triggers the stress response, or whether the same CCX induced effect/s regulate the synthesis of grps and hsps in different cell lines. It is interesting however, that the only sensitive cell line examined, Fg 293, was exceptional in the low level of grp 78 made in drug treated cells.

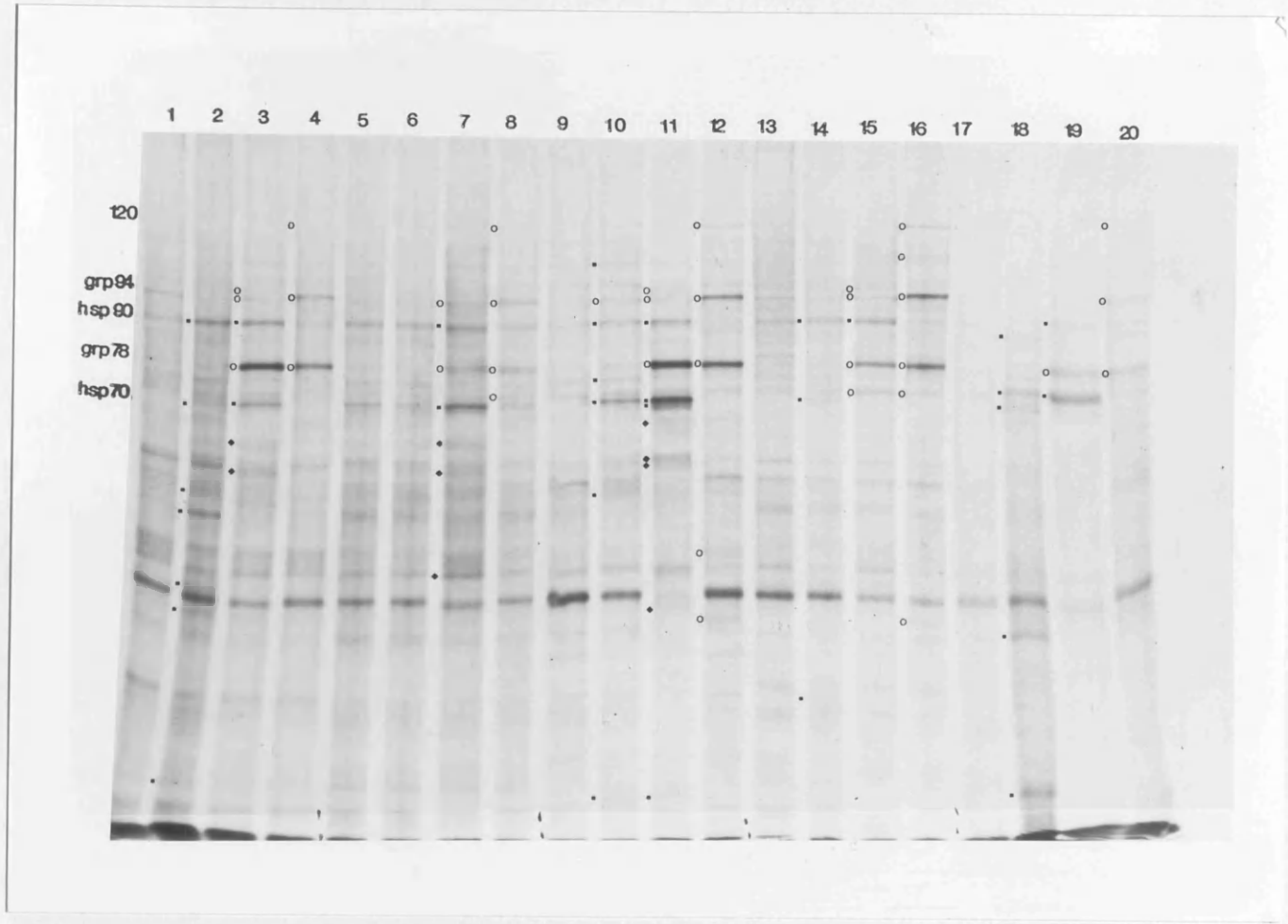

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
120
grp94
hsp90
grp78
hsp70

Figure 18

The synthesis of cellular stress proteins after 18h treatment with CCX.

SDS PAGE autoradiograph of HeLa (lanes 1-4); Fg 293 (lanes 5-8); Flow 2002 (lanes 9-12); BS-C-1 (lanes 13-16) and BHK-21 cells (lanes 17-20).

Cells were either drug free (lanes 1,5,9,13 and 17), or treated for 18h with 0.3uM disulfirum (lanes 2,6,10,14 and 18), CCX (lanes 3,7,11,15) or 5ug/ml tunicamycin (lanes 4,8,12,16 and 20). The CCX concentrations which produced the strongest stress response varied between different cell lines: HeLa and BS-C-1 (300uM); Flow 2002 and BHK-21 (200uM) and Fg 293 (100uM).

Novel protein bands produced in the presence of disulfirum (■); tunicamycin (○); CCX (◆).
grp 94 and 78K, and hsp 90 and 70K are identified.
(Disulfirum has also stimulated the expression of some grps).

3.2. VIRUS SURVEY

3.2.1. CCX INFECTIOUS YIELD DOSE-RESPONSE CURVES AND PLAQUE REDUCTION ASSAYS

Cicloxolone sodium (CCX), is a synthetic analogue of glycyhrrhizic acid (GA), which itself displays broad antiviral activity *in vitro* : Vaccinia, HSV-1, NDV, VSV, Influenza A and B and HIV (Pompei *et al.*, 1979, 1983; Ito *et al.*, 1987). Dargan and Subak-Sharpe (1985, 1986a and b), demonstrated that CCX exhibited anti-HSV activity (more potent activity than GA), and postulated that CCX operates by disturbing the normal function of host cell membranes. This proposed general mechanism, suggested that the replication of several unrelated viruses could be affected by the drug.

To test this proposition, the wider antiviral range of CCX was studied using HSV-1 and HSV-2 as positive controls. Dose-response experiments scoring infectious virus yield (2.2.5.3.) were performed with the viruses shown in Table 13. For technical reasons, in the cases of human cytomegalovirus (HCMV) and varicella zoster virus (VZV), plaque reduction assays and not infectious yield assays were performed.

The dose-response curve obtained for VSV (Fig 19a), demonstrates that CCX has a strong antiviral effect: drug treatment reduced the infectious yield about 10,000 fold at 300uM CCX. The magnitude of this reduction is similar to that previously reported for HSV-1 and HSV-2 i.e. from 1,000 to 100,000 fold (Dargan and Subak-Sharpe, 1985). In all the experiments the mock-infected controls show that cell viabilty was not significantly affected.

When two non-human herpesviruses were tested the infectious virus yields of both EHV-1 and BHV-1 were progressively reduced (Fig 19b). In the case of EHV-1, this resulted in a 1,000 fold reduction in infectious virus yield with 300uM CCX. BHV-1 proved very sensitive exhibiting a 5000 fold reduction in infectious yield with 100uM CCX, with infectivity completely abolished when cultures were treated with 300uM CCX. Similar reduction has been obtained in analogous experiments performed in MDBK cells infected with BHV-1 (Field, H.J. and S.K. Bandyopadhyay; personal communication). The antiviral activity against EHV-1 and

BHV-1 parallels the previously reported observations made with the human herpesviruses HSV-1 and HSV-2 and and suggests that sensitivity to CCX may be a property common to the herpesvirus family.

Figure 19c shows the curve obtained for Influenza A. While 50uM CCX had no detectable effect, higher doses result in a progressive reduction in infectious virus yield, reaching 100 fold with 300uM CCX. Typical dose-response curves for two Bunyaviruses, Bunyamwera and Germiston, are shown in Figure 20a; while treatment with 50uM CCX had no significant effect, there was a progressive reduction in infectious virus yield for both viruses, with 100uM and 200uM CCX dropping the titre to about a thirtieth with little further effect on increasing the CCX dose to 300uM. The dose-response curve for Polio-1 is shown in Figure 20b. CCX treatment of Polio-1 virus-infected cells results in a moderate 35 fold decrease in infectious virus yield with the antiviral activity consistently reaching a plateau at 150uM CCX. Also shown in this figure is the dose-response curve for SFV, the straight line obtained indicating total resistance to the drug.

The dose-response curve for Reovirus-3 is given in Figure 20c. There is great sensitivity to the presence of even small amounts of the drug resulting in a sharp drop in yield to a thirtieth with 50uM CCX; subsequent reduction in infectious virus yield continued to 100 fold with 150uM CCX at which concentration the dose-response curve reaches its plateau level. Additional experiments in which concentrations lower than 50uM CCX have been used, show that treatment with only 20uM already produces a significant effect (Fig 20c). Adenovirus-5 behaves similarly, giving the dose-response curve shown in Figure 20d; infectivity was reduced about 80 fold and the plateau level reached with 100uM CCX.

Plaque reduction assays were performed in the case of HCMV and VZV, as it is difficult to obtain cell-free infectious virus in these cases. ED_{50} values of 28uM and 52uM were obtained for HCMV and VZV respectively (Figure 21). Analogous experiments with HSV-1 and HSV-2 produced ED_{50} values of 186uM and 118uM respectively. Therefore, both VZV and HCMV have significantly lower ED_{50} values than

either HSV-1 or HSV-2, indicating greater sensitivity to CCX treatment.

These investigations of the antiviral range of CCX allowed placement of the viruses into three broad classes depending upon their dose-related response to the drug (Table 23). The first class is composed of VSV, Influenza A and the Herpesviruses HSV-1, HSV-2, EHV-1 and BHV-1, all of which exhibit a continuous drug dose-dependent reduction in virus infectivity. The extent of the drop varies for individual viruses, from 100 fold (Influenza A) to greater than 100,000 fold (BHV-1). VZV and HCMV probably fall into the same class, but here only the ED_{50} values which were 50uM and 28uM respectively could be ascertained. Since these values are lower than the ED_{50} values for HSV-1 or HSV-2, it would seem highly likely that both these important herpesviruses also belong to this class.

The second class contains Bunyamwera and Germiston virus, Polio-1, Reovirus-3 and Adenovirus-5. Members in general exhibit a less extensive total CCX response, infectivity being reduced by only 20-100 fold even with 300uM CCX. All the dose-response curves reach their plateau level with concentrations between 100 and 200 uM CCX. However, this second class falls into two sub-divisions : whereas both Adenovirus-5 and Reovirus-3 exhibit great sensitivity to low doses of drug and then reach their plateau values, Polio, Bunyamwera and Germiston are little affected by doses less than 50uM and only then respond to increased doses until a plateau is reached between 150-200uM CCX.

The third class, does not manifest any effect of CCX treatment irrespective of drug concentration. Presently only SFV falls into this non-responsive class.

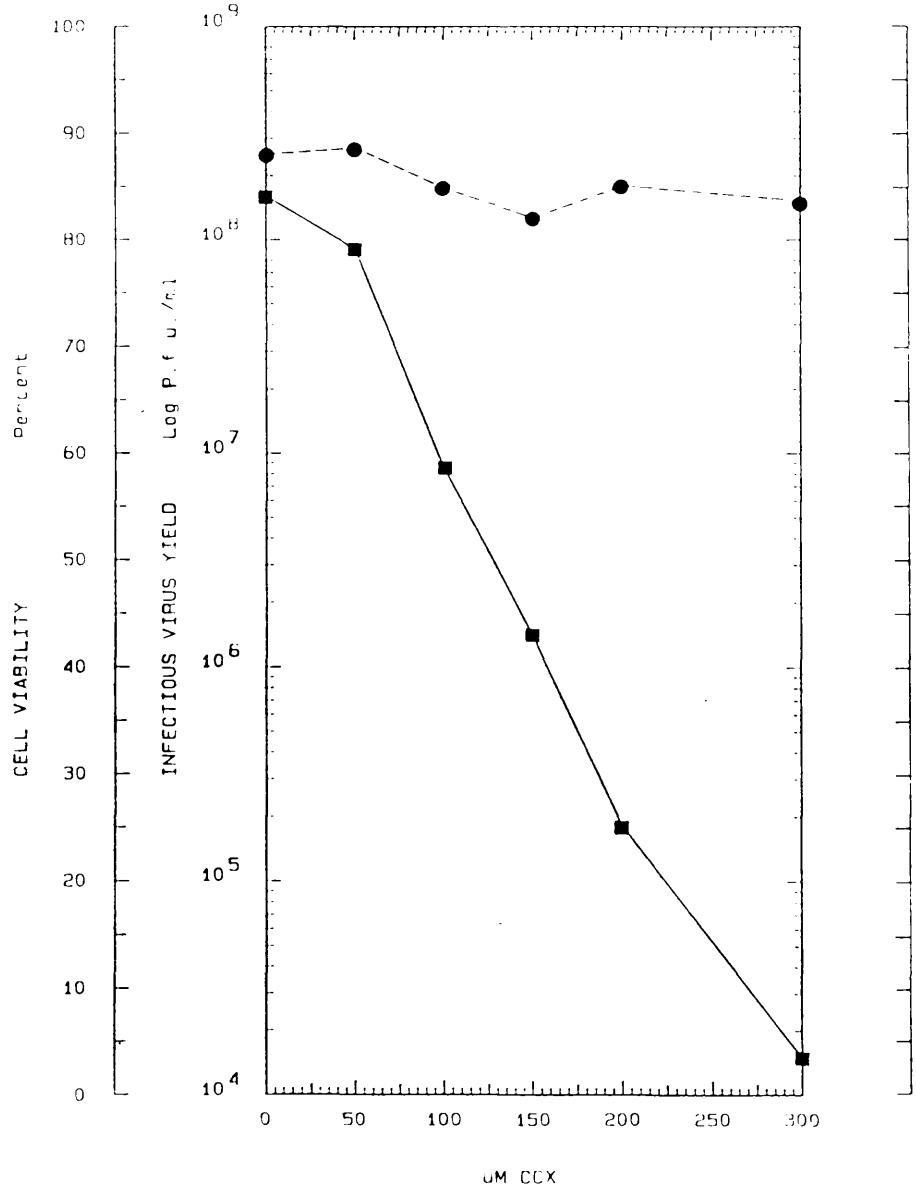

CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD
Log P.f.u./ml
100
90
80
70
60
50
40
30
20
10
0
10^9
10^8
10^7
10^6
10^5
10^4
0
50
100
150
200
250
300
uM CCX

Figure 19a

The effect of increasing concentrations of CCX on the infectious virus yield from BS-C-1 cells infected with VSV (Indiana).

Virus dose-response curve (■).

Viability of uninfected BS-C-1 cells from control cultures treated in parallel with increasing concentrations of CCX (●). Cell viability is plotted on a linear scale while virus yield is plotted on a $\log_{10}$ scale.

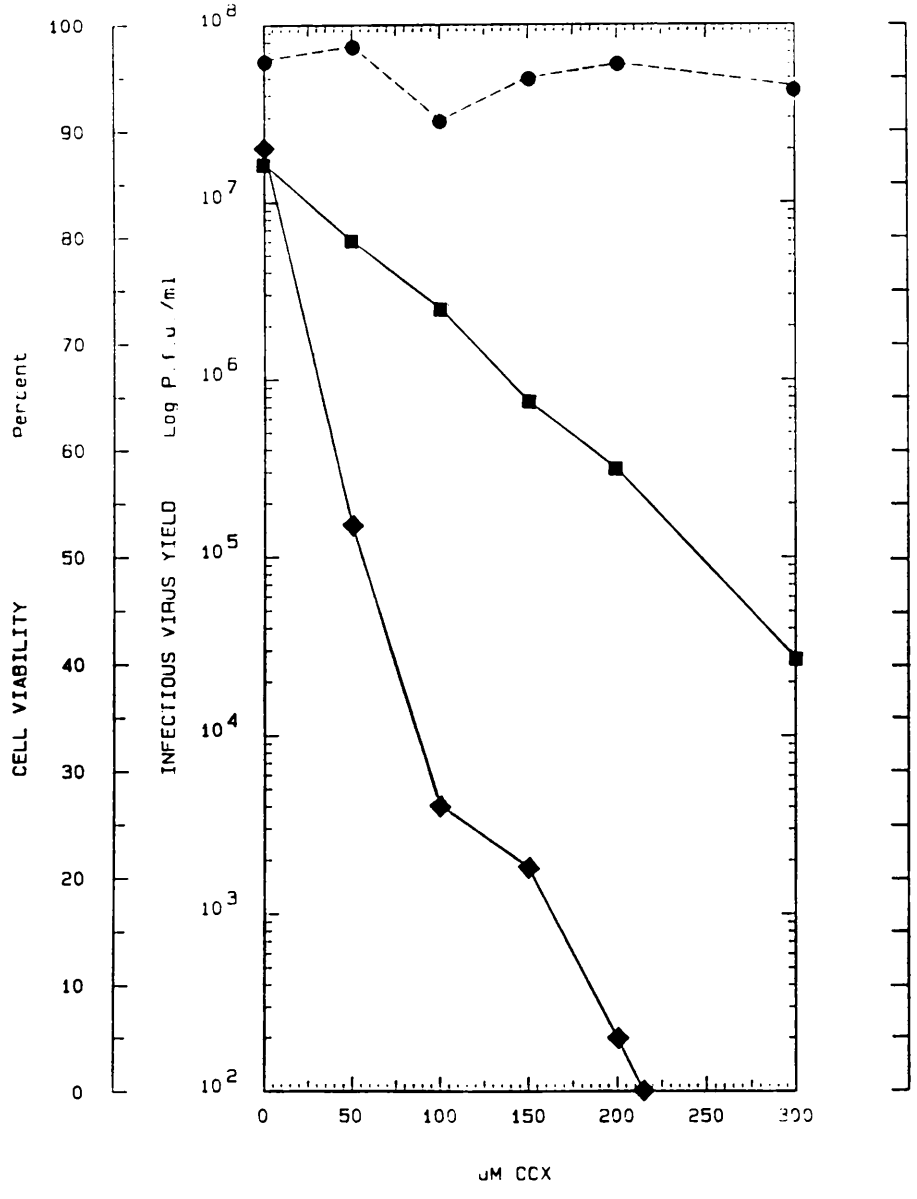

CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD
Log P.f.u /ml
uM CCX
100
90
80
70
60
50
40
30
20
10
0
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
50
100
150
200
250
300

Figure 19b

The effect of increasing concentrations of CCX on the infectious virus yield from RK-13 cells infected with EHV-1 and BHV-1.

Virus dose-response curves: EHV-1 (■);BHV-1 (◆).

Viability of uninfected RK-13 cells from control cultures treated in parallel with increasing concentrations of CCX (●).

Cell viability is plotted on a linear scale while virus yield is plotted on a $\log_{10}$ scale.

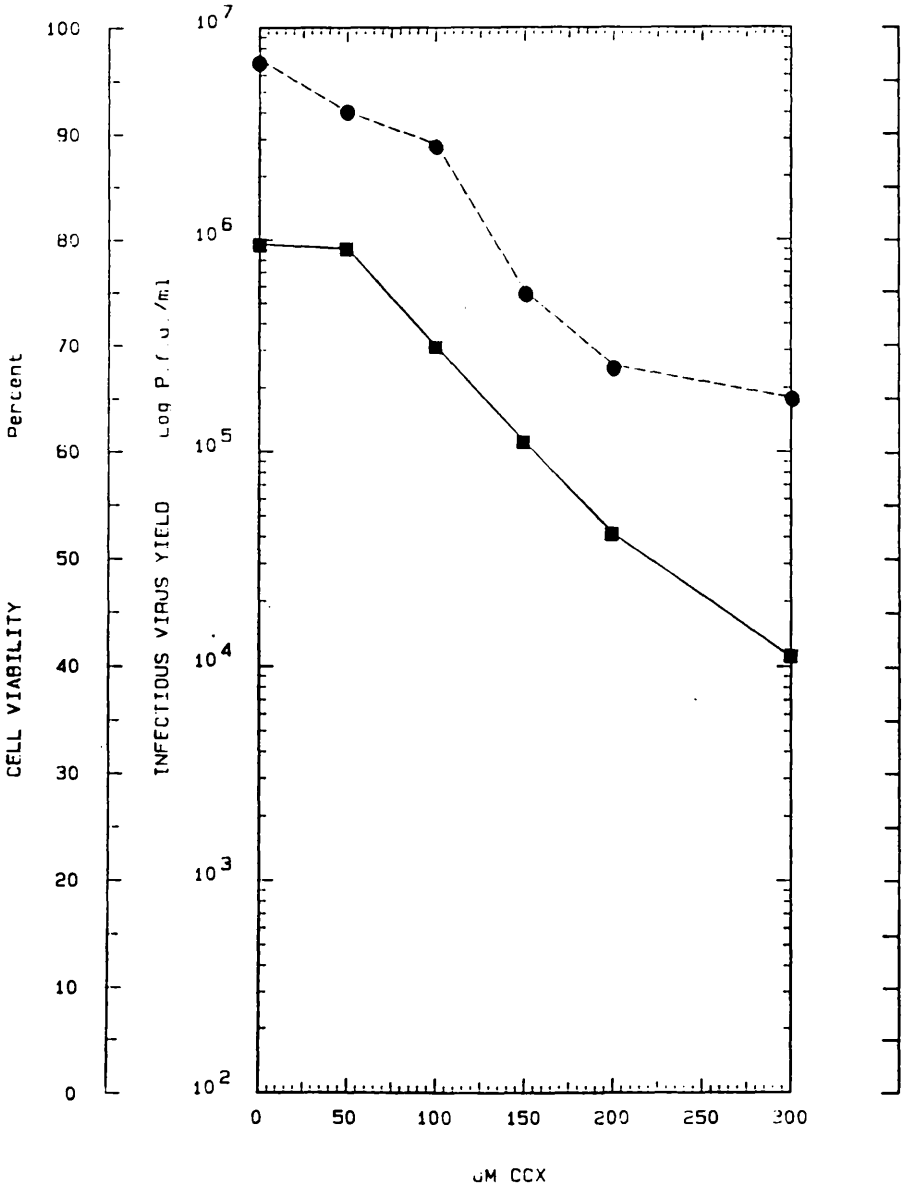

CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD
Log P.f.u./ml
100
90
80
70
60
50
40
30
20
10
0
10^7
10^6
10^5
10^4
10^3
10^2
0
50
100
150
200
250
300
uM CCX

Figure 19c

The effect of increasing concentrations of CCX on the infectious virus yield from MDCK cells infected with Influenza A.

Virus dose-response curve (■).

Viability of uninfected MDCK cells from control cultures treated in parallel with increasing concentrations of CCX (●).

Cell viability is plotted on a linear scale while virus yield is plotted on a $\log_{10}$ scale.

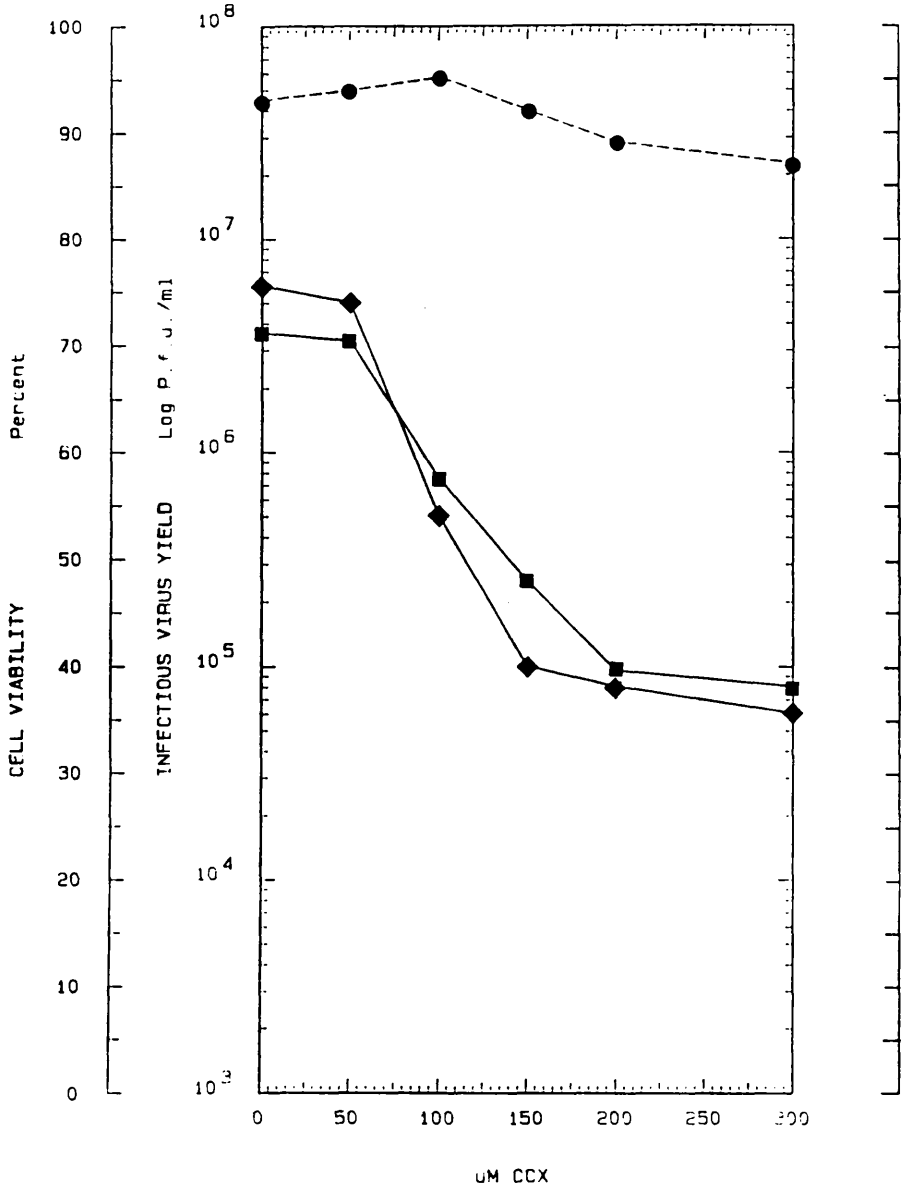

CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD
Log P.f.u./ml
uM CCX
100
90
80
70
60
50
40
30
20
10
0
10^8
10^7
10^6
10^5
10^4
0
50
100
150
200
250
300

Figure 20a

The effect of increasing concentrations of CCX on the infectious virus yield from BS-C-1 cells infected with Bunyamwera and Germiston virus.

Virus dose-response curves: Bunyamwera (■); Germiston (◆).

Viability of uninfected BS-C-1 cells from control cultures treated in parallel with increasing concentrations of CCX (●).

Cell viability is plotted on a linear scale while virus yield is plotted on a $\log_{10}$ scale.

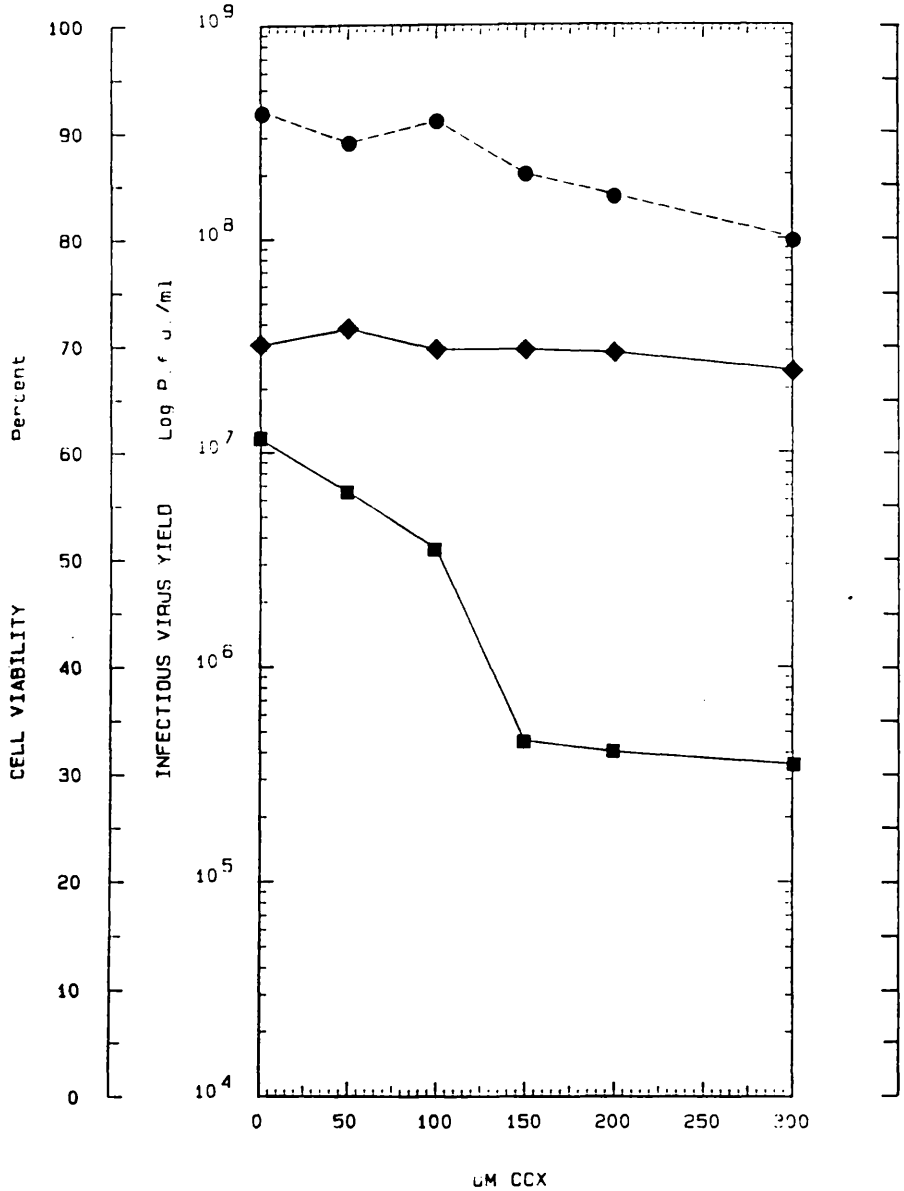

CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD
Log P.f.u./ml
100
90
80
70
60
50
40
30
20
10
0
10^9
10^8
10^7
10^6
10^5
10^4
0
50
100
150
200
250
300
uM CCX

Figure 20b

The effect of increasing concentrations of CCX on the infectious virus yield from BS-C-1 cells infected with Polio-1 and SFV.

Virus dose-response curves: Polio-1 (■); SFV (◆).

Viability of uninfected BS-C-1 cells from control cultures treated in parallel with increasing concentrations of CCX (●).

Cell viability is plotted on a linear scale while virus yield is plotted on a $\log_{10}$ scale.

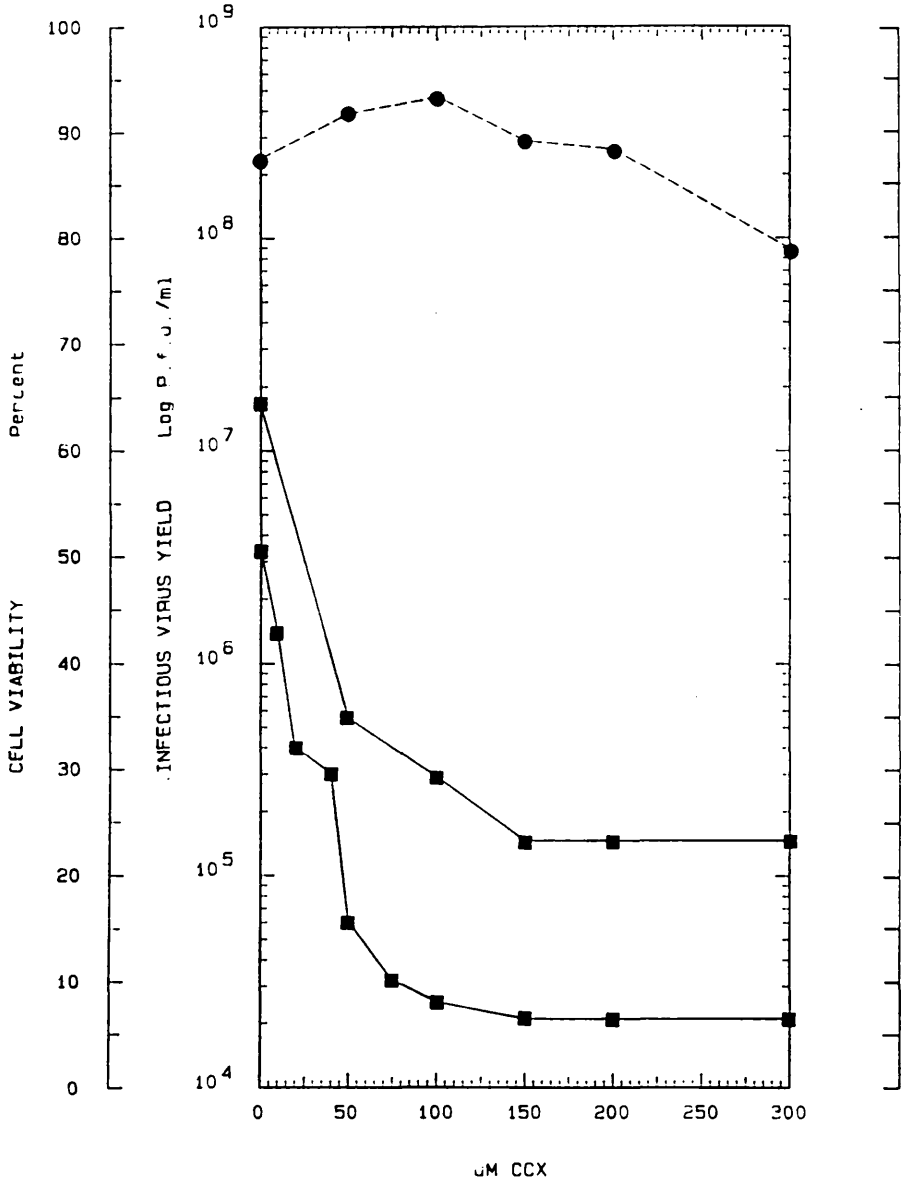

CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD
Log p.f.u./ml
100
90
80
70
60
50
40
30
20
10
0
10^9
10^8
10^7
10^6
10^5
10^4
0
50
100
150
200
250
300
uM CCX

Figure 20c

The effect of increasing concentrations of CCX on the infectious virus yield from BS-C-1 cells infected with Reovirus-3.

Virus dose-response curves (■). The second curve, where additional low concentrations of drug were used, has been transposed down by half a log unit to allow clearer presentation.

Viability of uninfected BS-C-1 cells from control cultures treated in parallel with increasing concentrations of CCX (●). Cell viability is plotted on a linear scale while virus yield is plotted on a $\log_{10}$ scale.

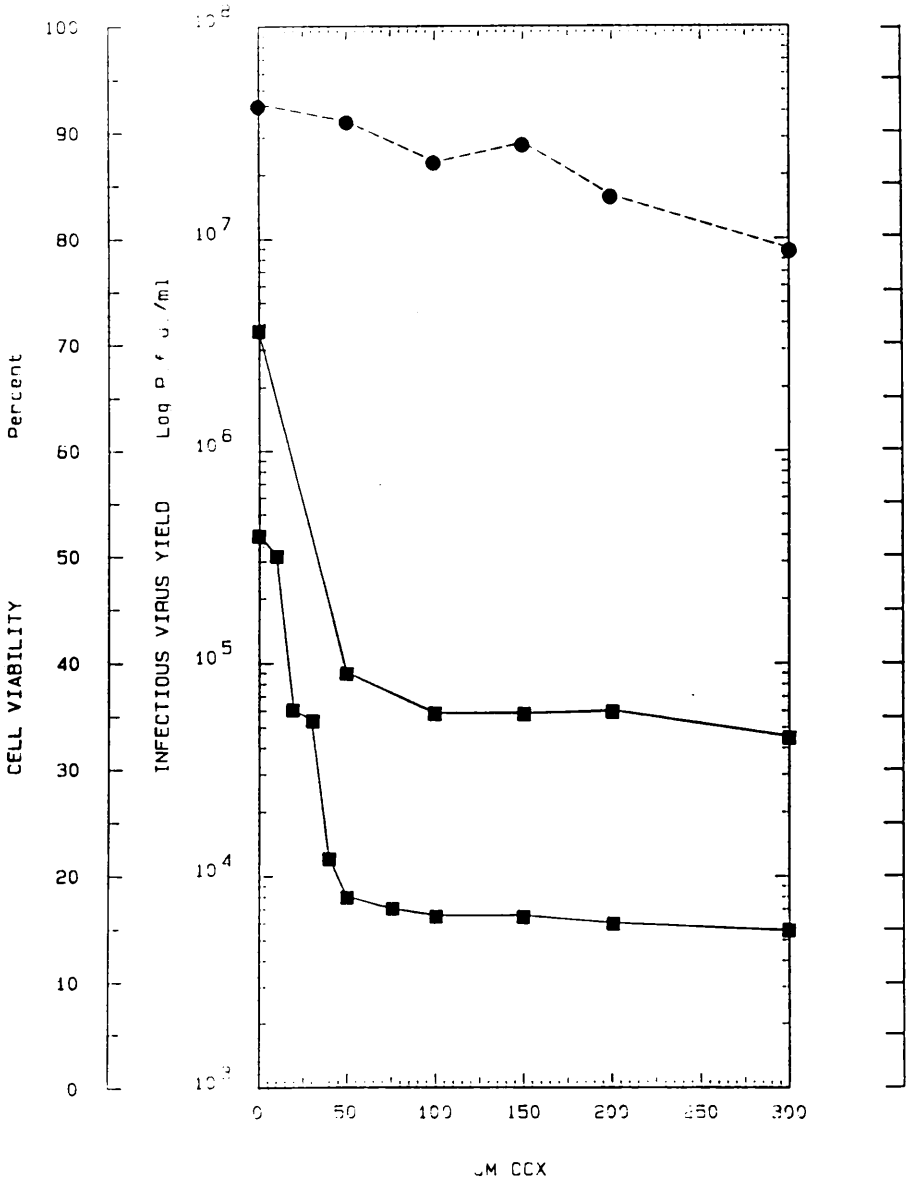

CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD
100
90
80
70
60
50
40
30
20
10
0
10^7
10^6
10^5
10^4
0
50
100
150
200
250
300
JM CCX

Figure 20d

The effect of increasing concentrations of CCX on the infectious virus yield from HeLa cells infected with Adenovirus-5.

Virus dose-response curve (■).

Viability of uninfected HeLa cells from control cultures treated in parallel with increasing concentrations of CCX (●).

Cell viability is plotted on a linear scale while virus yield is plotted on a $\log_{10}$ scale.

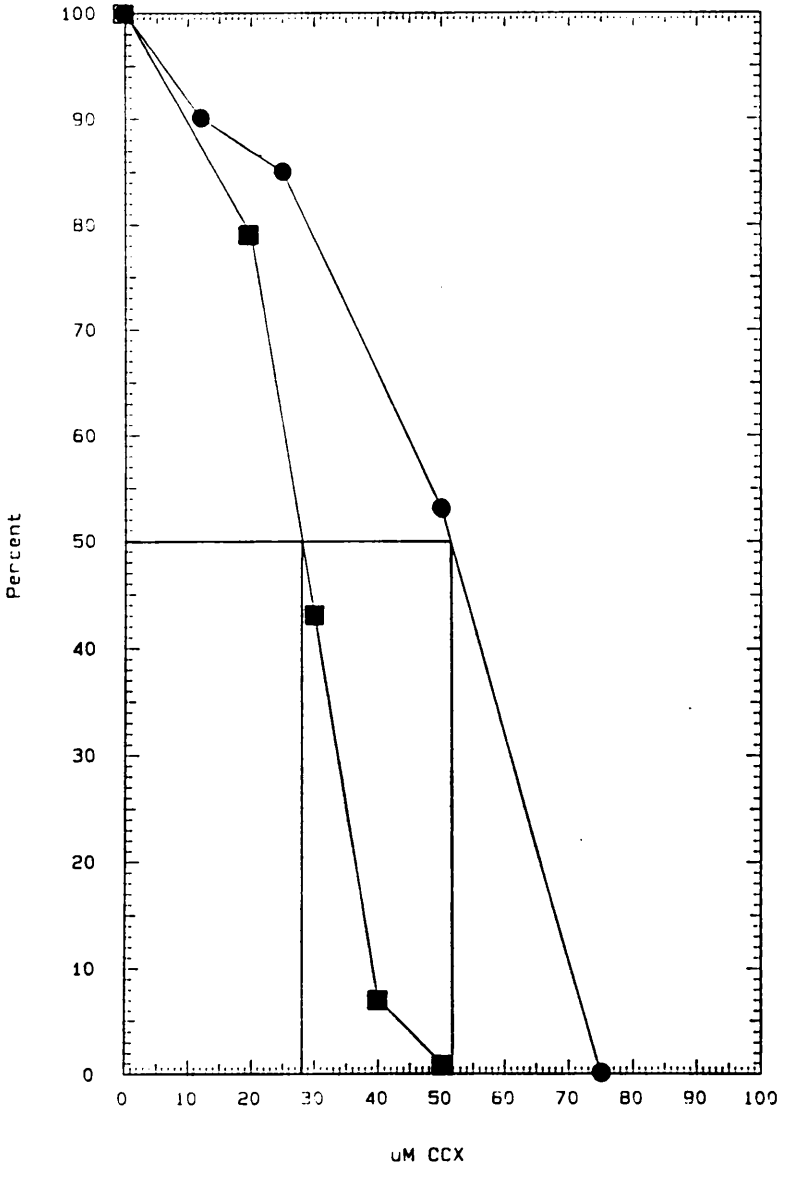

100
90
80
70
60
50
40
30
20
10
0
Percent
0
10
20
30
40
50
60
70
80
90
100
uM CCX

Figure 21

The effect of increasing concentrations of CCX on the number of plaques formed by HCMV and VZV on Flow 2002 cells.

Plaque reduction obtained with HCMV (■).
Plaque reduction obtained with VZV (●).

The number of plaques counted on drug free plates were 658 and 500, for HCMV and VZV respectively.
ED_{50} values obtained from these curves are shown.

PROGRESSIVE REDUCTION	PLATEAU	RESISTANT
100->10,000 FOLD REDUCTION IN VIRUS INFECTIVITY	10->100 FOLD REDUCTION IN VIRUS INFECTIVITY	< 10 FOLD REDUCTION IN VIRUS INFECTIVITY
HERPESVIRUSES HSV-I/2 EHV-I BHV-I probably VZV and HCMV	ADENOVIRUS-5 REOVIRUS-3 POLIOVIRUS-I	SEMLIKI FOREST VIRUS
VESICULAR STOMATITIS VIRUS (INDIANA)	BUNYAVIRUSES-BUNYAMWERA GERMISTON	
INFLUENZA A		

Table 23

Classification of response of viruses to CCX treatment.

3.2.2. DIASTEREOISOMER STUDY

Comparative dose-response experiments were performed with CCX and diastereoisomers of CCX, "690" and "688". It was hoped this might reveal information concerning the active form of the drug and allow inferences concerning its' mode of antiviral action versus toxicity. Three viruses each a member of a different group (as classified in section 3.2.1.) were chosen for the study : VSV (group 1), Germiston (Group 2) and SFV (Group 3). All virus infections were performed simultaneously in BS-C-1 cells and controlled for cytotoxicity. The results of dye exclusion performed on mock-infected BS-C-1, set up in parallel to the virus infected cells are shown in Table 24.

In agreement with previous observations, treatment with up to 300uM CCX, had little significant effect on cell viablity (resulting in a drop of only 6%), but did inhibit cell replication to some degree, with total cell number rising to 77% of the control level. Cell viability was not affected by treatment with up to 300uM "690" and only a very slight effect on cell replication was observed, with the total cell number in cultures treated with 300uM, reaching 88% of the control level. "690" is therefore less toxic to cells than CCX, allowing uncoupling of any antiviral and cytotoxic effects. In contrast, treatment with diastereoisomer "688" had a very marked effect on cell viability, which was reduced 20% with only 50uM. At 100uM "688", just over half the cells were viable, dropping to only 24% viability with 300uM "688". Cell replication was also markedly reduced with increasing concentrations of "688", resulting in complete inhibition with 300uM. Therefore, "688" is much more toxic to cells than CCX, making separation of antiviral and cytotoxic effects difficult at concentrations over 100uM.

Corresponding virus dose-response curves are shown in Figures 22, 23 and 24. In these experiments, virus yields were divided into cell-associated (CA) and cell-released (CR) fractions. Figure 22 shows the curves obtained for VSV. The CCX dose-response curve (Fig 22a), reveals that the CA fraction was reduced by 2 logs, compared with a 4 log reduction in the CR fraction. Diastereoisomer "690" (Fig 22b), hardly affected VSV CA yields even at concentrations

as high as 300uM. Although the reduction in CR infectious virus yields was less dramatic than that achieved with similar concentrations of CCX, the reduction of almost 3 logs achieved with 300uM "690" is still significant. Treatment with "688" (Fig 22c), resembles CCX in that there is a more dramatic reduction in the CR fraction compared to the CA fraction. However, the increase in knockdown of infectious virus yields is probably due to the cytotoxic effect of "688" on the cells (Table 24).

Figure 23 shows the curves obtained for Germiston virus. Treatment with up to 300uM CCX progressively reduces the CA fraction by almost 2 logs (Fig 23a). The CR fraction was reduced by just over a log with 100uM CCX, addition of higher concentrations having little further affect. "690" treatment had very little significant effect on infectious virus yields of either the CA or CR fractions, achieving an overall reduction of only 0.5 log (Fig 23b).However, the reduction was complete at low concentrations of "690"; 50uM in the case of the CA fraction and 100uM in the case of the CR fraction. As expected, due to its' cytotoxic effect, diastereoisomer "688" reduced infectious virus yields more markedly than CCX (Fig 23c) : the CA fraction was reduced by almost 3 logs and the CR fraction by just under 2 logs.

The dose-response curves for SFV are shown in Figure 24. Increasing concentrations of CCX, resulted in a progressive increase in the CA fraction of about 1 log, with a concomitant decrease in the CR fraction (Fig 24a). This agrees with the apparent resistance of SFV to CCX treatment when both fractions were pooled before titration. Increasing concentrations of "690", also resulted in a slight increase in the CA fraction (0.5 log), with a larger reduction in the CR fraction (1 log) (Fig 24b). The results for "688" again are distorted by the cytotoxic affect on the cells, yielding a reduction of over 3 logs in the CR fraction and 2 logs in the CA fraction (Fig 24c). It is interesting that a small rise in the CA fraction did occur, at the less cytotoxic concentrations of 50 and 100uM.

In conclusion, the extent of reduction in infectious virus yields, appears to be linked to the cytotoxic affect on cells with "690" < CCX < "688". The cytotoxicity of "688" at relatively low concentrations, renders it unsuitable for

use as an antiviral. Although "690" had no significant effect on infectious yields of Germiston virus or SFV, it did reduce infectious VSV yields by 3 logs in the CR fraction and 1 log in the CA fraction . It therefore displays significant anti-VSV activity although this is less potent than that exhibited by CCX.

DRUG	TIME (h)	CONCENTRATION (uM)	CELL VIABILITY(%)	TOTAL CELL NUMBER (%)
	0		94	54
		0	96	100
		50	96	98
CCX	24	100	97	96
		200	92	89
		300	90	77
	0		97	49
		0	96	100
		50	97	97
690	24	100	96	94
		200	96	90
		300	95	88
	0		95	48
		0	96	100
		50	76	92
688	24	100	62	81
		200	42	65
		300	24	52

Table 24

The effect of CCX and diastereoisomers of CCX ("690" and "688") on BS-C-1 cell viability and replication over 24h (performed in parallel to virus dose-response experiments with CCX and diastereoisomers).

Total cell number: numbers are the total cell counts relative to the 0 drug control given as a percentage.

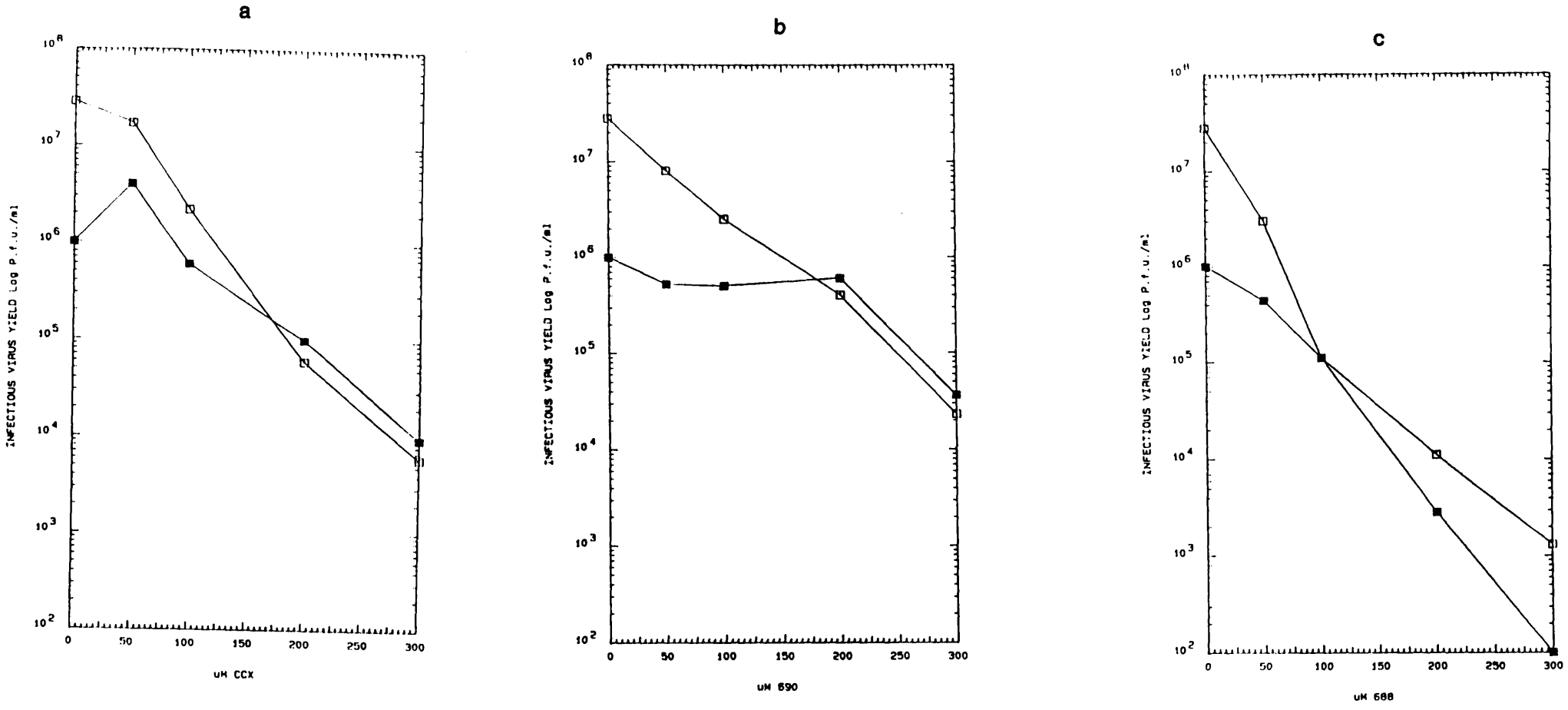

a
b
c
INFECTIOUS VIRUS YIELD Log P.f.u./ml
uM CCX
uM 690
uM 688
0
50
100
150
200
250
300

Figure 22

The effect of increasing concentrations of CCX (a), "690" (b) or "688" (c) on the cell-associated (CA) (■) and cell-released (CR) (□) infectious yields of VSV (Indiana), from BS-C-1 cells.

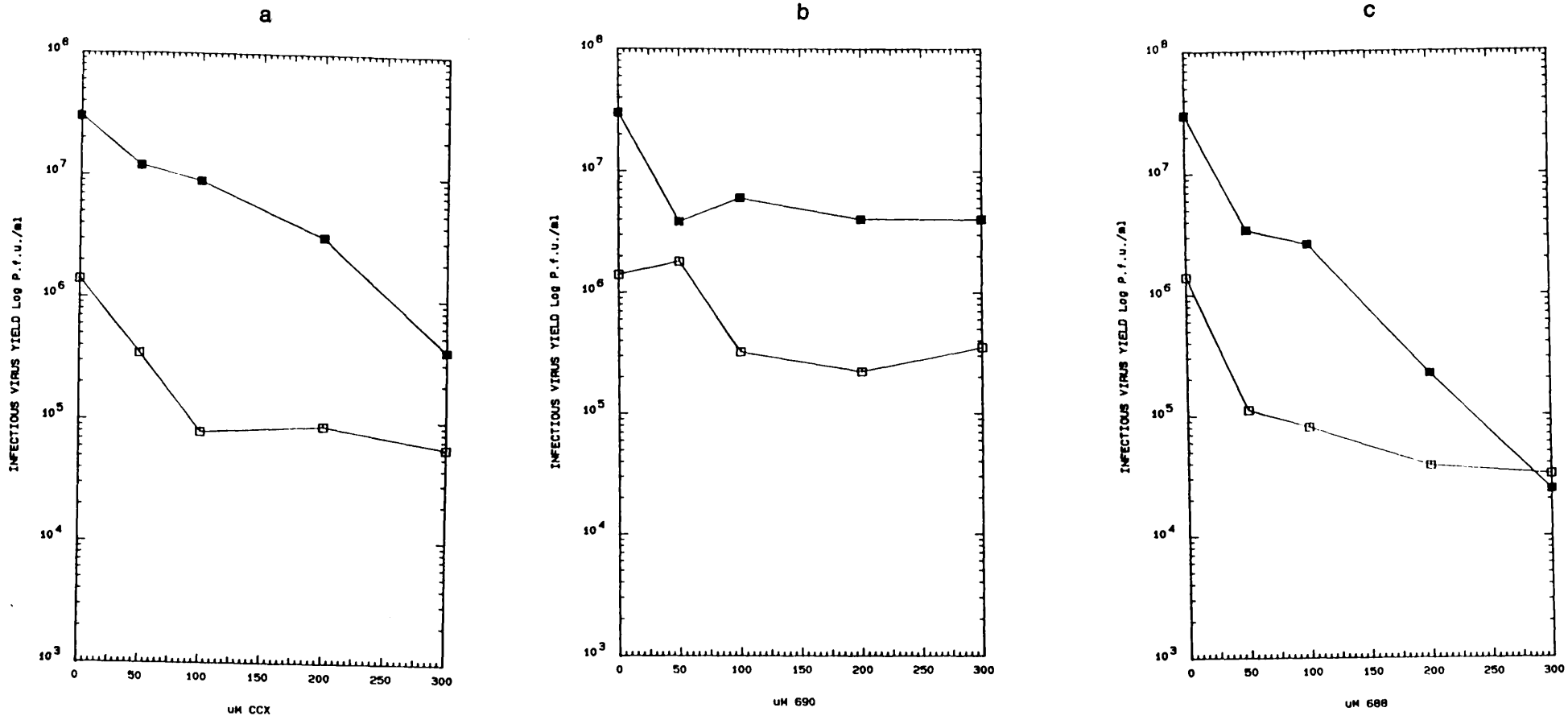

a
b
c
INFECTIOUS VIRUS YIELD Log P.f.u./ml
10^8
10^7
10^6
10^5
10^4
10^3
0
50
100
150
200
250
300
uM CCX
uM 690
uM 688

Figure 23

The effect of increasing concentrations of CCX (a), "690" (b) or "688" (c) on the cell-associated (CA) (■) and cell-released (CR) (□) infectious yields of Germiston virus, from BS-C-1 cells.

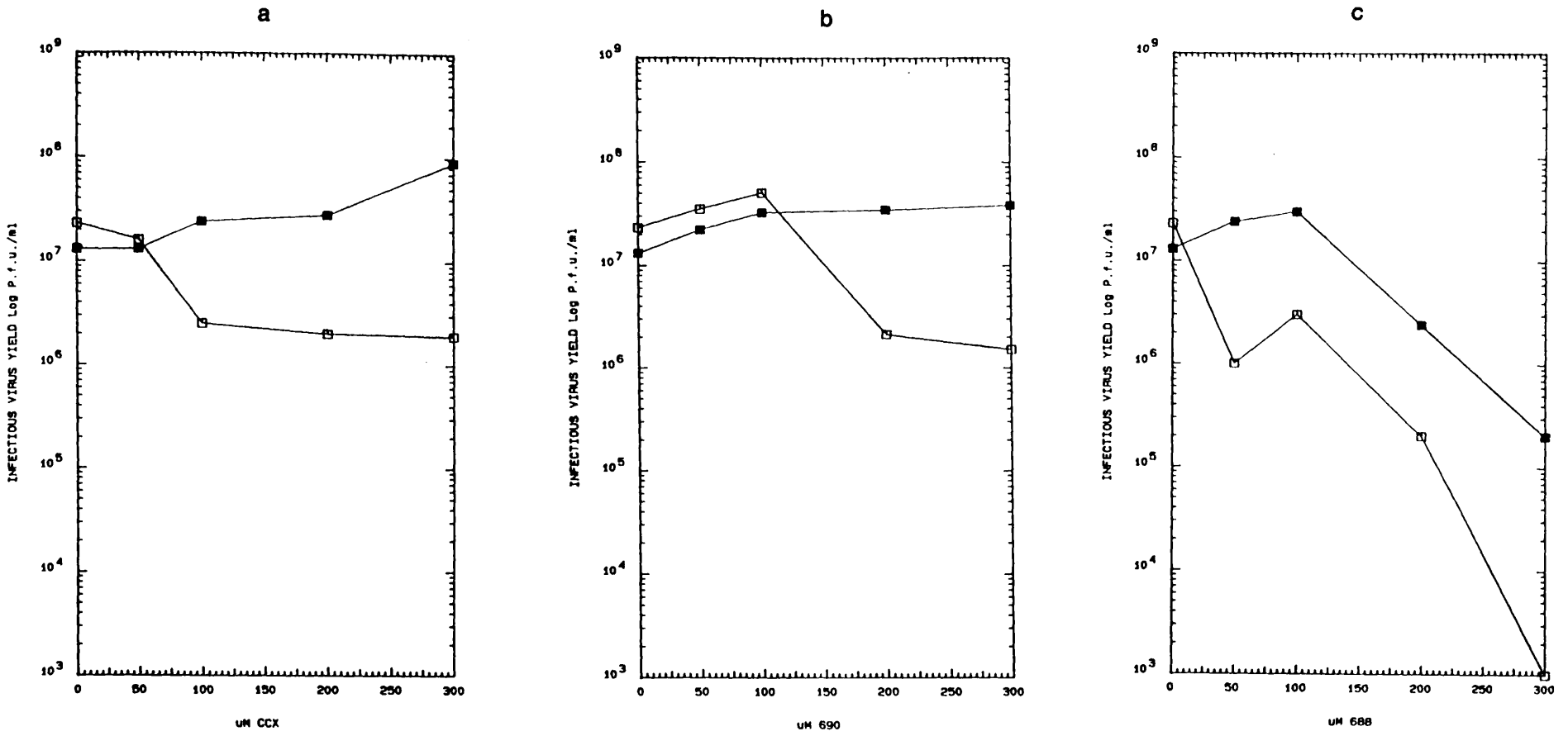

a
b
c
INFECTIOUS VIRUS YIELD Log P.f.u./ml
10^9
10^8
10^7
10^6
10^5
10^4
10^3
0
50
100
150
200
250
300
uM CCX
uM 690
uM 688

Figure 24

The effect of increasing concentrations of CCX (a), "690" (b) or "688" (c) on the cell-associated (CA) (■) and cell-released (CR) (□) infectious yields of SFV, from BS-C-1 cells.

3.3. THE EFFECT OF CCX ON THE REPLICATION OF VSV (INDIANA) : REPRESENTING THE CLASS 1 RESPONSE

3.3.1. VSV REPLICATION IN THE PRESENCE OF CCX

A one-step growth curve was performed at 37°C in monolayers of BS-C-1 cells in the presence and absence of CCX (Fig 25). VSV failed to replicate in the presence of 300uM CCX with no evidence that any new progeny virus was produced during the 36h period studied. The virus yield observed is probably just cell adsorbed virus that failed to penetrate from the initial infecting innoculum.

3.3.2. THE EFFECT OF INCREASING CONCENTRATIONS OF CCX ON VSV PARTICLE NUMBER AND PARTICLE / P.F.U. RATIOS

Virus particle counts were performed by electron microscopy (2.2.6.1.) on total virus yields from dose-response experiments. Table 25 shows the results obtained from three independent experiments. The data show that increasing CCX concentrations resulted in a significant reduction in particle number, accompanied by a somewhat greater decrease in infectious yields, resulting in elevated particle/p.f.u. ratios (typically 10 fold).

These results therefore indicate, that the anti-VSV effect of CCX operates by inhibiting virus particle production and in addition by lowering the quality of those virus particles which are produced.

3.3.3. POLYPEPTIDE ANALYSIS OF VSV PARTICLES PRODUCED IN BS-C-1 CELLS TREATED WITH CCX

^{35}S methionine radiolabelled VSV yields from CCX treated and untreated BS-C-1 cells were purified by passing through Ficoll gradients, and the structural proteins analyzed by SDS PAGE (Fig 26). The mobility of the G band is marginally increased in CCX treated tracks, suggesting some effect on the glycosylation of this protein. Apart from this, the polypeptide composition of VS virions produced in the presence and absence of 150uM CCX are similar. This is in contrast to HSV virions produced in the presence of CCX, which have abnormal polypeptide composition, with some protein bands under-represented and others over-represented (Dargan and Subak-Sharpe, 1986a and b). There is no evidence

The drug-treated tracks also contains 2 bands (of unknown origin) below M band.

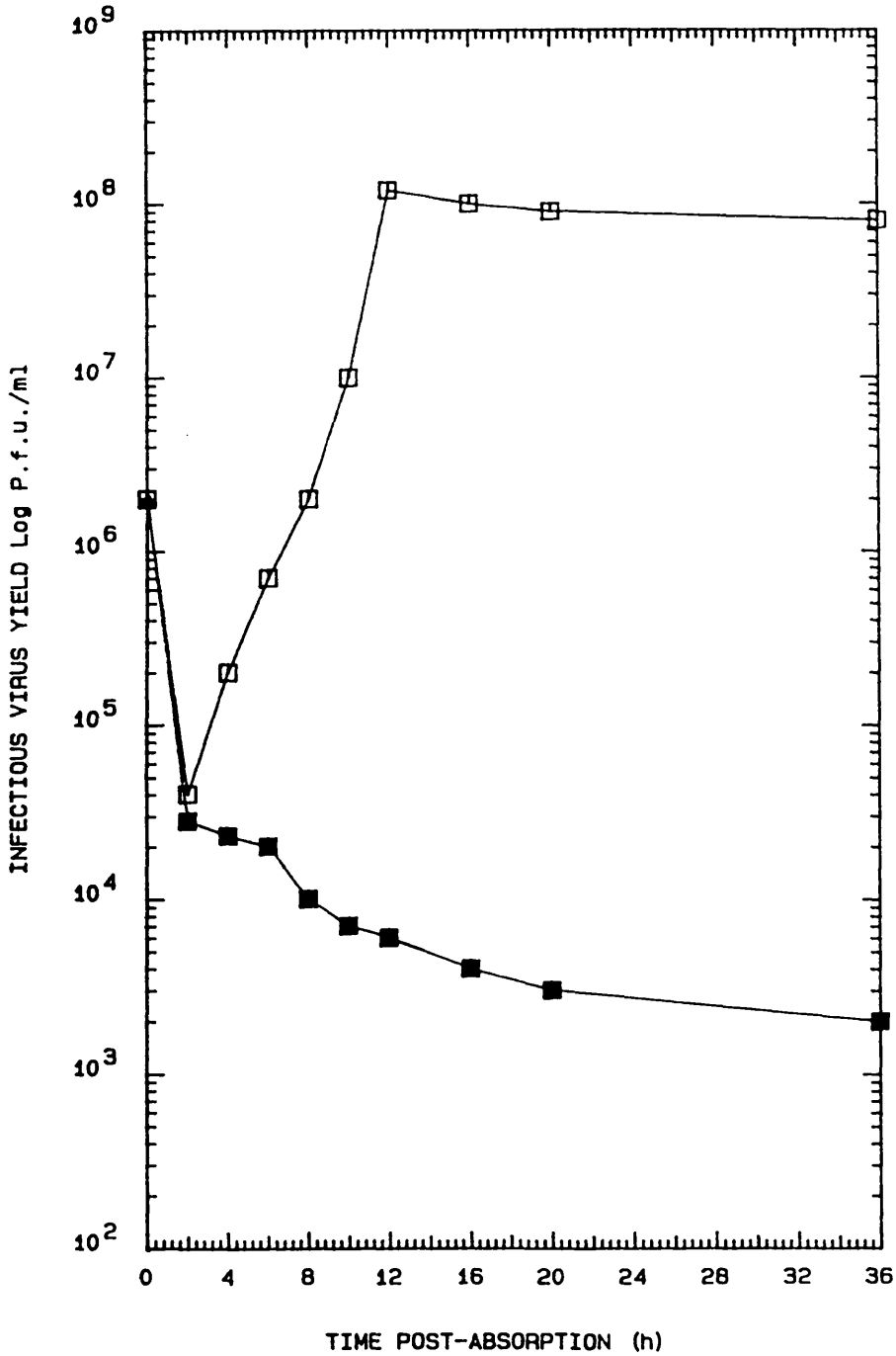

INFECTIOUS VIRUS YIELD Log P.f.u./ml
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
4
8
12
16
20
24
28
32
36
TIME POST-ABSORPTION (h)

Figure 25

VSV replication in BS-C-1 cells in the presence of CCX.

One-step growth curve obtained without drug (□) or in the presence of 300uM CCX (■).
The m.o.i. was 5 p.f.u./cell and incubation was at 37°C.

	CCX (uM)	PARTICLE NUMBER	P.F.U	PARTICLE/P.F.U RATIO
	0	$1.2*10^{10}$	$2.6*10^{8}$	46
1	150	$7.5*10^{8}$	$2.2*10^{6}$	325
	300	$<1.0*10^{8}$	$5.2*10^{4}$	ND
	0	$1.35*10^{11}$	$2.6*10^{9}$	51.9
2	150	$5.0*10^{8}$	$3.9*10^{6}$	128.0
	300	$<1.0*10^{8}$	$7.2*10^{4}$	ND
	0	$1.0*10^{10}$	$2.38*10^{8}$	42
	50	$1.0*10^{10}$	$2.12*10^{8}$	47
3	100	$7.0*10^{8}$	$2.16*10^{6}$	324
	200	$1.43*10^{8}$	$6.0*10^{5}$	238
	300	$<1.0*10^{8}$	$1.6*10^{4}$	ND

Table 25

The effect of increasing concentrations of CCX on VSV particle numbers, infectious virus yields (p.f.u.) and particle/p.f.u. ratios, after 24h.

1,2 and 3 represent data from 3 independent experiments.

N.D. not determined

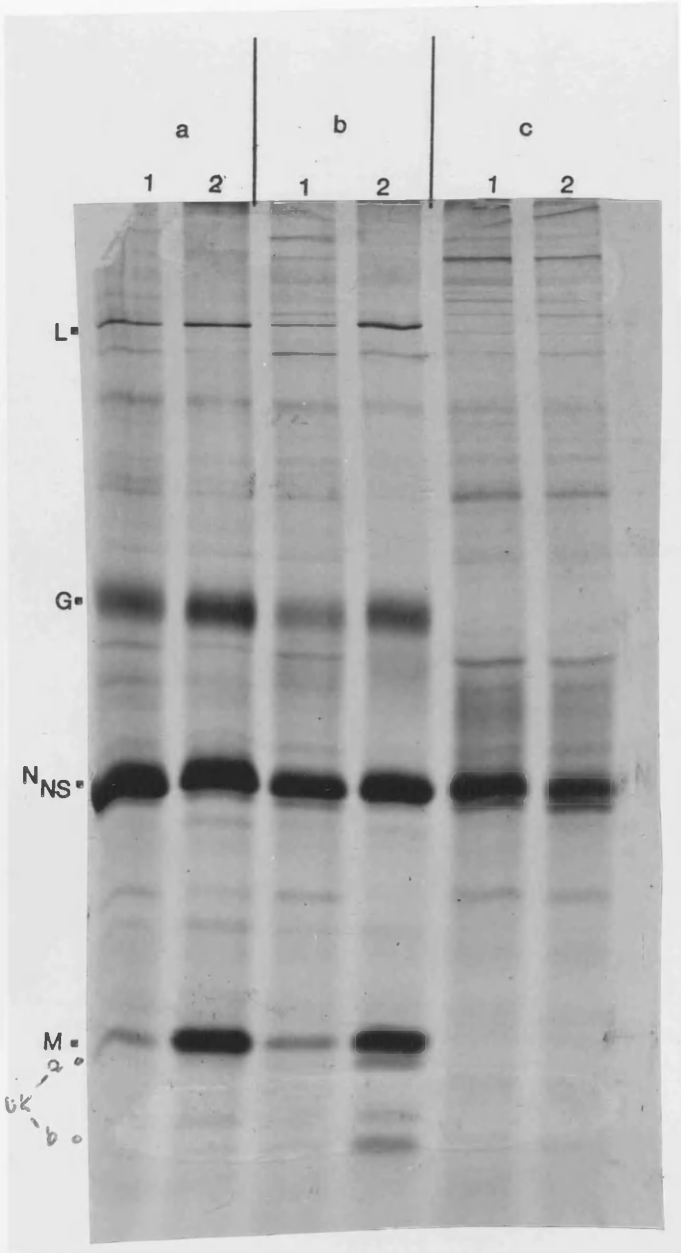

a
b
c
1
2
1
2
1
2
L
G
N
NS
M
a
b

Figure 26

SDS PAGE profile on a 10% gel of the structural proteins of VSV produced in BS-C-1 cells treated with 0 (a), 150uM CCX (b) or 300uM CCX (c).

The m.o.i. was 5 p.f.u./cell and yields were harvested at 24h p.i. and purified by centrifugation through 5-15% Ficoll gradients. 2 bands were collected; upper band containing D.I. particles (which contain short, defective RNA) (1) and a lower band containing purified VSV particles (which contain normal length RNA) (2).

of production of any VSV proteins in the 300uM samples.

3.3.4. DIRECT EFFECT ON INFECTIVITY OF VSV PARTICLES IN SUSPENSION

To determine whether CCX directly inactivated virus particles, suspensions of virus were incubated with either mock drug or 300uM CCX (2.2.5.7.), at 4°C or 37°C for 24h and infectious yields titrated before and after drug addition. This experiment was performed several times, yielding varying results, from almost no effect to almost a log reduction in infectivity. The results from two separate experiments, representing the two extremes are shown in Table 26.

3.3.5. ADSORPTION OF VSV TO BS-C-1 CELLS PRE-TREATED WITH VARYING CONCENTRATIONS OF CCX

Adsorption experiments (2.2.5.6.), were performed with VSV and BS-C-1 cells pre-treated with varying concentrations of CCX (Fig 27). Although there was some slight effect on VSV adsorption to pre-treated cells, at concentrations as high as 900uM CCX, virus was clearly still able to bind to receptors. In addition, adsorption had not gone to completion (plateau) during the 40 minute observation period. It is therefore unlikely that inhibition of adsorption of virus to pre-treated cells could play a significant part in limiting the spread of infection at least *in vitro*.

a

TIME (hr)	TEMP (C)	DRUG	TITRE	% AGE	REDUCTION
0	-	ORIGINAL STOCK	$3.2*10^{7}$		
24	4	MOCK DRUG	$1.75*10^{7}$	100	14.7
24	4	300 uM CCX	$1.5*10^{7}$	85.7	
24	37	MOCK DRUG	$2.0*10^{6}$	100	37.5
24	37	300 uM CCX	$1.25*10^{6}$	62.5	

b

TIME (hr)	TEMP (C)	DRUG	TITRE	% AGE	REDUCTION
0	-	ORIGINAL STOCK	$3.6*10^{7}$		
24	4	MOCK DRUG	$2.85*10^{7}$	100	90.0
24	4	300 uM CCX	$2.85*10^{6}$	10	
24	37	MOCK DRUG	$6.0*10^{6}$	100	85.9
24	37	300 uM CCX	$8.5*10^{5}$	14.1	

Table 26

Direct effect on VSV particle infectivity by CCX.

a) and b) represent data from 2 separate experiments.

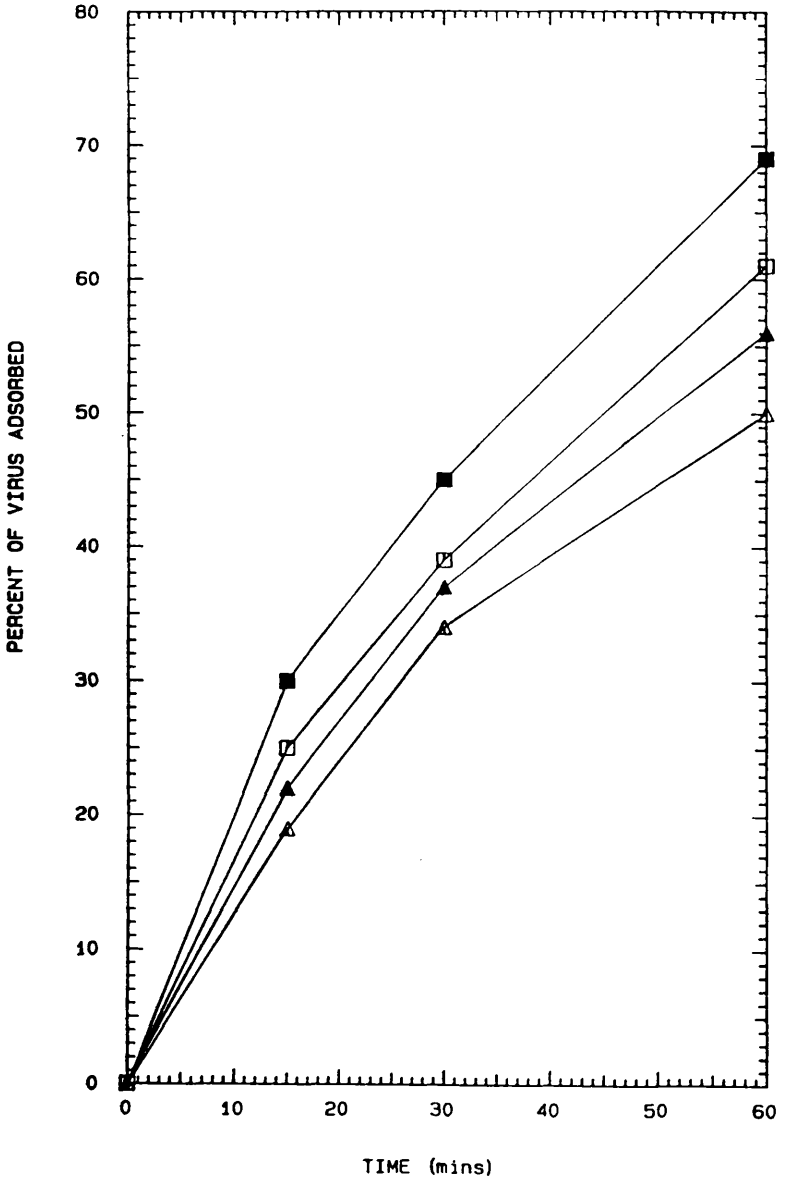

80
70
60
50
40
30
20
10
0
PERCENT OF VIRUS ADSORBED
0
10
20
30
40
50
60
TIME (mins)

Figure 27

Adsorption of VSV particles to BS-C-1 cells pre-treated for 1h at 37°C with 0 (■), 300uM CCX (□), 600uM CCX (▲) or 900uM CCX (△).

3.3.6. THE EFFECT ON INFECTIOUS VSV YIELDS FROM BS-C-1 CELLS BY ADDITION OF CCX AT VARIOUS TIMES POST-ADSORPTION

CCX to 300uM was added to the growth medium of VSV-infected BS-C-1 cells at the times indicated in Figure 28. The curve obtained indicates that the effect of CCX was progressive throughout the life cycle, with larger yield reductions achieved the earlier the drug was added. As expected there was no significant effect on infectious yields if CCX was added 12h or later after adsorption, as the virus life cycle should be complete at this time. The small decrease in titre observed may be due to direct inactivation of virus particles.

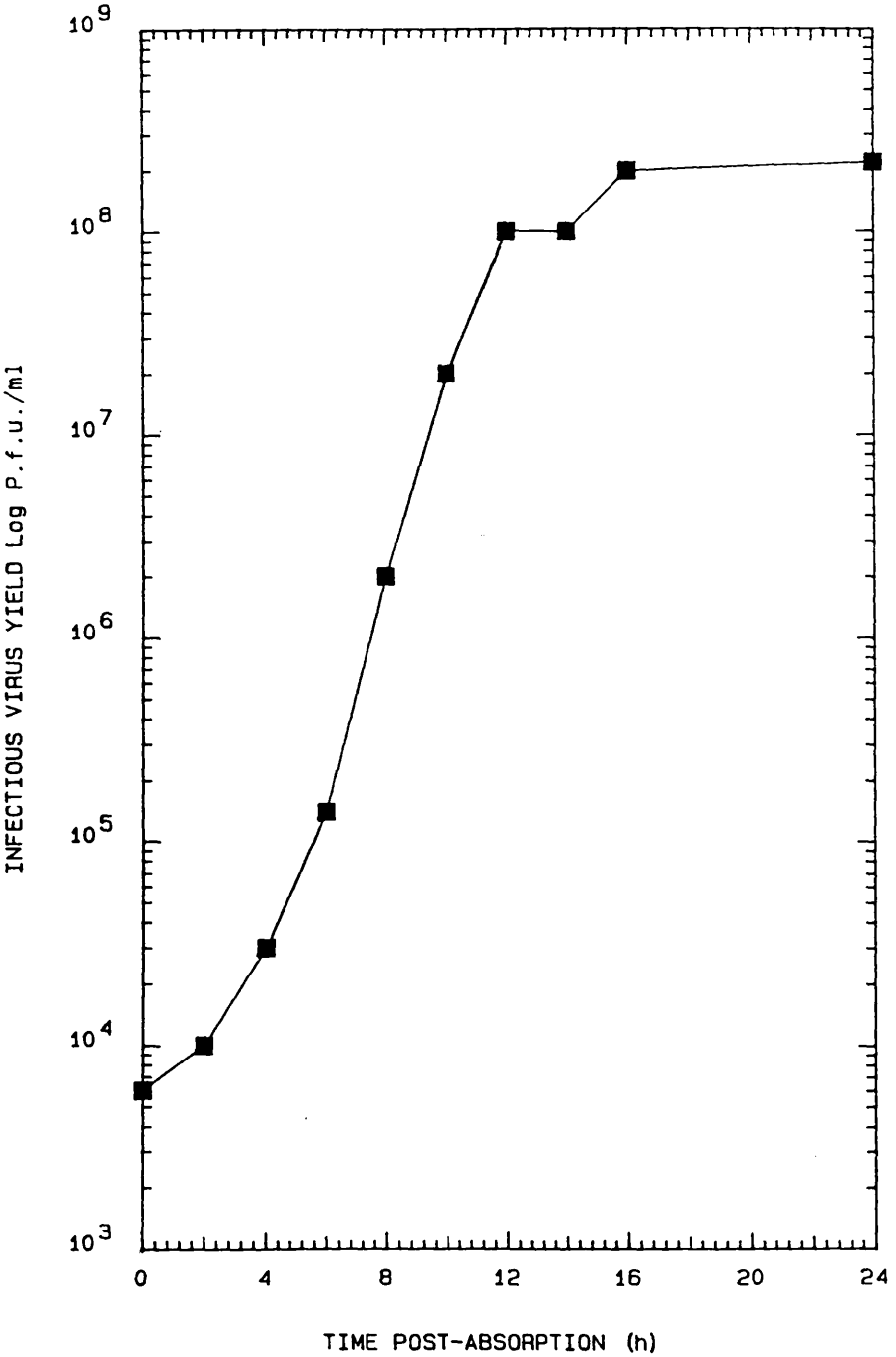

INFECTIOUS VIRUS YIELD Log P.f.u./ml
10^9
10^8
10^7
10^6
10^5
10^4
10^3
0
4
8
12
16
20
24
TIME POST-ABSORPTION (h)

Figure 28

Effect on infectious VSV yields from BS-C-1 cells (m.o.i. 5 p.f.u./cell), by addition of CCX at various times post-adsorption.

CCX (to 300uM) was added at 2,4,6,8,10,12,14,16 and 24 hours post-adsorption and infectious virus yields estimated at 24h.

3.3.7. THE EFFECT OF CCX ON RNA AND PROTEIN SYNTHESIS

VSV TRANSCRIPTION

VSV transcription occurs in two distinct stages, termed primary and secondary transcription :

Primary transcripts are synthesised from infecting VSV genomes, by the viral transcriptase, which is a structural component of the virus particle (thought to comprise L, NS and N) (Marcus et al., 1971)

Secondary transcripts are synthesised from either infecting parental VSV genomes or progeny genomes and is carried out by the newly made viral transcriptase i.e. consisting of L, NS and N proteins synthesised from primary transcripts. Secondary transcription is dependent on protein synthesis (Flamand and Bishop, 1974).

The products of primary and secondary transcription are identical and consist of a leader RNA and 5 monocistronic mRNAs (Fig.29) (Moyer et al., 1975; Moyer and Banerjee, 1975; Freeman et al., 1977; Rhodes et al., 1977).

VSV TRANSLATION

VSV mRNAs are efficiently translated on host cell ribosomes. Transcriptional rather than translational control is normally used to regulate the quantity of each VSV protein synthesized; thus the amount of each protein synthesized is proportional to the amount of each mRNA. Since VSV transcription is both sequential and polar, the N mRNA and hence the N protein are most abundant, while the L mRNA and L protein least abundant (Villarreal et al., 1976).

An experiment was designed to yield three pieces of information :

1.) Does CCX affect VSV transcription; primary and/or secondary? What is the effect on transcription in mock-infected BS-C-1 cells?

2.) Does CCX affect protein synthesis in VSV-infected and/or mock-infected BS-C-1 cells?

3.) Does CCX affect transcription and/or translation?

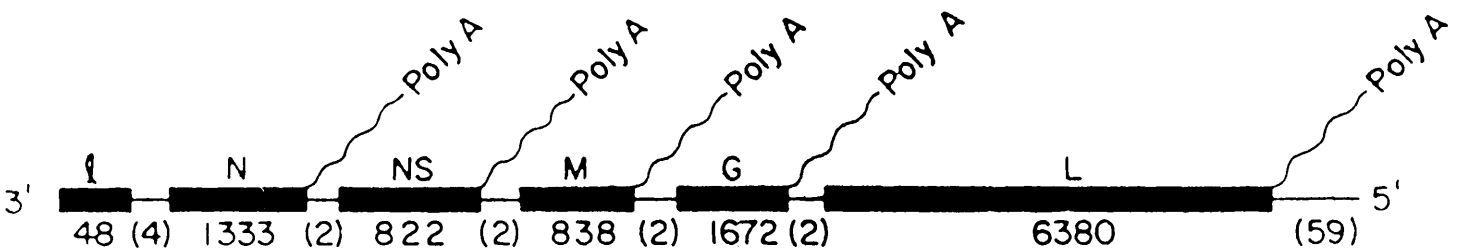

Poly A
Poly A
Poly A
Poly A
Poly A
3'
l
N
NS
M
G
L
5'
48 (4) 1333 (2) 822 (2) 838 (2) 1672 (2) 6380 (59)

Figure 29

Diagram of the genome of VSV

The gene order is shown above the genome, while the numbers in brackets represent the number of nucleotides not found in the transcription products. Transcribed regions are shown as heavy black lines with the length of the transcript in nucleotides given below.

This diagram is not drawn to scale.

EXPERIMENTAL DESIGN (Table 27)

RADIOLABELLING :

TIME

VSV primary transcripts, cellular transcripts and polypeptides synthesized in VSV-infected and mock-infected BS-C-1 cells were all labelled in parallel from 1-8h p.i.

VSV secondary transcripts , cellular transcripts and polypeptides synthesised in VSV-infected and mock-infected BS-C-1 cells were all labelled in parallel from 4-24h p.i. (Also see one-step growth curve, Fig. 25).

RADIOLABELS

VSV primary transcripts were labelled with ^{3}H uridine, VSV secondary transcripts and cellular transcripts with ^{32}P orthophosphate and all polypeptides with ^{35}S methione. Radiolabelling was performed as described in 2.2.7.3.

USE OF INHIBITORS

Labelling of VSV primary transcripts was performed in the presence of cycloheximide (CHX), which inhibits protein synthesis and therefore blocks the production of secondary transcripts.

Actinomycin D (Act.D) was used when labelling both primary and secondary transcripts to selectively suppress host RNA synthesis (blocks DNA directed RNA synthesis, but not RNA directed RNA synthesis)

Polypeptides synthesized in CCX treated infected cells were therefore labelled in the presence and absence of Act.D. to determine 1.) how CCX affected polypeptide synthesis 2.) whether addition of Act.D. in any way modifies the action of CCX and 3.) to allow comparison of mRNA and protein levels.

	1 - 8 h pi		4 - 24 h pi	
RNA	VSV PRIMARY TRANSCRIPTS	ACT-D CHX	VSV SECONDARY TRANSCRIPTS	ACT-D
	MOCK-INFECTED CELLULAR TRANSCRIPTS	-	MOCK-INFECTED CELLULAR TRANSCRIPTS	-
PROTEIN	POLYPEPTIDES SYNTHESISED IN VSV INFECTED AND MOCK INFECTED CELLS	+/- ACT-D	POLYPEPTIDES SYNTHESISED IN VSV INFECTED AND MOCK INFECTED CELLS	+/- ACT-D

Table 27

Determining the affect of CCX on RNA and protein synthesis: experimental design.

ACT.D. Actinomycin D

CHX Cycloheximide

3.3.7.1. THE EFFECT OF INCREASING CONCENTRATIONS OF CCX ON RNA AND PROTEIN SYNTHESIS IN MOCK-INFECTED AND VSV-INFECTED BS-C-1 CELLS FROM 1-8h p.i.

THE EFFECT OF CCX ON RNA SYNTHESIS IN MOCK-INFECTED BS-C-1 CELLS

Fig 30a shows a reduction in radiolabelled cellular transcripts with only 100uM CCX, becoming more marked with 200 and 300uM CCX. The reduction in radioactive label with 200uM CCX, was in part due to the underloading of this lane (Fig 30b, lane 3). However this is not the case with 300uM CCX, as this lane had in fact been overloaded (Fig 30b, lane 4). Therefore the results show that the production of cellular cytoplasmic RNA was progressively inhibited with increasing concentrations of CCX.

THE EFFECT OF CCX ON VSV PRIMARY TRANSCRIPTION IN INFECTED BS-C-1 CELLS

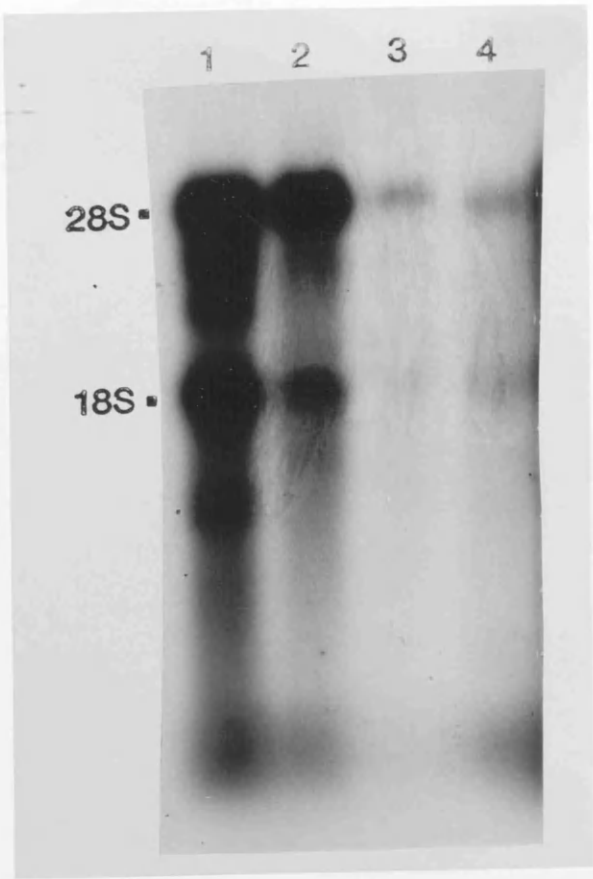
The extraction of VSV primary transcripts was not successful: samples were subjected to electrophoresis on a 1.2% agarose / formaldehyde gel, but RNA was not detected after soaking the gel in EtBr and iluminating with U.V. (260nm). Radiolabelled RNA was not detected after exposing the gel to X-Omat film. This experiment was only attempted once and must therefore be repeated.

THE EFFECT OF CCX ON POLYPEPTIDE SYNTHESIS IN MOCK-INFECTED AND VSV-INFECTED BS-C-1 CELLS IN THE PRESENCE AND ABSENCE OF ACT D.

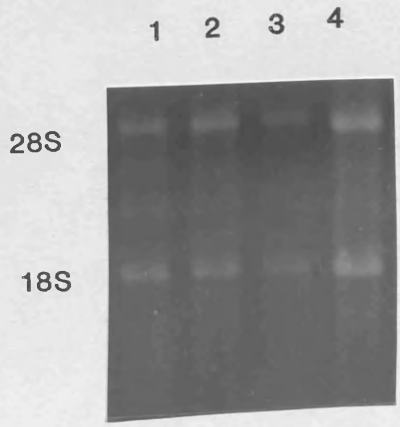
MOCK-INFECTED CELL EXTRACTS

- ACT.D.

Host cell protein synthesis was progressively inhibited with increasing concentrations of CCX, in the absence of Act.D., shown by the progressive reduction in T.C.A. precipitable counts with increasing CCX to 61.8% of the drug free control with 300uM (Table 28, Fig 32). Analysis by SDS PAGE, revealed no apparent qualitative change in the polypeptides induced in CCX treated, mock-infected BS-C-1 cells over this time (Fig 31b, lanes 6-9)



a



b

Figure 30a and b

The effect of 8h treatment with increasing concentrations of CCX on cellular cytoplasmic levels

32-P orthophosphate labelled cytoplasmic RNA was isolated from mock-infected cells treated with either no drug (lane 1) or 100,200,300uM CCX (lanes 2-4), from 1-8h p.i. RNA was analyzed by electrophoresis on a 1.2% agarose/formaldehyde gel, equal quantities of RNA loaded onto each track (yields were estimated by measuring O.D. at 260nm).

The radiolabelled RNA in each track was visualized by exposing the gel to X-Omat S film (a).
The total RNA added to each track was visualized by soaking the gel in EtBr and illuminating under short wave U.V. (260nm) (b). (This allowed estimation of loading errors).

The position of the 28S and 18S rRNA are indicated.

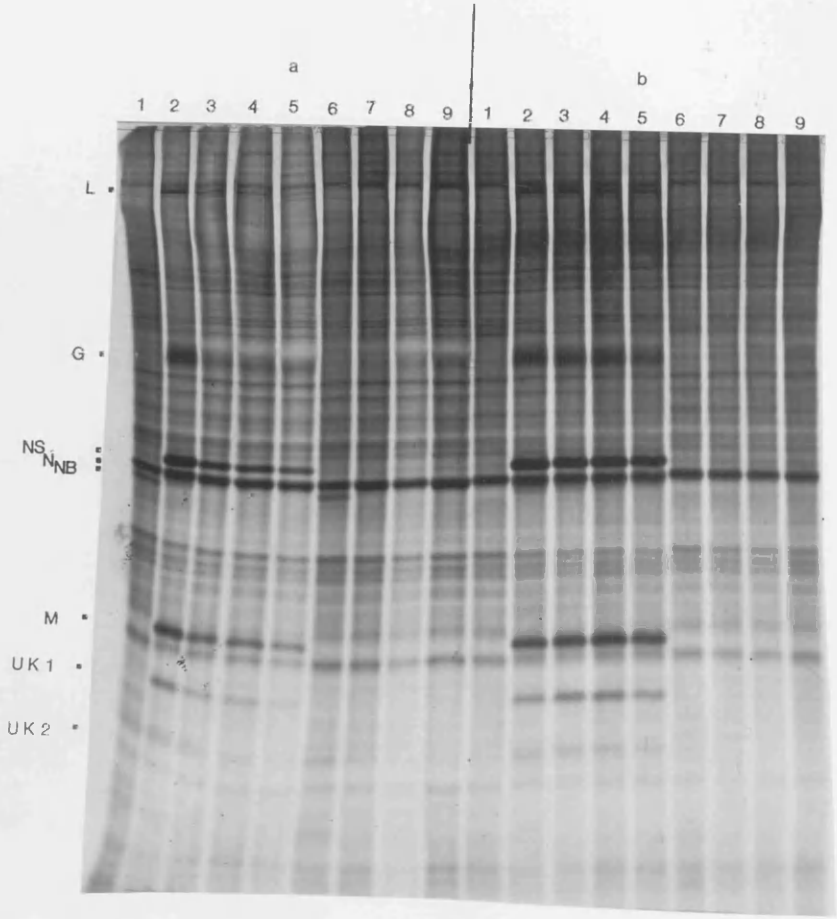

a
b
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9
L
G
NS
N
NB
M
UK1
UK2

Figure 31a

SDS PAGE profile on a 10% gel of the infected cell polypeptides induced in BS-C-1 cells treated with either no CCX (lanes 1,2 and 6) or 100,200 or 300uM CCX (lanes 3-5 and 7-9), from 1-8h p.i.
Cells were also pre-treated for 2h before infection, during ?infection and for 8h p.i. with 5ug/ml ACT.D.

Cells were either mock-infected (lanes 1 and 6-9) or infected with VSV at m.o.i. of 50 p.f.u./cell (lanes 2-5) and ^{35}Smet added from 1-8h p.i.

Equal numbers of counts were loaded onto each track. Letters on the left indicate the positions of known virus-encoded proteins (L,G,NS,N and M), a low m.wt. band of unknown origin present in virus-infected but not mock-infected cell extracts (UK1) and a novel cellular band induced in mock-infected cells in the presence of ACT. D.

Figure 31b

SDS PAGE profile on a 10% gel of the infected cell polypeptides induced in BS-C-1 cells treated with either no CCX (lanes 1,2 and 6) or 100,200 or 300uM CCX (lanes 3-5 and 7-9), from 1-8h p.i.

Cells were either mock-infected (lanes 1 and 6-9) or infected with VSV at a m.o.i. of 50 p.f.u./cell (lanes 2-5) and ^{35}Smet added from 1-8h p.i.

Equal numbers of counts were loaded onto each track. Letters on the left indicate the positions of known virus-coded proteins (L,G,NS,N and M) and 2 low m.wt. bands of unknown origin present in virus-infected but not mock-infected cell extracts (UK1 and UK2).

	CCX (uM)	+ ACT.D.	-ACT.D.
	0	105,008	130,270
VSV-INF.	100	55,852	124,566
BSC-I	200	37,279	85,987
CELLS	300	45,400	80,545
	0	89,365	304,016
MOCK-INF	100	80,787	233,043
BSC-I	200	79,932	186,967
CELLS	300	42,919	136,164

	CCX (uM)	L	G	N	NS	M	UK1
ACT. D.	0	328 (100)	552.3 (100)	367.4 (100)	316.1 (100)	226.7 (100)	94.8 (100)
	100	176.4 (54)	196 (35)	125.9 (34)	158.2 (50)	72.1 (32)	33.6 (35)
	200	111.3 (34)	86.7 (16)	69.4 (19)	97.6 (31)	39.2 (17)	ND
	300	59.0 (18)	102.6 (18)	61.4 (17)	94.4 (30)	35.2 (16)	14.18(15)
-ACT. D.	0	459.4 (100)	604.2 (100)	404.9 (100)	321.3 (100)	198.3 (100)	73.8 (100)
	100	399.2 (87)	557.4 (92)	361.6 (89)	308.5 (96)	238.0 (120)	92.9 (126)
	200	348.0 (76)	510.0 (84)	310.0 (76)	256.33 (80)	225.7 (114)	75.7 (103)
	300	324.0 (71)	442.77(73)	250.4 (62)	217.92 (68)	174.6 (88)	60.2 (82)

Table 28

Incorporaton of ^{35}S-methionine into VSV infected or mock infected cells (i.e. T.C.A. precipitable counts), treated with increasing concentrations of CCX, in the presence and absence of ACT.D., from 1-8h p.i.

Table 29

Densitometer scan of polypeptide bands in VSV infected tracks: tracks were scanned and the surface areas of each band divided by the volume added to each track, in order to standardise in terms of protein and not radioactive counts.

Numbers in parentheses are percentages: in each case the 0 value is 100%.

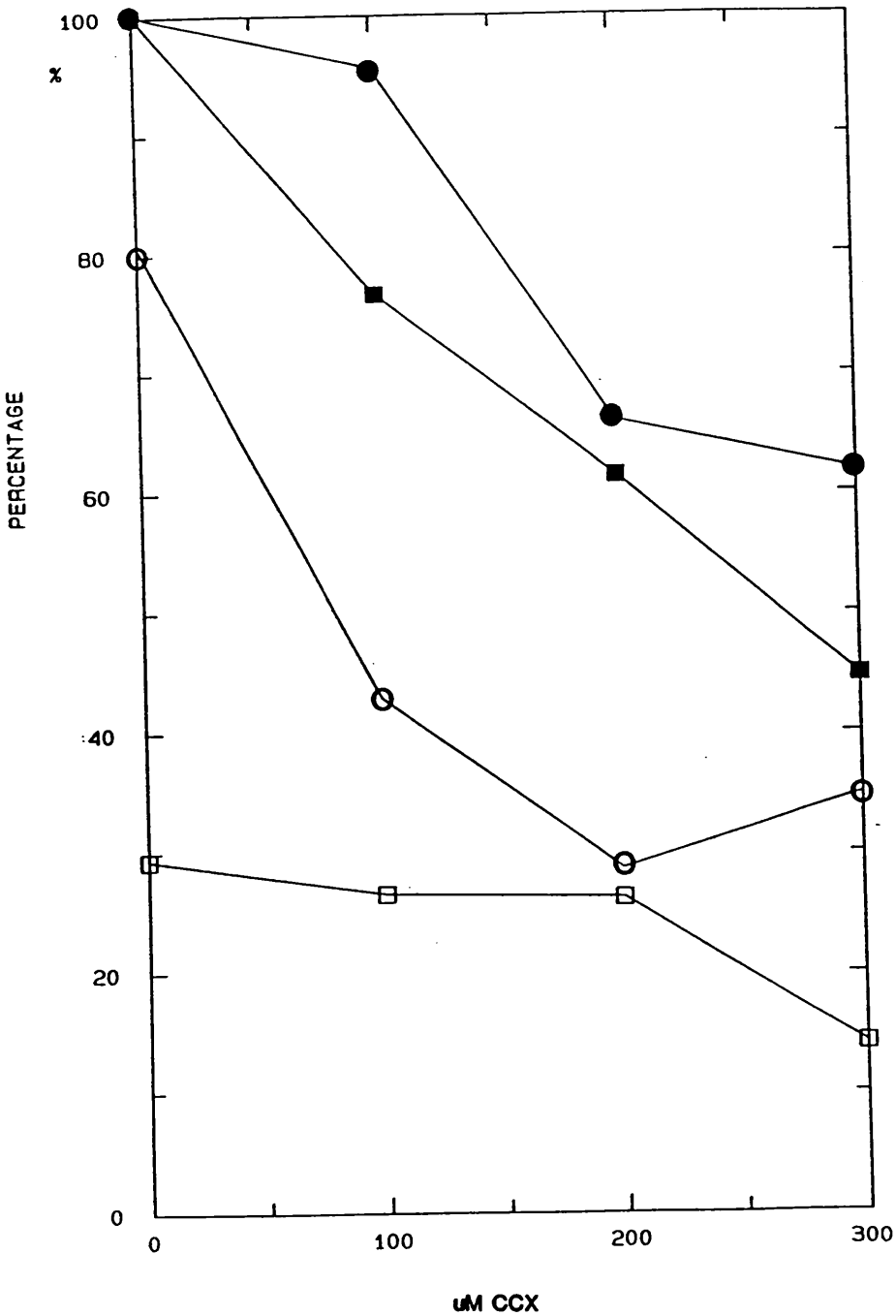

100
%
80
60
40
20
0
PERCENTAGE
0
100
200
300
uM CCX

Figure 32

The effect of increasing concentrations of CCX on the incorporation of ^{35}Smet into polypeptides induced in VSV-infected (○ , ●) and mock-infected (□ , ■) BS-C-1 cells in the presence (○ , □) and absence (● , ■) of ACT.D. (5ug/ml).

35Smet counts shown in Table 28, plotted as a percentage of the drug free controls.

+ ACT.D.

As expected T.C.A. precipitable counts in ACT. D. treated cell extracts were lower than in untreated cell extracts, falling to 29.3% of - Act.D. drug free control (Table 28, Fig 32) In the presence of Act.D., counts remained constant with 100 and 200uM CCX. However, treatment with 300uM CCX in the presence of Act.D. resulted in a fall of approximately 50%, similar to the decrease seen at this concentration in the absence of Act.D. (Fig 32). Analysis of polypeptides by SDS PAGE, revealed that treatment with Act.D. alone induced a novel band (NB), just below the actin band, which disappeared on the addition of CCX (Fig 31a, lanes 6-9).

VSV-INFECTED CELL EXTRACTS

Labelled infected cell extracts were analyzed by SDS PAGE (Fig 31a and b) and the virus-infected tracks densitometrically scanned and standardised for equal amounts of protein in each track (Table 29).

EFFECT OF ACT.D.

Analysis by SDS PAGE, revealed no qualitative difference in viral polypeptide bands induced in VSV-infected BS-C-1 cells in the presence of Act.D. (Fig 31a, lane 2), or in the absence of either drug (Fig 31b, lane 2). Densitometric scanning of the tracks also revealed no significant difference in the relative abundance of viral protein bands within each track (Table 29). Thus, Act.D. by itself had no significant effect on viral protein synthesis during the period 1-8h p.i.

EFFECT OF CCX

Treatment with increasing concentrations of CCX, did not significantly inhibit the synthesis of any viral protein with levels remaining above 60% of the drug free control in all cases (Table 29). However CCX treatment did result in slight changes in the relative abundance of bands; L, G, N and NS all progressively decreased with increasing CCX to 70.5%, 73.2%, 61.8% and 67.8% of drug free levels respectively in the presence of 300uM CCX (Table 29). In contrast, levels of M were increased with both 100 and 200uM

CCX to 120% and 113.8% respectively, falling to just below drug-free levels (88%) with 300uM CCX. UK_1 (an unknown band present in VSV-infected but not mock-infected cell extracts), increased very slightly with 100uM CCX to 125.8% of drug free levels, fell to drug free levels with 200uM CCX (102.8%) and to below drug free levels (81.5%) with 300uM CCX (Table 29). Another band designated UK_2, which also appeared in virus-infected but not mock-infected cell extracts, was too faint to scan, but appeared to increase and decrease with UK_1, with increasing CCX concentrations (Fig 31b).

There was also a slight shift downwards in polypeptide band G, with all CCX concentrations, indicating some effect on glycosylation (Fig 31b).

EFFECT OF ACT.D. AND CCX

It is clear that the synthesis of all viral polypeptides was markedly inhibited at all concentrations of CCX, in the presence of Act.D. (Table 29). The downward shift in the G band is greater than that seen with CCX in the absence of Act.D., suggesting a greater effect on glycosylation (Fig 31b). However, as the gel was standardised to contain equal counts in each track; more BSA was added to tracks 3-5 in Fig 31a than the same tracks in Fig 31b. BSA can cause distortion of the gel in this area and may explain at least in part, the apparent marked shift in this band.

CONCLUSIONS

CELLULAR TRANSCRIPTION AND POLYPEPTIDE SYNTHESIS

8h treatment with increasing concentrations of CCX, resulted in a progressive decrease in levels of cellular cytoplasmic RNA and protein. Whether the decrease in polypeptide levels was entirely due to decreased levels of transcript, or was also a consequence of a separate effect of CCX on the translation process is not clear and will be discussed more fully later.

VSV PRIMARY TRANSCRIPTION AND POLYPEPTIDE SYNTHESIS

When Act.D or CCX were added together viral protein synthesis was inhibited much more dramatically than in the presence of either compound alone. Therefore, the presence of Act. D. affected the action of CCX over this time (1-8h p.i.). Since primary transcripts were labelled from 1-8h p.i. in the presence of both these compounds,no conclusions could be drawn from the result of this experiment, regarding the effect of CCX on primary transcription.

A significant reduction in primary transcription seems unlikely as all viral proteins were synthesised in substantial amounts in infected cells treated with CCX from 1-8h p.i. However, there was some change in the relative intensity of viral polypeptide bands with some slightly decreased (L, G, N and NS) and others increased (M, UK_1 and UK_2). These changes could in theory be due to differential effects on transcription (primary and/or secondary).

3.3.7.2. THE EFFECT OF INCREASING CONCENTRATIONS OF CCX ON RNA AND PROTEIN SYNTHESIS IN MOCK-INFECTED AND VSV-INFECTED BS-C-1 CELLS FROM 4-24h p.i.

THE EFFECT OF CCX ON RNA SYNTHESIS IN MOCK-INFECTED BS-C-1 CELLS

Treatment with 100 and 200uM CCX (Fig 33a, lanes 2 and 3), resulted in increased incorporation of radiolabel into the 28S band, and decreased incorporation of radiolabel into the 18S band. However, both these tracks had been overloaded with RNA (Fig 33b, lanes 2 and 3), indicating that the overall reduction in radiolabelled transcripts was greater than that suggested by Fig 33a.

All radiolabelled transcripts were markedly reduced with 300uM CCX (Fig 33a, lane 4). EtBr staining revealed that this track had also been overloaded with RNA (Fig 33b, lane 4), therefore the actual reduction in radiolabelled transcripts was again greater than that suggested by Fig 33a.

This experiment therefore revealed, that the production of cellular cytoplasmic RNA was progressively inhibited with increasing concentrations of CCX.

THE EFFECT OF CCX ON SECONDARY TRANSCRIPTION IN VSV-INFECTED BS-C-1 CELLS

There was a marked reduction in radiolabelled VSV secondary transcripts with 100 and 200uM CCX; L mRNA was virtually undetectable in extracts labelled with only 100uM or 200uM CCX (Fig 34a, lanes 2 and 3). Radiolabelled transcripts disappeared completely with 300uM CCX (Fig 34a, lane 4). However, EtBr staining of this gel (Fig 34b, lane 4) showed that considerably less RNA had been loaded onto this track. Thus, the great reduction in radiolabel could have been largely due to a loading error. Lanes 2 and 3 of Fig 34 also contained less RNA than lane 1, however, in this case the difference was not great enough to account for the very pronounced decrease in radiolabelling observed. It is concluded therefore, that VSV secondary transcription becomes progressively inhibited by increasing concentrations of CCX.

THE EFFECT OF CCX ON POLYPEPTIDE SYNTHESIS IN MOCK-INFECTED

AND VSV-INFECTED BS-C-1 CELLS IN THE PRESENCE AND ABSENCE OF ACT.D.

MOCK-INFECTED CELL EXTRACTS

Host cell protein synthesis was progressively reduced with increasing concentrations of CCX, irrespective of Act. D., as shown by the progressive decrease in T.C.A. precipitable counts; the counts fell to 11% of the drug-free level in the presence and absence of Act.D., with 300uM CCX (Table 30, Fig 36). Analysis of polypeptides by SDS PAGE, revealed that in the absence of Act. D., 100, 200 and 300uM CCX induced polypeptide bands previously identified as host cell stress proteins (Fig 35b, lanes 8 and 9). Furthermore, the novel band observed after 8h Act.D. treatment (Fig 31a, lane 6), was not detectable at 24h (Fig 35a, lane 6).

VSV-INFECTED CELL EXTRACTS

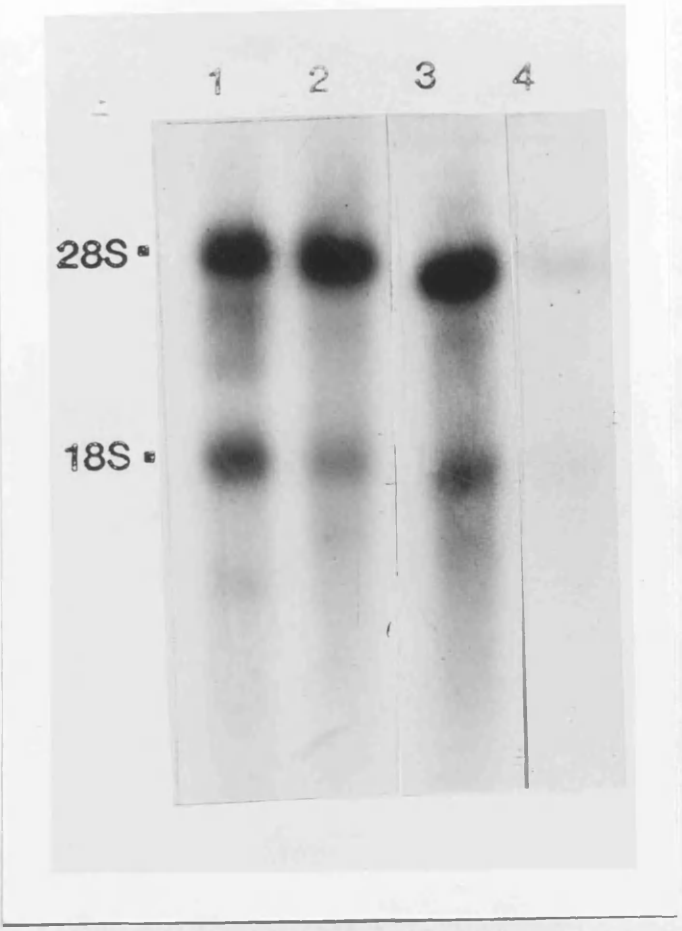
Labelled infected cell extracts were analyzed by SDS PAGE (Fig 35a and b), the virus-infected tracks densitometrically scanned and standardized for equal amounts of protein in each track (Table 31).

EFFECT OF ACT.D.

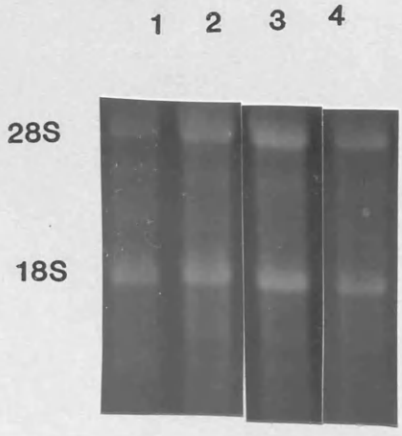
24h treatment had no significant qualitative or quantitative effect on viral polypeptides induced in VSV-infected BS-C-1 cells (Fig 35, compare a and b, lane 2; Table 31).

EFFECT OF CCX

Only polypeptide bands L and N progressively decreased with increasing concentrations of CCX. L fell to 83.7% of drug free controls at 100uM CCX, becoming too faint to scan at 200 and 300uM CCX, while N fell to 41.6% of drug free levels with 300uM CCX. (Fig 35b, Table 31).Band UK_1 was greater than drug-free levels at 100uM CCX (154% of drug free control), falling to below drug-free levels with 200 and 300uM CCX (to 53.3% and 41.2% respectively). Bands G and UK_2 were increased over drug-free levels at both 100 and 200uM CCX (to 135.5% and 188% respectively) only falling below drug-free levels (to 53% and 40% respectively) at 300uM CCX. Bands NS, M and UK_3 were greater than drug-free



a



b

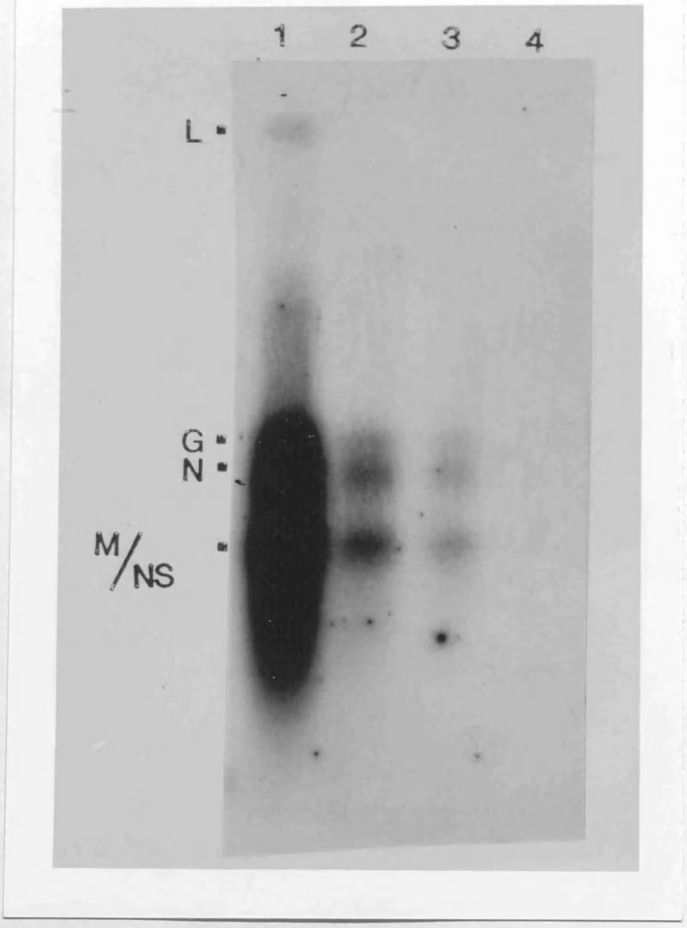
Figure 33a and b

The effect of 24h treatment with increasing concentrations of CCX on cellular cytoplasmic RNA levels.

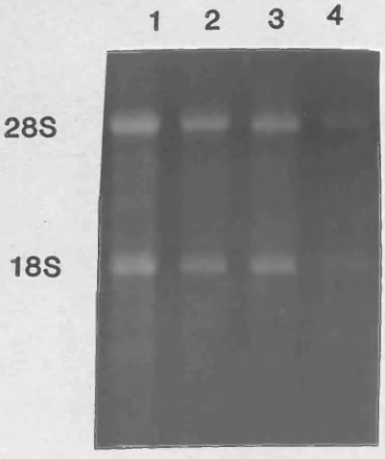
Mock-infected cells were treated with either no CCX (lane 1) or 100,200 or 300uM CCX (lanes 2-4) from 1-24h p.i. ^{32}P-orthophosphate was added from 4-24h p.i., then cytoplasmic RNA extracted and analyzed on a 1.2% agarose/formaldehyde gel.

Approximately,
Equal quantities of RNA were added to each track. (Yields were estimated by measuring O.D. at 260nm).
The radiolabelled RNA in each track was visualised by exposing the gel to X-Omat S film (a).
The total RNA added to each track was visualised by soaking the gel in EtBr and illuminating under short wave U.V. (260nm) (This allowed estimation of loading errors).

The positions of the 28S and 18SrRNA are indicated.



a



b

Figure 34a and b

VSV specific secondary transcription in CCX treated and untreated BS-C-1 cells.

Cells were pre-treated for 2h with 5ug/ml ACT.D. and in the continued presence of ACT.D., VSV infected (m.o.i. 50 p.f.u./cell) cells were treated with either no CCX (lane 1) or 100,200 or 300uM CCX (lanes 2-4). ^{32}-P orthophosphate was added from 4-24h p.i., then cytoplasmic RNA extracted and analyzed by electrophoresis on a 1.2% agarose/formaldehyde gel.

Approximately
Equal quantities of RNA were added to each track. (Yields were estimated by measuring O.D. at 260nm).
The radiolabelled RNA in each track was visualized by exposing the gel to X-Omat S film (a). The position of the virus coded transcripts L,G,NS,N and M are indicated and the sizes of the transcripts (in kb) are shown in brackets.

The total RNA added to each track was visualised by soaking the gel in EtBr and illuminating under short wave U.V. (260nm). (This allowed estimation of loading errors).
The positions of the 28S and 18srRNA are indicated.

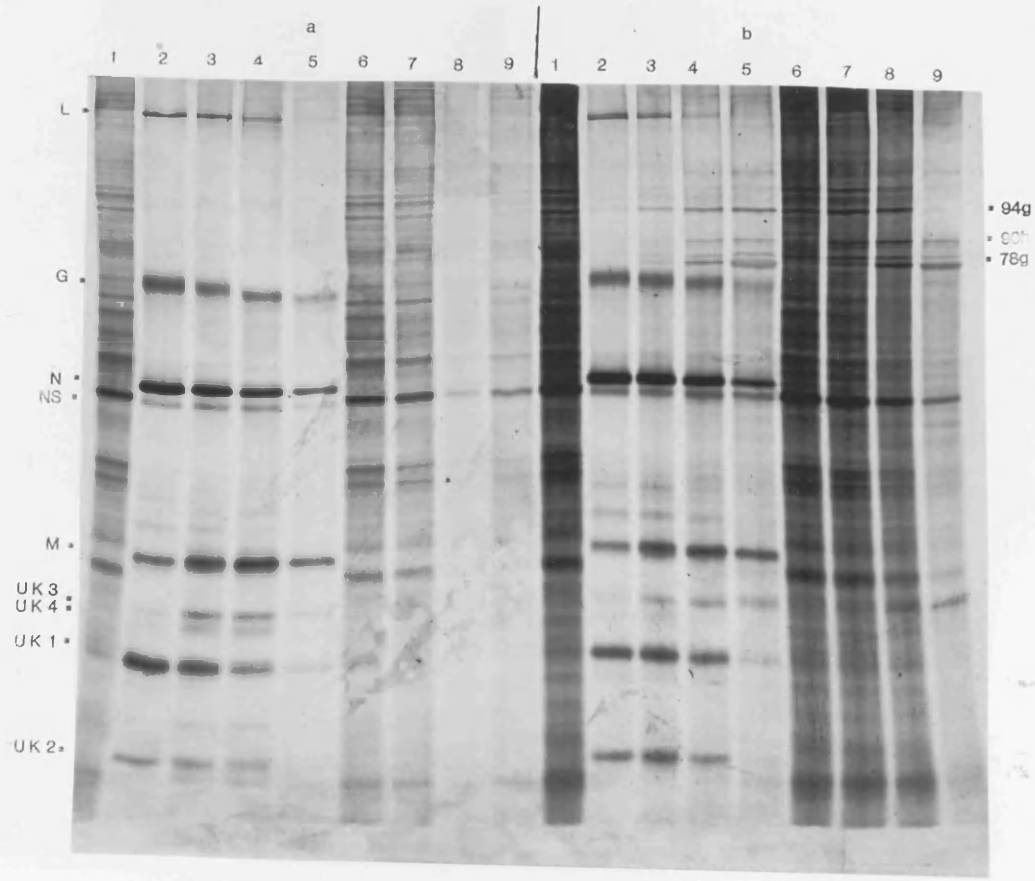

a
b
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
L
G
N
NS
M
UK3
UK4
UK1
UK2
94g
78g

Figure 35a

SDS PAGE profile on a 10% gel of the infected or mock-infected cell polypeptides induced in BS-C-1 cells treated and pre-treated for 2h before infection, during infection and for 24h p.i. with 5ug/ml ACT.D. and either no CCX (lanes 1,2 and 6) or 100,200 or 300uM CCX (lanes 3-5 and 7-9), from 1-24h p.i.

Cells were either mock-infected (lanes 1 and 6-9) or infected with VSV at m.o.i. of 50 p.f.u./cell (lanes 2-5) and ^{35}Smet was added from 4-24h p.i.
Equal amounts of counts were loaded onto each track.
Letters on the left indicate the positions of known virus coded proteins (L,G,NS,N and M) and 4 low m.wt. bands of unknown origin present in the virus-infected but not mock-infected cell extracts (UK1, UK2, UK3 and UK4).

Figure 35b

SDS PAGE profile on a 10% gel of the infected or mock-infected cell polypeptides induced in BS-C-1 cells treated with either no CCX (lanes 1,2 and 6) or 100,200 or 300uM CCX (lanes 3-5 and 7-9), from 1-24h p.i.

Cells were either mock-infected (lanes 1 and 6-9) or infected with VSV at m.o.i. of 50 p.f.u./cell (lanes 2-5) and ^{35}Smet was added from 4-24h p.i.
Equal numbers of counts were loaded onto each track.
Letters on the left indicate the positions of known virus-coded proteins (L,G,NS,N and M), 4 low m.wt. bands of unknown origin present in virus-infected but not mock-infected cell extracts (UK1, UK2 ~~and~~ UK3, UK4) and cellular stress proteins (94grp, 90hsp and 78grp).

	CCX (uM)	+ ACT.D.	-ACT.D.
	0	145,385	172,060
VSV-INF.	100	94,481	159,065
BSC-I	200	65,027	159,433
CELLS	300	28,637	96,904
	0	289,189	603,696
MOCK-INF.	100	106,767	473,653
BSC-I	200	68,717	300,971
CELLS	300	31,506	70,736

	CCX (uM)	L	G	N	NS	M	UK1	UK2	UK3	UK4
+ACT.D.	0	73.4 (100)	201.7 (100)	321.3 (100)	36.9 (100)	93.4 (100)	237.1 (100)	44.9	-	-
	100	75.8 (103)	132.1 (65)	274.0 (85)	63.0 (170)	222.3 (76)	179.6(76)	54.0	51.9	48.4
	200	37.8 (51)	71.7 (36)	124.7 (40)	39.4 (107)	150.8 (161)	57.6(24)	27.7	34.27	24.7
	300	10.2 (14)	15.3 (8)	30.2 (9)	6 (16)	30.3 (32)	-	-	-	-
-ACT.D.	0	86 (100)	160.5 (100)	409 (100)	46.4 (100)	83.5 (100)	146.1(100)	77.7 (100)	-	-
	100	72 (84)	217.2 (135)	405.8 (99)	64.6 (139)	248.5 (297)	225.5(154)	146.2(188)	57.4	-
	200	-	165.9 (103)	329.2 (80)	122.9(265)	220.1 (263)	77.9 (53)	137.7(177)	84.3	-
	300	-	85.1 (53)	170.5 (42)	89.4 (193)	119.3 (143)	60.3 (41)	31.5 (40)	119.3	-

Table 30

Incorporation of ^{35}S-methionine into polypeptides induced in VSV-infected or mock-infected cells treated with increasing concentrations of CCX, in the presence or absence of ACT.D. from 1-24h p.i. (^{35}S-met was added from 4-24h p.i.)

Table 31

Densitometer scan of polypeptide bands in VSV-infected tracks (4-24h p.i.): tracks were scanned and the surface areas of each band divided by the volume added to each track in order to standardise in terms of protein and not radioactive counts.

Numbers in parentheses are percentages: in each case the 0 value is 100%.

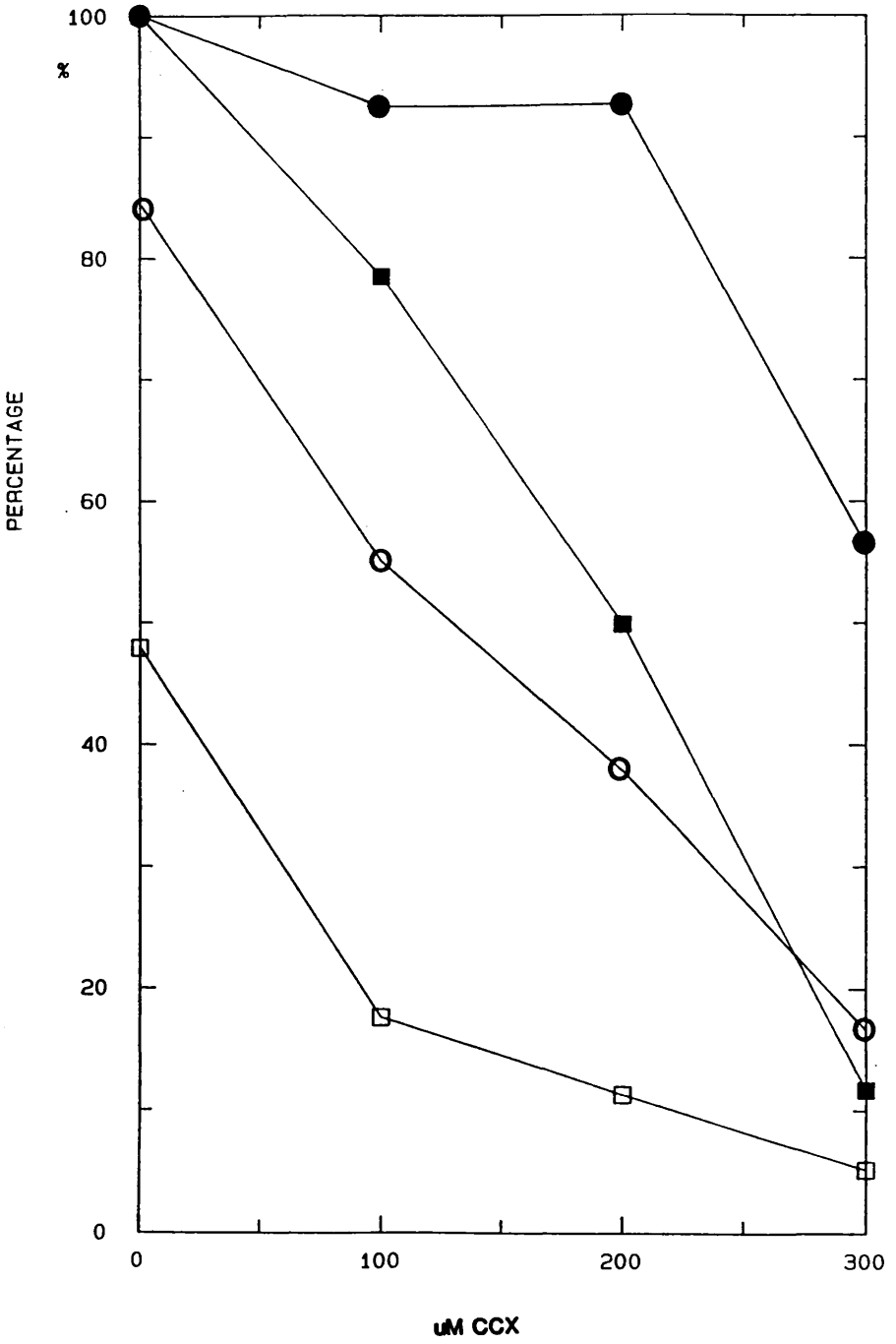

100
%
80
60
40
20
0
PERCENTAGE
0
100
200
300
uM CCX

Figure 36

The effect of increasing concentrations of CCX on the incorporation of ^{35}Smet into polypeptides induced in VSV infected (○ , ●) and mock-infected (□ , ■) BS-C-1 cells in the presence (○ , □) and absence (● , ■) of ACT.D. (5ug/ml).

^{35}S met counts shown in Table 30, plotted as a percentage of the drug free controls.

levels at all concentrations of CCX, NS peaking at 200uM (264.8% of drug free levels), M at 100uM (297% of drug free levels) and UK_3 at 300uM CCX (0 band too faint to scan) (Table 31).

In addition, the mobility of the G band was increased at all CCX concentrations, resulting in a downward shift of this band. This is most likely due to an effect on the glycosylation of this protein (Fig 35).

EFFECT OF ACT.D. AND CCX

In the presence of Act.D., polypeptide bands L, G, N, UK_1 and UK_2 all progressively decreased with increasing CCX concentrations, to 13.8%, 7.5%, 9.3% (UK_1 and UK_2 were too faint to scan) of drug free levels respectively with 300uM CCX.Polypeptide bands M, UK_3 and UK_4 (a band only present in infected cell extracts treated with both CCX and Act. D.) all increased to 100uM CCX (M to 238% of drug free levels while UK_3 and UK_4 were too faint to scan),then fall dropping to below drug-free levels (M to 32.4%)at 300uM CCX, although in all cases levels are greater than drug-free (M to 161.4%) at 200uM CCX. Levels of band NS are greater than drug free at 100uM CCX (170% of drug free control), and 200uM CCX (106.7%), falling to below drug free levels with 300uM CCX (to 16.2%).(Table 31, Fig 35).

The increase in the mobility of the G band with increasing CCX concentrations, appeared greater in the presence of Act.D. As previously stated, this may be due to a greater effect on glycosylation or greater amounts of BSA in these tracks.

CONCLUSIONS

CELLULAR TRANSCRIPTION AND POLYPEPTIDE SYNTHESIS

24h treatment with CCX resulted in decreased levels of cellular cytoplasmic RNA and proteins. Cytoplasmic RNA levels may be reduced by blocking transport of RNA from the nucleus to the cytoplasm , by directly affecting the transcription process itself or by more rapid degradation in the cytoplasm. Levels of cytoplasmic and total RNA in CCX treated cells should be compared to differentiate between these possibilities.

Whether the reduction in cellular polypeptides was entirely due to reduced levels of mRNA is not clear and a separate effect on translation by CCX cannot be dismissed. Transcriptional and translational effects could be more clearly separated by *in vitro* assays; utilising nucleocapsids isolated from CCX treated VSV-infected cells in *in vitro* transcription systems and then the resultant transcripts in *in vitro* translation systems.

VSV SECONDARY TRANSCRIPTION AND POLYPEPTIDE SYNTHESIS

Levels of NS, M and UK_3 polypeptide bands were greater than drug-free levels with 100 and 200uM CCX, in the presence of Act.D. and at all CCX concentrations in the absence of Act.D. Therefore, the general trend was the same although the increase was greater in the absence of Act.D. In contrast, transcript levels were less than drug-free levels at all CCX concentrations; therefore the accumulation of these polypeptides must be a consequence of some post-transcriptional effect of CCX. There are several possible explanations which include 1.) differential mRNA stability 2.) preferential translation of these transcripts 3.) differential secretion of polypeptides and /or 4.) differential polypeptide stability in the presence of CCX.

Although bands G, UK_1 and UK_2 increase to varying degrees with CCX in the absence of Act.D., all of these bands progressively decrease with increasing CCX in the presence of Act.D. The contrasting results obtained in the presence and absence of Act.D. do not allow direct comparison between mRNA and protein levels in these cases and therefore does not allow separation of transcriptional

and post-transcriptional effects of CCX.

L and N mRNA and polypeptide levels progressively decreased with increasing CCX in the presence and absence of Act. D. This therefore allows comparison of mRNA and polypeptide levels and meaningful conclusions drawn ~~regarding~~ regarding the action of CCX. It seems likely that the reduced polypeptide levels were a consequence of reduced levels of mRNA, although additional post-transcriptional effects cannot be ruled out.

The mobility of polypeptide band G was increased in the presence and absence of Act.D., indicative of an effect on glycosylation. The increase in mobility was greater in the presence of Act.D.; this may be due to greater effect on glycosylation or because these tracks contained more BSA, than Act.D. free tracks (due to standardisation of radioactive counts).

3.3.8. THE EFFECT OF CCX ON POST-TRANSLATIONAL MODIFICATIONS

3.3.8.1. THE EFFECT OF CCX ON PHOSPHORYLATION

There was no detectable effect on the phosphorylation of proteins in CCX treated mock-infected (Fig 37a) or VSV-infected (Fig 37b) BS-C-1 cells. It should be noted however, that the NS protein was not markedly phosphorylated, the predominant phosphorylated band in infected cells being a low m.wt. band. This band occurs only in VSV-infected tracks and may be one of the low m.wt. bands previously observed in ^{35}S methionine labelled VSV-infected BS-C-1 cells.

3.3.8.2. THE EFFECT OF CCX ON GLYCOSYLATION

SDS PAGE analysis of CCX treated and untreated VSV-infected BS-C-1 cells revealed that the G protein band in CCX treated tracks was increased in mobility, suggesting some effect on the glycosylation of this protein. In addition, Dargan and Subak-Sharpe have previously shown that glycosylation was affected in HSV-1 and HSV-2 infected and mock-infected Flow 2002 and BHK-21 cells. To determine if and to what extent glycosylation was affected in VSV-infected and mock-infected BS-C-1 cells, comparative studies were performed using monensin, which disrupts Golgi functions where processing of sugars occurs and tunicamycin, which completely blocks addition of core sugars normally occurring in the ER.

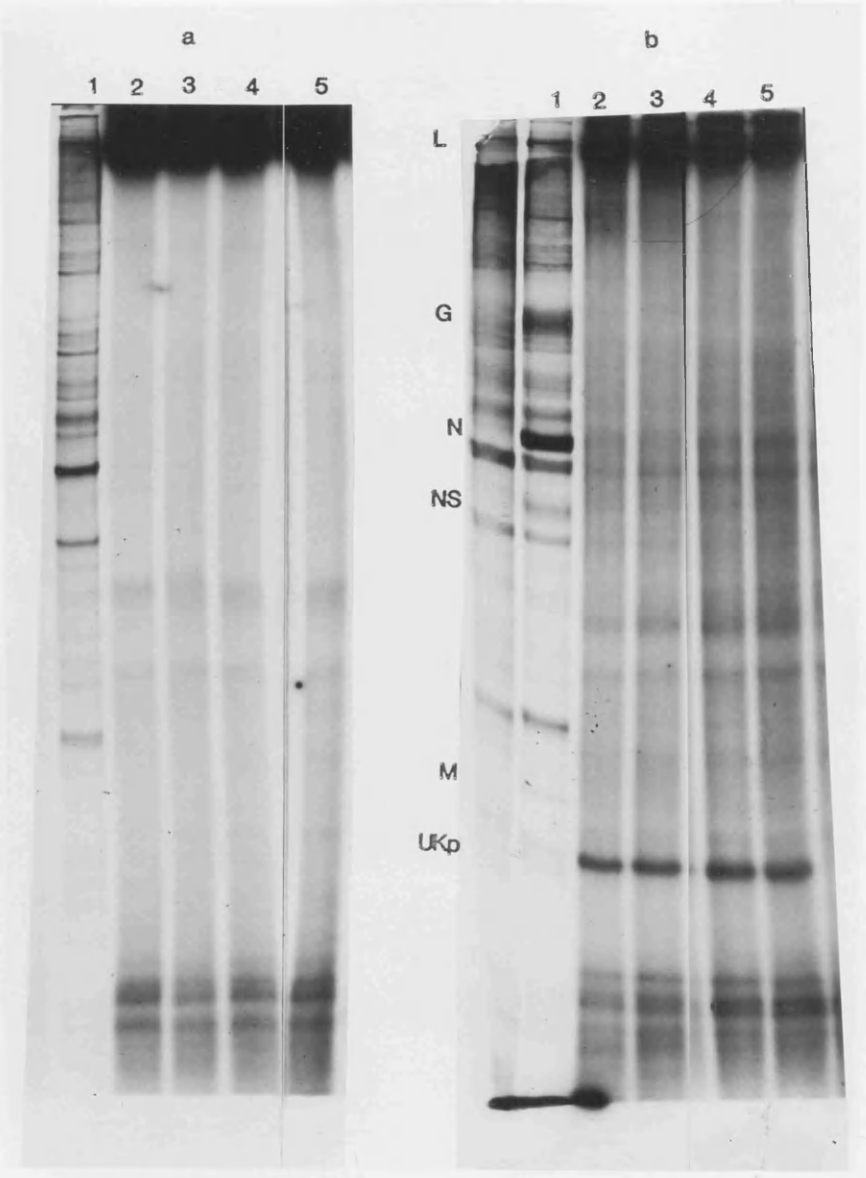

a
1 2 3 4 5
b
1 2 3 4 5
L
G
N
NS
M
UKp

Figure 37

SDS PAGE profile obtained on a 10% gel of ^{32}P-labelled polypeptides induced in mock-infected (a) and VSV-infected (m.o.i. 5 p.f.u./cell) (b) BS-C-1 cells treated with 1,100,200 or 300uM CCX (lanes2-5), from 1-24h p.i. Lane 1 shows ^{35}Smet-labelled polypeptides induced in drug-free mock-infected (a) and VSV-infected (m.o.i. 5 p.f.u./cell) (b) BS-C-1 cells.

^{32}P-orthophosphate and ^{35}S-methionine were added 2-24h p.i. Equal volumes of sample were added to each track. The positions of the known VSV-encoded proteins L,G,N,NS and M and the unknown low m.wt. phosphorylated band, present in VSV-infected but not mock-infected BS-C-1 cells, designated UKp, are indicated.

SDS PAGE ANALYSIS OF 35S-METHIONINE VSV-INFECTED AND MOCK-INFECTED BS-C-1 CELLS TREATED WITH CCX,MONENSIN OR TUNICAMYCIN

MOCK-INFECTED CELL EXTRACTS

PROTEIN SYNTHESIS (Table 32)

T.C.A. precipitable ^{35}S-met counts progressively decreased with increasing concentrations of CCX, falling to 62.8% of drug free levels with 300uM CCX (Table 32). Treatment with 10uM monensin resulted in a slight increase in T.C.A. precipitable counts (121.1% of drug free control), while the counts obtained with tunicamycin were almost identical to those obtained in the absence of any drug (100% of drug free control). Therefore, while CCX inhibited protein synthesis, monensin caused a slight increase and tunicamycin had no effect.

STRESS RESPONSE (Fig 39)

Analysis by SDS PAGE revealed that in this experiment, neither CCX (Fig 39; lanes 10,11,12) or monensin (Fig 39, lane 13) induced any stress response. However, tunicamycin induced the synthesis of glucose-related stress proteins; grp 94 and 78 (Fig 39, lane 14).

VSV-INFECTED CELL EXTRACTS

^{35}S-met labelled infected cell extracts were analyzed by SDS PAGE (Fig 39), the virus-infected tracks densitometrically scanned and standardised for equal amounts of protein in each track (Table 33).

RELATIVE ABUNDANCE OF VIRAL PROTEIN BANDS (Table 33)

L, N, and NS all progressively decreased with increasing concentrations of CCX, falling to 31.6%, 31.4% and 31.7% of drug free levels respectively, with 300uM CCX (Table 33). Levels of M and G were greater than drug free at all CCX concentrations, peaking at 200uM CCX (231% of drug free levels)in the case of the M protein band and 100uM CCX (239% of drug free levels)in the case of the G protein band. Low m.wt. bands UK_1, UK_2 and UK_3 were too faint to scan. However, Fig 39 shows that UK_3 increased with increasing

		CPM	
		VSV INFECTED BS-C-1	MOCK INFECTED BS-C-1
uM CCX	0	74,708	166,238
	100	58,167	157,269
	200	60,263	109,281
	300	38,078	104,443
uM MON	10	71,173	201,172
ug/ml TUN	2	78,316	166,333

		L	G	N	NS	M
uM CCX	0	663.5 (100)	131.8 (100)	2348.5(100)	359.1(100)	210 (100)
	100	507 (76)	316 (239)	2110 (72)	203 (56)	385 (183)
	200	394 (59)	307 (232)	1766 (60)	183 (51)	486 (231)
	300	210 (32)	278 (210)	928 (31)	114 (32)	326 (155)
uM MON	10	794 (119)	855 (648)	2307 (78)	238 (66)	686 (326)
ug/ml TUN	2	1029 (155)	925 (701)	2413 (82)	353 (98)	203 (97)

Table 32

T.C.A. precipitable 35S-methionine counts from VSV-infected and mock-infected cell extracts treated with 0, 100, 200, 300uM CCX, 10uM Monensin or 2ug/ml Tunicamycin.

Table 33

Densitometer scan of polypeptide bands in VSV-infected tracks on Figure 39: tracks were scanned and the surface areas of each band divided by the volume added to each track, in order to standardise in terms of protein and not radioactive counts.

Numbers in parentheses are percentages: in each case the 0 value is 100%.

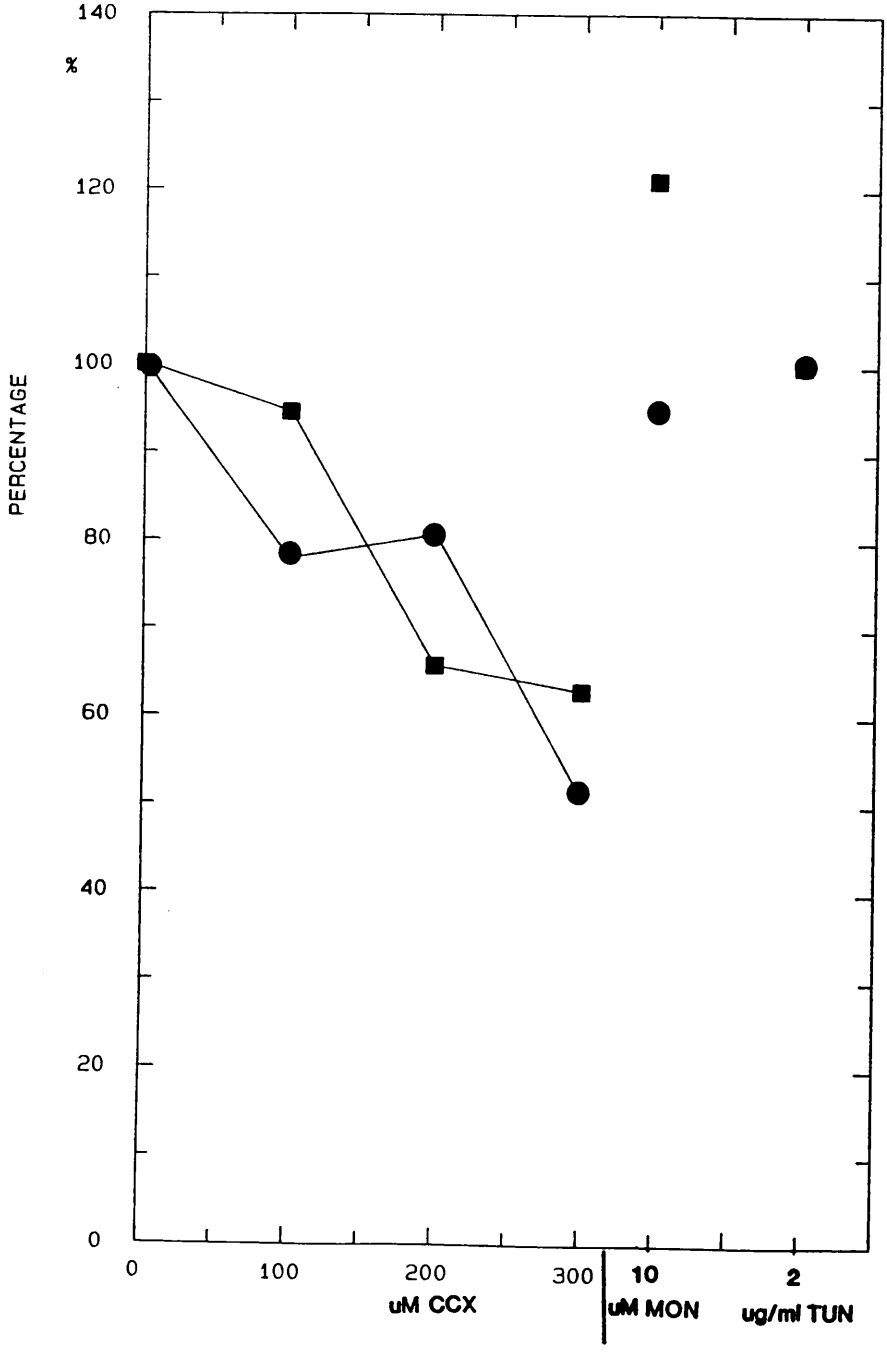

140
%
120
100
80
60
40
20
0
PERCENTAGE
0
100
200
300
10
2
uM CCX
uM MON
ug/ml TUN

Figure 38

The effect of 100, 200 and 300uM CCX, 10uM monensin and 2ug/ml tunicamycin on the incorporation of ^{35}Smet into polypeptides induced in VSV infected (●) and mock-infected (■) BS-C-1 cells.

^{35}S met counts shown in Table 32, plotted as a percentage of the mock-infected drug free control.

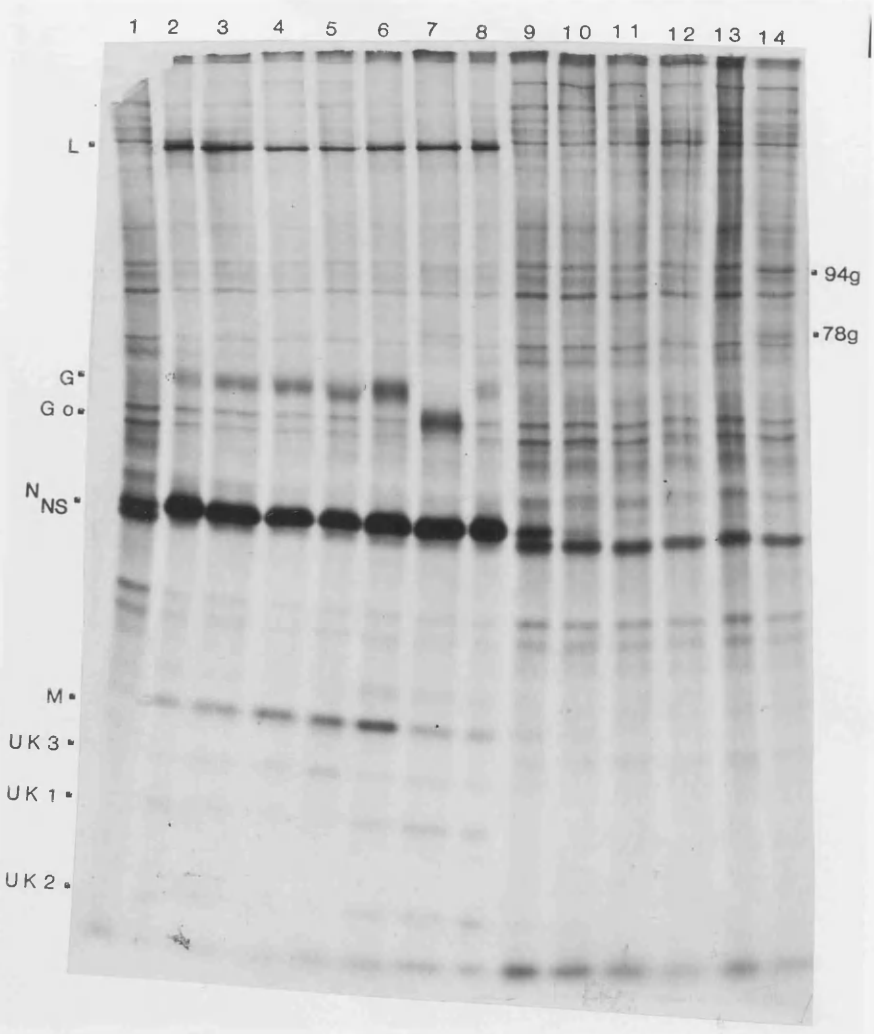

1 2 3 4 5 6 7 8 9 10 11 12 13 14
L
94g
78g
G
Go
N
NS
M
UK 3
UK 1
UK 2

Figure 39

SDS PAGE profile on a 10% gel of polypeptides induced in infected BS-C-1 cells treated with 0 (lanes 1,2,8 and 9), 100uM CCX (lanes 3 and 10), 200uM CCX (lanes 4 and 11), 300uM CCX (lanes 5 and 12), 10uM monensin (lanes 6 and 13) or 2ug/ml tunicamycin (lanes 7 and 14).

Cells were either mock-infected (lanesl,9-14), or infected with VSV at m.o.i. 5 p.f.u./cell (lanes 2-8).
^{35}Smet was added from 1-24h p.i.
Letters on the left indicate the positions of known virus-coded proteins (L,G,N,NS and M) and 3 low m.wt. bands of unknown origin present in virus-infected but not mock-infected cell extracts (UK1,UK2 and UK3). Go denotes the position of the unglycosylated form of G.
Also marked are glucose-related stress proteins present in tunicamycin treated BS-C-1 cells; 94grp and 78grp.
Equal numbers of counts were loaded onto each track.

concentrations of CCX, while UK_1 and UK_2 decreased.

Levels of bands L, N and NS in monensin tracks were 119%, 78.2% and 66.2% of those in drug free tracks respectively (Table 33). Monensin treatment resulted in a 6 fold increase in the level of protein band G (to 648% of drug free levels) and a 3 fold increase (326% of drug free levels) in the level of protein band M. While low m.wt. band UK_3, may have increased slightly, both UK_1 and UK_2 levels were comparable to those in drug-free tracks. (Fig 39).

In tunicamycin tracks, levels of bands N, NS and M were comparable to levels in drug free tracks (81.8%, 98.3% and 96.6% of drug free levels respectively) while the level of band L rose to 155% of the drug free control. The unglycosylated form of the G protein, designated G_o, produced in the presence of tunicamycin appeared to accumulate in infected cells; levels of band G_o were seven times greater than levels of band G in the drug free track (rising to 70% of drug free levels).

MOBILITY OF G BAND (Fig 39)

In agreement with previous observations, treatment with CCX caused a slight increase in the mobility of G (Fig 39, lanes 3-5). Treatment with monensin also resulted in only a slight shift downward of this band (Fig 39, lane 6). In contrast, the unglycosylated form of G, produced in the presence of tunicamycin runs considerably lower than the fully glycosylated form of the protein (Fig 39, lane 7). Therefore, although CCX may have affected processing of sugars occurring in the Golgi, it did not completely inhibit sugar addition.

CONCLUSIONS

1. In this experiment, levels of the NS band decreased with increasing concentrations of CCX. This does not agree with the previous experiment (Fig 35; Table 31), where NS actually increased with increasing CCX. This difference may be related to a variable inducement of the stress response as in this experiment, again in contrast to Fig 35, no stress response was induced. (It is interesting that m.o. in Fig 35 was 50 p.f.u./cell compared to 5 p.f.u./cell in this experiment) This will be discussed more fully later.

2. In agreement with previous observations, CCX treatment resulted in increased levels of G, M and UK_3 in infected cells. It is interesting that monensin treatment also resulted in increased levels of these bands, suggesting some similarity in the mode of action. The fact that levels of L, N and NS were constant in monensin treated cells, yet reduced in CCX treated cells, may be due to differential effects on protein synthesis (Note that monensin caused a slight increase in T.C.A. precipitable counts in mock-infected cells, while counts were progressively decreased with increasing concentrations of CCX).

3. It is clear that CCX did not completely block addition of sugar onto the VSV G protein, as treatment with CCX resulted in only a ~~slight~~ slight downward shift in this band. In contrast, the unglycosylated form of this protein, produced in tunicamycin treated infected cells runs considerably lower than the fully glycosylated form. Monensin treatment also results in only a slight shift downward.

Therefore, CCX did not completely block sugar addition, but may have disrupted processing steps occurring in the Golgi apparatus.

SDS PAGE ANALYSIS OF 14C-GLU AND 35S-MET LABELLED VSV-INFECTED AND MOCK-INFECTED BS-C-1 CELLS TREATED WITH CCX,MONENSIN AND TUNICAMYCIN

To establish more clearly how CCX affects sugar addition, VSV-infected and mock-infected BS-C-1 cells treated in parallel with CCX, monensin or tunicamycin were labelled with ^{14}C-glucosamine and ^{35}S-methionine. Labelling with ^{14}C-galactose was also attempted but was not successful.

35S-MET LABELLED CELL EXTRACTS

35S-MET LABELLED MOCK-INFECTED CELL EXTRACTS

PROTEIN SYNTHESIS

T.C.A. precipitable ^{35}S-met counts progressively decreased with increasing concentrations of CCX, falling to 41.9% of drug free levels with 300uM CCX. In this experiment, counts were also decreased in monensin treated extracts, falling to 77.7% and 64.3% of drug free levels with 5 and 10uM respectively. Tunicamycin again had no significant effect on T.C.A. precipitable counts, falling to 95.4% Of drug free levels. (Table 34)

Therefore, CCX progressively inhibited host cell protein synthesis. Although monensin also inhibited protein synthesis at both 5 and 10uM, the reduction was less than that achieved with CCX. In agreement with previous observations, tunicamycin had little if any effect on protein synthesis.

STRESS RESPONSE

Analysis by SDS PAGE revealed that in this experiment, stress proteins 70h, 94g and 78g were all induced with 150, 200 and 300uM CCX, the response becoming more marked with increasing CCX (Fig 40a, lanes 6,7 and 8). In contrast, there was no notable inducement of stress proteins in monensin treated cell extracts (Fig 40a, lanes 9and 10). Glucose related stress proteins, 94g and 78g and ~~a~~ high m.wt. band, and a 40K protein were strongly induced in tunicamycin treated cells (Fig 40a, lane 11). Therefore, CCX induced both hsps and grps, tunicamycin only grps and monensin no stress proteins.

35S-MET LABELLED VSV-INFECTED INFECTED CELL EXTRACTS

^{35}S-met labelled infected cell extracts were analyzed by SDS PAGE (Fig 41a), adding equal volumes of sample onto each track, and the virus-infected tracks densitometrically scanned (Table 35).

RELATIVE ABUNDANCE OF VIRAL BANDS (Table 35)

Levels of polypeptide band L, progressively decreased with increasing concentrations of CCX to 31.9% of drug free levels with 300uM CCX. Levels of band NS also fell progressively with increasing CCX concentrations to 200uM CCX (to 33.8% of drug free levels). At 300uM CCX, levels of band NS increased but were still below drug free levels (to 74.6%)

In this experiment, levels of bands G, M and UK_3, progressively increased with increasing CCX. Levels of G and M at 300uM CCX were 385% and 575% of drug free levels respectively, while drug free levels of UK3 were too faint to scan. There was little significant change in the levels of band UK_1 at most CCX concentrations, falling to 63.9% of drug free levels.

Levels of bands L, N and NS in the 5uM monensin track were only slightly greater than levels in drug free tracks (110.3%, 105% and 125.5% of drug free levels respectively). Levels of L and NS rose slightly in the 10uM monensin track (to 124.1% and 114.4% respectively), while levels of N were almost identical to those in drug free tracks (99.8%). Levels of band UK_1 were increased slightly with 5uM monensin (to 133.3% of drug free levels) and decreased slightly with 10uM monensin (to 88.8% of drug free levels). Although levels of these bands were changed, the differences were small and their significance, if any, unclear. Levels of bands G and M were increased with both 5uM (to 543% and 322% of drug free levels respectively) and 10uM (to 516% and 244% respectively) monensin, with the increase being slightly greater with 5uM monensin. The increase in these bands was much greater than the increase in any other viral bands. Levels of G and M were approximately five and three times greater than drug free levels respectively. Band UK_3 was also increased over drug free levels, but was too faint to scan (Fig 41).

In tunicamycin tracks, levels of band L were almost

			CPM			
			VSV INFECTED BS-C-1		MOCK INFECTED BS-C-1	
			35S met	14C glu	35S met	14C glu
C C X	uM	0	47,736 (100)	3,888 (100)	434,567 (100)	4,239 (100)
		25	28,996 (61)	4,099 (105)	362,944 (84)	6,239 (147)
		50	29,683 (62)	3,834 (99)	329,863 (76)	3,633 (86)
		75	26,143 (55)	2,533 (65)	214,434 (49)	5,287 (125)
		100	29,371 (62)	2,502 (64)	215,247 (50)	5,995 (141)
		150	32,263 (68)	1,901 (49)	196,833 (45)	3,555 (84)
		200	44,764 (94)	1,292 (33)	159,580 (37)	4,871 (115)
		300	50,563 (106)	1,921 (49)	183,597 (42)	5,501 (130)
M O N		5	81,189 (170)	2,044 (52)	337,764 (78)	2,549 (60)
		10	73,313 (153)	5,959 (153)	279,674 (64)	6,320 (149)
TUN	ug/ml	2	55,605 (116)	2,829 (73)	414,623 (95)	3,490 (82)

Table 34

T.C.A. precipitable ^{35}S-methionine and ^{14}C-glucosamine counts from VSV-infected and mock-infected cell extracts treated with 0, 25, 50, 75, 100, 150, 200, 300uM CCX, 5, 10uM Monensin or 2ug/ml Tunicamycin.

The numbers in parentheses are percentages: in each case the 0 value is 100%.

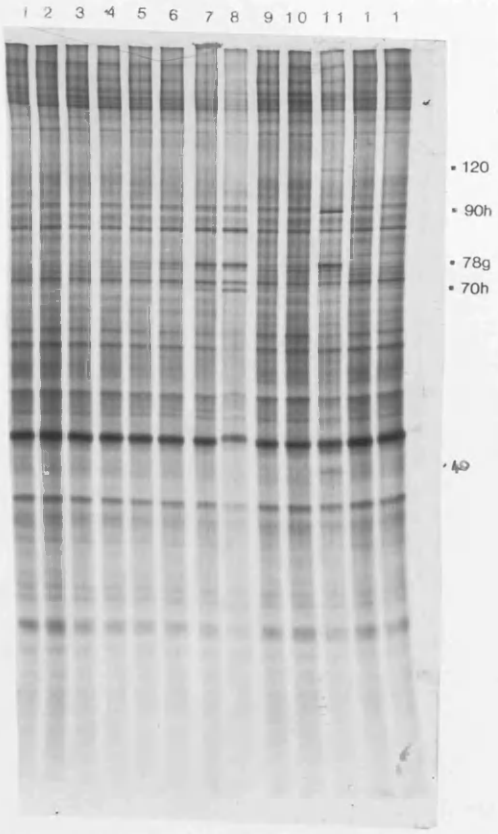
			L	G	N	NS	M	UK1	UK3
CCX	uM	0	1180 (100)	757 (100)	3546 (100)	1137 (100)	548 (100)	869 (100)	-
		25	1327 (112)	787 (103)	3601 (95)	955 (84)	439 (80)	815 (94)	-
		50	838 (71)	878 (115)	3281 (92)	815 (72)	952 (173)	811 (93)	-
		75	973 (82)	1227 (162)	3886 (109)	745 (66)	1410 (257)	1237 (142)	118
		100	728 (62)	1083 (143)	3329 (94)	433 (38)	1635 (298)	833 (96)	142
		150	629 (53)	1610 (212)	3476 (98)	385 (34)	1895 (345)	934 (107)	184
		200	687 (58)	1705 (225)	3512 (99)	ND	3056 (559)	1061 (122)	481
		300	377 (32)	2917 (385)	3426 (97)	849 (75)	3155 (575)	556 (64)	493
MON	nM	5	1302 (110)	4118 (543)	3726 (105)	1427 (126)	1770 (322)	1159 (133)	-
		10	1467 (124)	3913 (516)	3542 (99)	1301 (114)	1341 (244)	772 (89)	-
TUN	ug/ml	2	1273 (108)	3229 (426)	4653 (131)	879 (77)	956 (174)	1518 (175)	-

Table 35

Densitometer scan of polypeptide bands in VSV-infected tracks in Figure 40a.

The numbers in parentheses are percentages: in each case the 0 value is 100%.

a



b

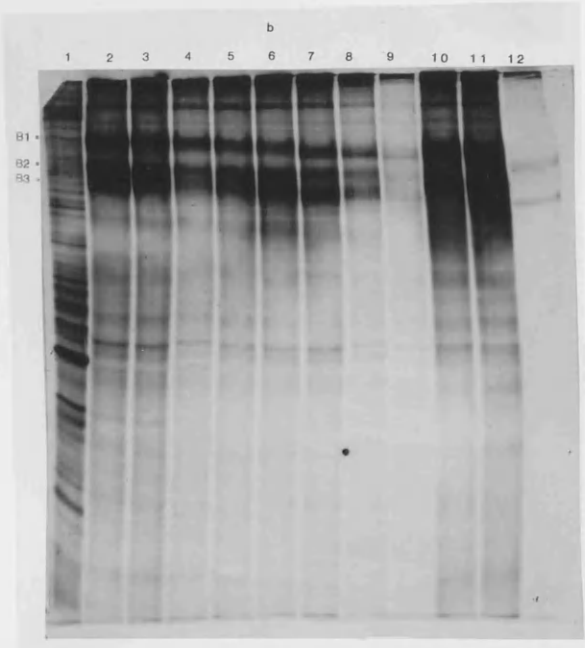


Figure 40

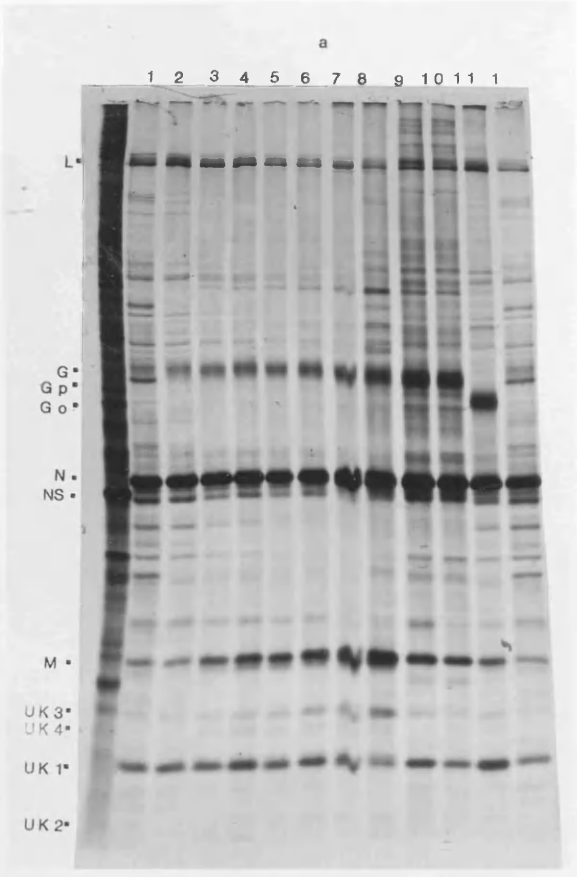
SDS PAGE profile on a 10% gel of ^{35}Smet-labelled (a) or ^{14}Cglu-labelled (b) polypeptides induced in mock-infected BS-C-1 cells. Radiolabel was added from 8-24h p.i.

(a) ^{35}Smet-labelled cell extracts
Mock-infected cells were treated with 0,25,50,75,100,150,200 or 300uM CCX (lanes 1-8), 5 or 10uM monensin (lanes 9 and 10) or 2ug/ml tunicamycin (lane 11).
Letters on the right indicate the positions of stress proteins; 94grp, 78grp and 70h.

(b) 14Cglu-labelled cell extracts
Mock-infected cells were treated with 0,25,50,75,100,150,200 or 300uM CCX (lanes 2-9), 5 or 10uM monensin (lanes 10 and 11) or 2ug/ml tunicamycin (lane 12).
B1, B2 and B3 denote 3 highly glycosylated cellular proteins.

Lane 1 shows ^{35}Smet-labelled mock-infected cell extract.

Equal volumes of sample were loaded onto each track.


a
1 2 3 4 5 6 7 8 9 10 11 1
L
G
Gp
Go
N
NS
M
UK3
UK4
UK1
UK2

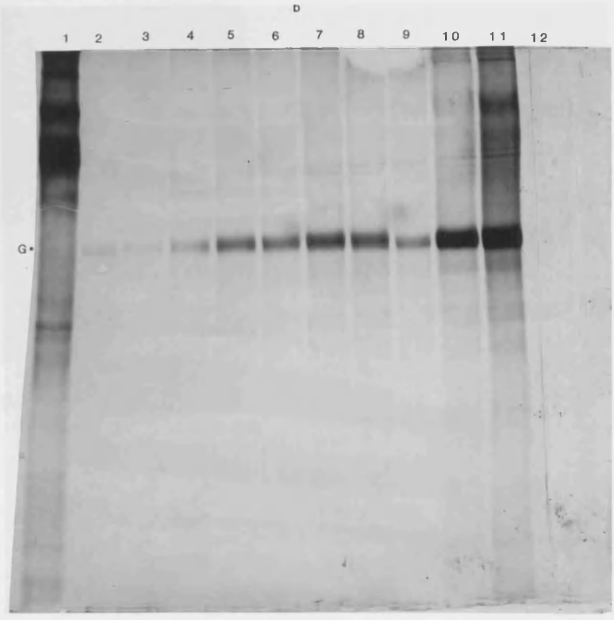

b
1 2 3 4 5 6 7 8 9 10 11 12
G

Figure 41

SDS PAGE profile on a 10% gel of ^{35}Smet-labelled (a) or ^{14}Cglu-labelled (b) polypeptides induced in VSV-infected (m.o.i. 5 p.f.u./cell), BS-C-1 cells. Radiolabel was added from 8-24h p.i.

(a) ^{35}Smet-labelled cell extracts

VSV-infected cells were treated with 0,25,50,75,100,150,200 or 300uM CCX (lanes 1-8), 5 or 10uM monensin (lanes 9 and 10) or 2ug/ml tunicamycin (lane 11).

Letters on the left indicate the position of known virus-coded proteins (L,G,N,NS and M) and 3 low m.wt. bands of unknown origin present in virus-infected, but not mock-infected cell extracts (UK1,UK2 and UK3).

Gp denotes the position of the partially glycosylated G protein .

Go denotes the position of unglycosylated G protein.

A drug free mock-infected track is shown on the left hand side.

(b) ^{14}Cglu-labelled cell extracts

VSV-infected cells were treated with 0,25,50,75,100,150,200 or 300uM CCX (lanes 2-9), 5 or 10uM monensin (lanes 10 and 11) or 2ug/ml tunicamycin (lane 12).

Lane 1 shows ^{14}Cglu-labelled mock-infected cell extracts.

The position of VSV glycoprotein G is marked.

Equal volumes of sample were loaded onto each track.

the same as drug free levels (107.8% of drug free control),while levels of band N were slightly increased (to 131.2% of drug free levels) and NS slightly decreased (77.3% of drug free levels). Again, the changes in these cases was small and their significance, if any, unclear. The levels of the unglycosylated form of G designated Go, rose to 426% of drug free levels i.e. four times greater than levels of the fully glycosylated form of G produced in the absence of drug. In this experiment, levels of bands M and UK_1 in tunicamycin tracks were almost double those in drug free tracks (rising to 174.4% and 174.6% of drug free levels respectively).

MOBILITY OF G BAND (Fig 41a)

The mobility of the G band was progressively increased with increasing concentrations of CCX, resulting in a slight shift downward with 300uM CCX (Fig 41a, lanes 1-8). A similar shift in the position of the band was seen with both 5 and 10uM monensin (Fig 41a, lanes 9 and 10). As expected, G_0 - the unglycosylated form of G produced in tunicamycin treated cells runs considerably lower than the fully glycosylated form of the protein (Fig 41a, lane 11). This agrees with previous observations and indicates that although CCX may have affected sugar processing,it did not completely inhibit sugar addition.

^{14}C-GLU LABELLED CELL EXTRACTS

^{14}C-GLU LABELLED MOCK-INFECTED CELL EXTRACTS

^{14}C-glu labelled cell extracts were subjected to SDS PAGE, adding equal volumes of sample onto each track (Fig 40b). Tunicamycin, as expected, almost completely abolished glucosamine incorporation. Only two bands were evident in the tunicamycin track (Fig40b, lane 12) and are most probably glycoprotein precursors (Blo and B3o), the shift in m.wt. explained by the almost complete block in sugar addition.

Monensin treatment had no detectable effect on glucosamine incorporation into bands Bl and B3, while band B2 could not be detected (Fig 40b, lanes 10 and 11). However, there was a slight downward shift in the mobility of bands Bl and B3 (Fig 40b, lanes 10 and 11) in monensin tracks.

Although there was no significant change in T.C.A. precipitable counts (Table 34), from CCX treated samples, analysis by SDS PAGE revealed a progressive reduction in glucosamine labelled proteins (Fig 40b, lanes 2-9). This is most probably predominantly due to the decrease in protein synthesis (Fig 40a, Table 34). However, bands Bl, B2 and B3 increased in mobility with increasing concentrations of CCX (Fig 40a, lanes 2-9), the shift in mobility (achieved with concentrations to 200uM) being similar to that seen in the monensin tracks. The shift in mobility obtained with monensin and CCX may be due to a block in the addition of sugars other than glucosamine e.g. galactose or possibly sialic acid, which are normally added after glucosamine. Attempts to label with ^{14}C-galactose however were unsuccessful and so this must remain speculation.

14-C GLU LABELLED VSV-INFECTED CELL EXTRACTS

^{14}C-glu labelled infected cell extracts were subjected to SDS PAGE, adding equal volumes of sample to each track (Fig 41b). ^{14}C-labelled G protein increased progressively with increasing CCX, to 200uM CCX, in agreement with the increase seen in ^{35}S-met labelled extracts, indicating that at these CCX concentrations there was no significant inhibition in incorporation of glucosamine (Fig 41a, lanes 1-7 and Fig 41b, lanes 2-8). However, while levels of band G increased with 300uM CCX in ^{35}S-met labelled extracts, levels of ^{14}C-glu labelled G actually decreased at this concentration, indicating some inhibition of glucosamine incorporation.

Levels of ^{14}C-glu and ^{35}S-met labelled G protein were both increased with 5 and 10uM monensin. Therefore, monensin had no effect on glucosamine incorporation. In contrast, ^{14}C-glu labelled G protein was not detectable in tunicamycin tracks, despite increased levels of ^{35}S-met labelled G_0. Therefore, tunicamycin, as expected completely abolished incorporation of glucosamine.

CONCLUSIONS

1. The aim of this experiment was to determine the affect of CCX on the VSV G protein. In agreement with the previous experiment, both CCX and monensin treatment resulted in increased levels of bands G, M and UK_3 (although the increase in band UK_3 was greater in CCX tracks). In addition there was a similar slight increase in the mobility of the G band with both CCX and monensin indicating some slight effect on glycosylation by both these compounds. In contrast, due to the absence of sugar on the G protein, resulting in a significant difference in m.wt., the mobility of the G band was greatly increased in tunicamycin tracks.

2. As expected, incorporation of ^{14}C-glucosamine was completely inhibited in tunicamycin treated infected cells. It has previously been shown that the unglycosylated form of the G protein accumulates in the ER. The accumulation of G_0 is therefore a consequence of non-glycosylation. M (and possibly UK_1) may associate with G_0 (unglycosylated form of G) in tunicamycin treated cells resulting in the accumulation of all these proteins.

In contrast, monensin and CCX to 200uM had no effect on glucosamine incorporation. However, these compounds may block the addition of sugars (e.g. galactose) occuring in the trans face of the Golgi. Labelling with ^{14}C-galactose however was not successful.

3. Levels of band N remained constant with increasing CCX concentrations, while band NS decreased progressively to 200uM CCX, then increased slightly at 300uM CCX. The changes in levels of N and NS, in the presence of CCX, varied between experiments; in two experiments levels of N progressively decreased and in one remained constant. Levels of NS were decreased in the presence of CCX in two experiments, but progressively increased to 200uM CCX in one other experiment. It is interesting that protein synthesis in host cells was most markedly affected and a very strong stress response induced where NS decreased, perhaps suggesting the involvement of the stress response in variable levels of NS.

In this and all other experiments, levels of the L

band consistently decreased with increasing CCX concentrations.

THE EFFECT OF CCX, MONENSIN AND TUNICAMYCIN ON PARTICLE NUMBERS, INFECTIOUS VIRUS YIELDS (P.F.U.) AND PARTICLE/P.F.U. RATIOS

This experiment was performed to directly compare the effects of these three compounds on infectivity, particle production and particle/p.f.u. ratios. Treatment of infected cells with CCX, reduced VSV particle production, however, the reduction was greater in the presence of CCX (>2 logs; no particles detected at 300uM CCX; Table 36), than in the presence of either monensin or tunicamycin (reduced particle numbers by 2 logs; Table 36). This greater reduction by CCX is most likely due to the additional effects of CCX on protein synthesis.

Virus particles which were produced from monensin or tunicamycin treated cells were as infectious as those produced under drug free conditions i.e. particle/p.f.u. ratios were essentially unchanged (Table 36). In contrast, virus yields produced from cells treated with higher concentrations of CCX (150, 200 and 300uM CCX) had elevated particle/p.f.u. ratios (10 fold or greater; Table 36). This elevation is most likely due to direct inactivation of VSV particles by CCX. (See Table 26)

DRUG		CONC.	PARTICLE NUMBER	P.F.U.	PARTICLE/P.F.U. RATIO
CCX	uM	0	$1.3*10^{10}$	$4.5*10^{8}$	29
		150	$8.5*10^{8}$	$2.5*10^{6}$	343
		200	$1.43*10^{8}$	$4.8*10^{5}$	298
		300	$<10^{8}$	$2.3*10^{4}$	ND
MON		10	$1.43*10^{8}$	$4.2*10^{6}$	34
TUN	ug/ml	2	$1.43*10^{8}$	$3.6*10^{6}$	40

Table 36

The effect of CCX, Monensin and Tunicamycin on VSV particle numbers, infectious virus yields (p.f.u.) and particle/p.f.u. ratios.

N.D. not determined.

3.4. THE EFFECT OF CCX ON THE REPLICATION OF SEMLIKI FOREST VIRUS (SFV) ; REPRESENTING THE CLASS 3 RESPONSE

3.4.1. DIRECT INACTIVATION OF SFV PARTICLES

Suspensions of SF virus particles were incubated with either mock drug or 300uM CCX (2.2.5.7.) at 4°C or 37°C for 24h. Infectious virus yields were titrated before and 24h after drug addition. The experiment was performed twice, and the results are shown in Table 37. CCX reduced the infectivity of SF virus yields by an average of 26% (There was only a 10% difference between the effect of CCX at 37°C and 4°C).

3.4.2. ADSORPTION OF SFV TO PRE-TREATED BS-C-1 CELLS

Fig 43 shows the adsorption curves obtained for SFV and BS-C-1 cells pretreated with 0, 300, 600 or 900uM CCX (as described in 2.2.5.6.). Although there was some slight effect on virus adsorption, the majority of virus was still able to adsorb even to cells pre-treated with 900uM CCX. Therefore, CCX had only a slight effect on subsequent SFV adsorption to BS-C-1 cells.

3.4.3. THE EFFECT OF CCX, MONENSIN AND TUNICAMYCIN ON INFECTIOUS SFV YIELDS FROM TREATED BS-C-1 CELLS

When SFV-infected BS-C-1 cells were treated with increasing concentrations of CCX, a progressive increase in cell-associated (CA) infectious virus yields (-1 log) resulted which was accompanied by a concomitant decrease in the cell-released (CR) fraction (-1 log) (Fig 44a). A similar result was obtained with increasing concentrations of monensin (Fig 44b). In contrast, tunicamycin treatment reduced the infectivity of both CA and CR fractions by -3 logs, indicating that the addition of core sugar is important for virus infectivity.

The increase in the cell-associated fraction observed in both CCX and monensin treated SFV-infected BS-C-1 cells could be due to retention of virus particles at the PM and/or budding of virus particles at intracellular membrane. The latter possibility seems most likely as budding of SFV particles into Golgi vacuoles has been observed in SFV-infected BHK cells treated with monensin (Griffiths,

Quinn and Warren, 1983).

a

TIME(hr)	TEMP (C)	DRUG	TITRE	%AGE	REDUCTION
0	-	ORIGINAL STOCK	$3.85*10^6$		
24	4	MOCK DRUG	$3.75*10^6$	100	20
24	4	300 uM CCX	$3.0*10^6$	80	
24	37	MOCK DRUG	$7.0*10^5$	100	28.6
24	37	300 uM CCX	$5.0*10^5$	71.4	

b

TIME(hr)	TEMP (C)	DRUG	TITRE	%AGE	REDUCTION
0	-	ORIGINAL STOCK	$4.20*10^6$		
24	4	MOCK DRUG	$3.60*10^6$	100	22.3
24	4	300 uM CCX	$2.8*10^6$	77.7	
24	37	MOCK DRUG	$9.2*10^5$	100	32.7
24	37	300 uM CCX	$6.2*10^5$	67.3	

Table 37

Direct inactivation of SFV particles by CCX.

a) and b) represent data from 2 separate experiments.

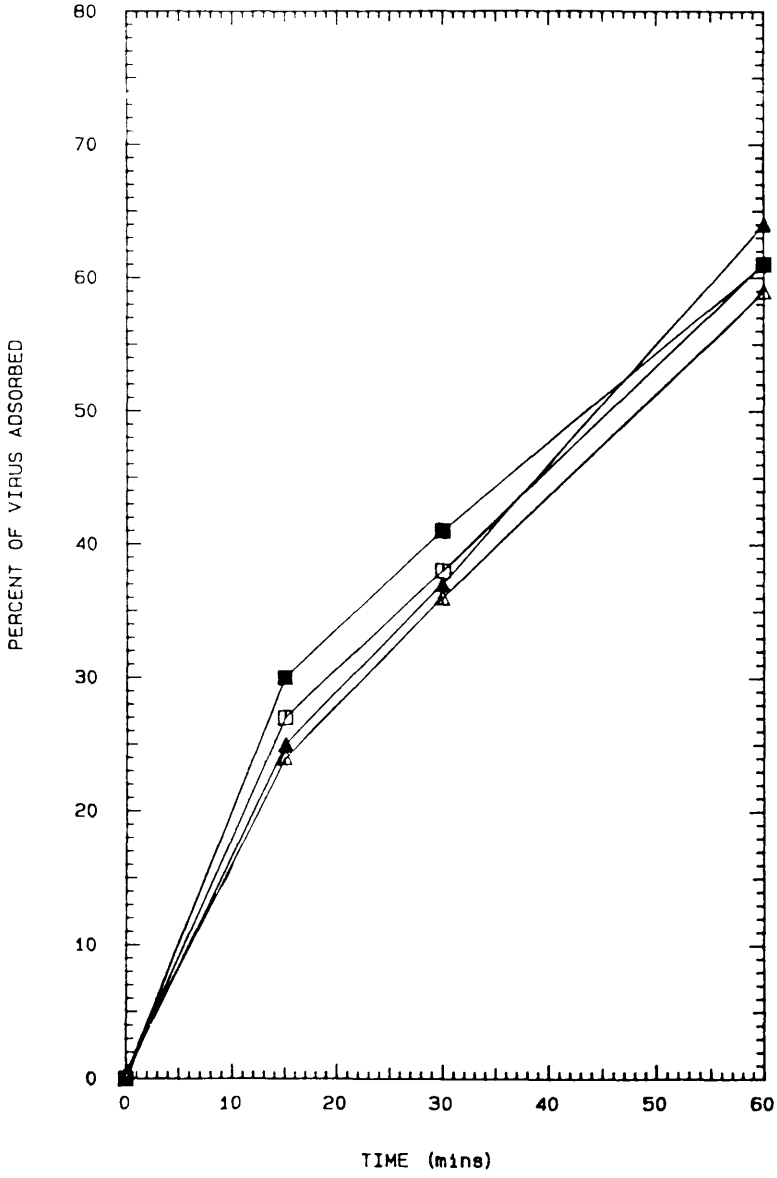
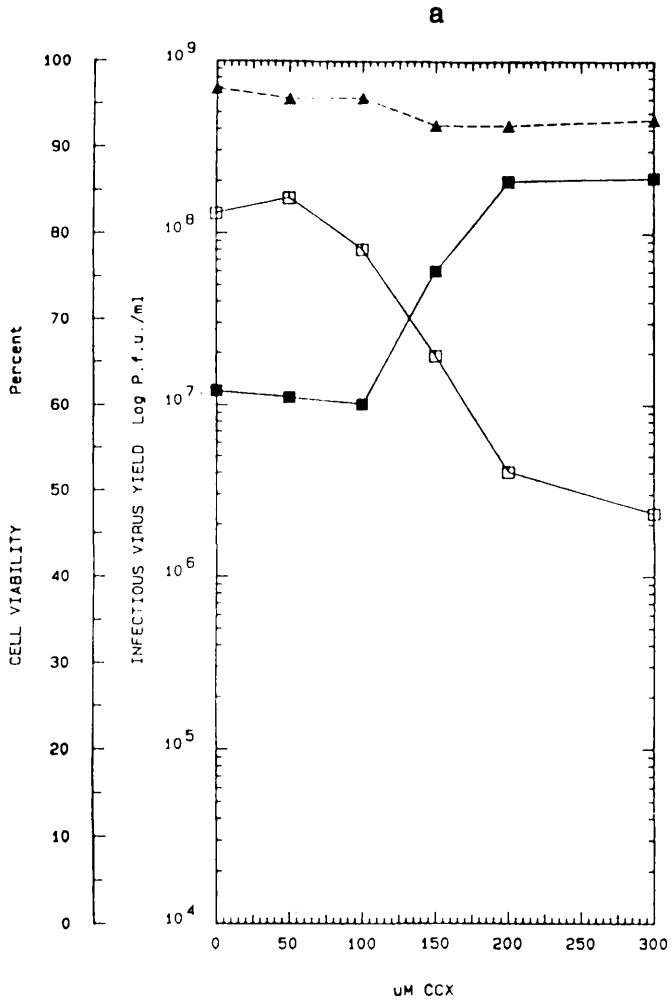

80
70
60
50
40
30
20
10
0
PERCENT OF VIRUS ADSORBED
0
10
20
30
40
50
60
TIME (mins)

Figure 43

Adsorption of Semliki Forest virus particles to BS-C-1 cells pre-treated with 0 (■), 300uM CCX (□), 600uM CCX (▲) or 900uM CCX (△).


a
CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD Log P.f.u./ml
uM CCX
0
50
100
150
200
250
300

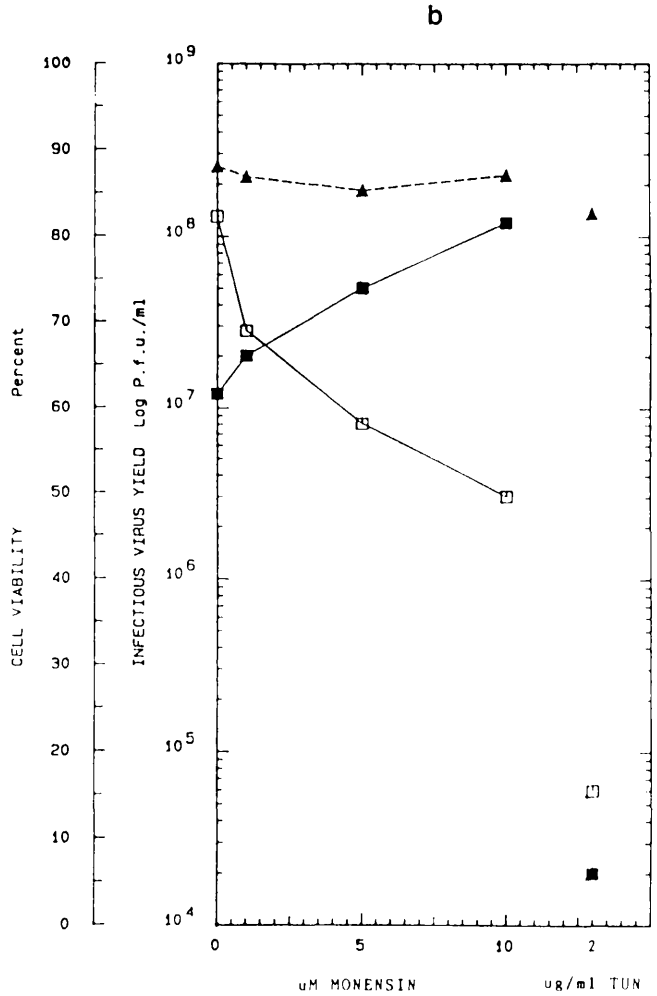

b
CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD Log P.f.u./ml
uM MONENSIN
ug/ml TUN
0
5
10
2

Figure 44

The effect of CCX (a), monensin or tunicamycin (b)on infectious yields from SFV-infected BS-C-1 cells.

The m.o.i. was 5 p.f.u./cell and virus yields were harvested at 24h p.i. and divided into cell-associated (■) and cell-released (□) fractions.

Viability of uninfected BS-C-1 cells from control cultures treated in parallel with CCX, monensin or tunicamycin (▲).

3.4.4. THE EFFECT OF CCX, MONENSIN AND TUNICAMYCIN ON POLYPEPTIDES INDUCED IN MOCK-INFECTED AND SFV-INFECTED BS-C-1 CELLS

MOCK-INFECTED CELL EXTRACTS (Fig 46a)

There was no significant effect on host cell protein synthesis, with CCX, monensin or tunicamycin. Glucose-related stress proteins, grp 74 and 78 ~~and a~~ high m.wt. band (120), and a low protein were induced in tunicamycin treated BS-C-1 cells (Fig 46a, lane 6). No stress response was induced in either CCX treated (Fig 46a, lanes 2-4), or monensin treated (Fig 46a, lane 5) BS-C-1 cells.

SFV-INFECTED CELL EXTRACTS (Fig 46b)

Only structural SFV proteins were detected. This may be due to the short half life of the non-structural proteins (Keranen and Ruohonen, 1983). Levels of glycoproteins p62, E1 (possibly E2; difficult to separate E1 and E2 as they are so close in m.wt.), increased with increasing CCX concentrations and also in the presence of monensin (Fig 25b, lanes 1-5). In both CCX and monensin treated tracks, the intensity of the capsid (C) was decreased. A high m.wt. band also accumulates in CCX, monensin and tunicamycin tracks. This band may be the glucose-related stress protein grp 94 (induced in mock-infected tunicamycin tracks). However, if this is so, it is difficult to explain the absence of this band in the mock-infected CCX and monensin treated tracks.

Levels of all viral bands were increased in tunicamycin tracks. As expected, due to the inhibition of sugar addition, there was a marked downward shift in glycoprotein bands p62, and E1 (and possibly E2).

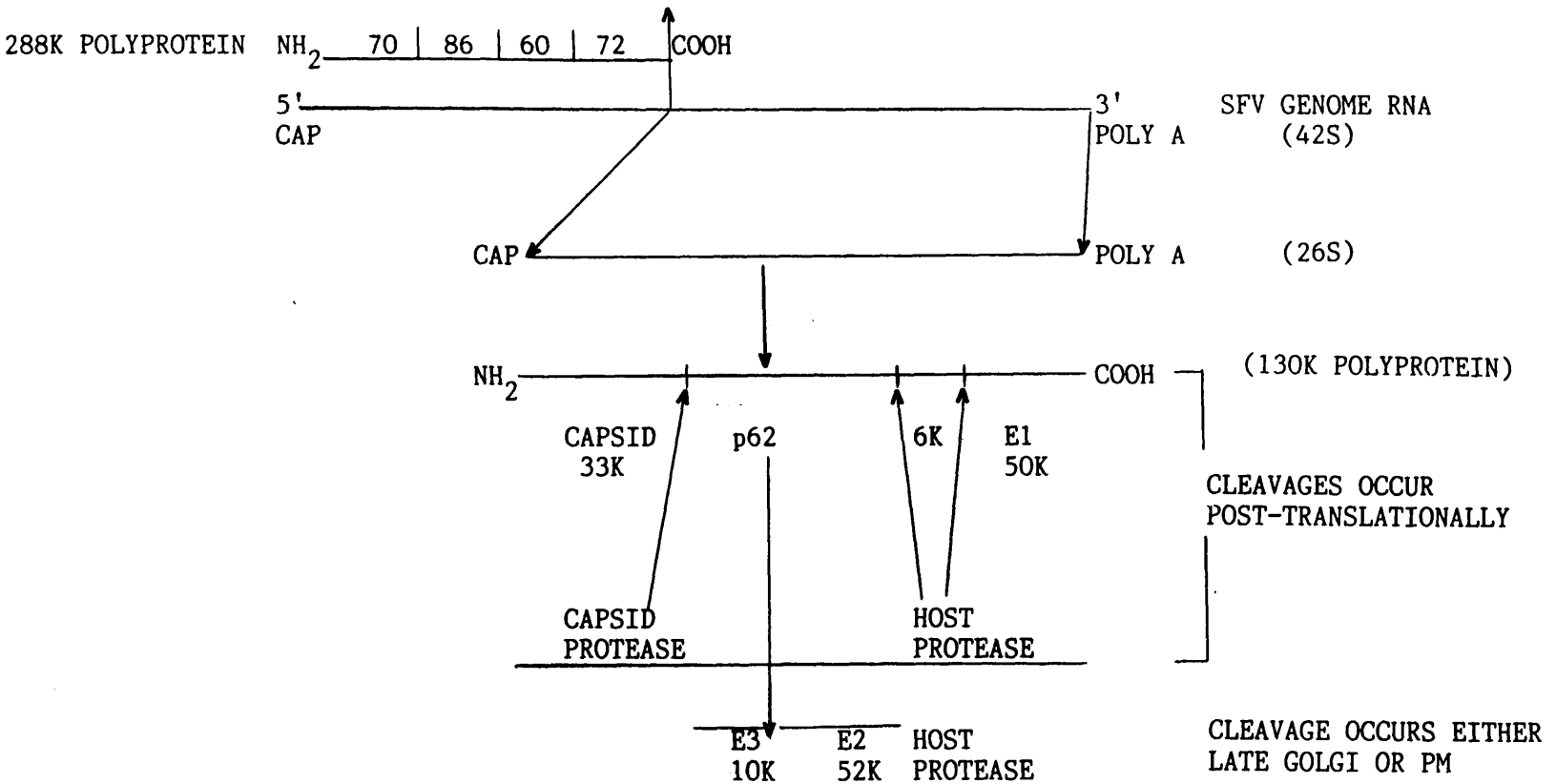

288K POLYPROTEIN
NH2
70
86
60
72
COOH
5'
CAP
3'
POLY A
SFV GENOME RNA
(42S)
CAP
POLY A
(26S)
NH2
COOH
(130K POLYPROTEIN)
CAPSID
33K
p62
6K
E1
50K
CLEAVAGES OCCUR
POST-TRANSLATIONALLY
CAPSID
PROTEASE
HOST
PROTEASE
E3
10K
E2
52K
HOST
PROTEASE
CLEAVAGE OCCURS EITHER
LATE GOLGI OR PM

Figure 45
Semliki Forest Virus: Proteins

The genome of SFV (and all other alphaviruses) is organized into 2 distinct regions. The 5' two thirds of the genome encodes the non-structural proteins, which are translated as a polyprotein from a messenger that is indistinguishable from the virion RNA.These precursors are then cleaved post-translationally to produce ultimately 4 products, non-structural proteins, nspl (70K), nsp2 (86K), nsp3 (60K) and nsp4 (72K) (Lachmi and Kaariarnen, 1976; Keranen and Ruohonen, 1983). These proteins function as the replicase/transcriptase for the virus (Clewley and Kennedy, 1976; Ranki and Kaariainen, 1979; Keranen and Kaarianinen, 1979; Gomatos et al., 1980).

The 3' third of the genome that encodes the structural proteins i.e. the polypeptide components of the mature virion (Garoff et al., 1980a,b; Rice and Strauss, 198la). Semliki Forest virus (SFV), consists of a nucleocapsid containing the single-stranded virus RNA complexed with a basic protein, surrounded by a lipid bilayer, containing 2 integral glycosylated membrane proteins (E2 and El) and a small peripheral protein (Simons and Garoff, 1980). The translation of the structural proteins is initiated at a single site on a subgenomic 26S mRNA. Three co-translational cleavage events release the capsid (33K), p62 (a precursor of E3 [10K]) and E2 [51K] proteins), a non-structural 6K protein and finally the El protein (50K) from a polyprotein (130).

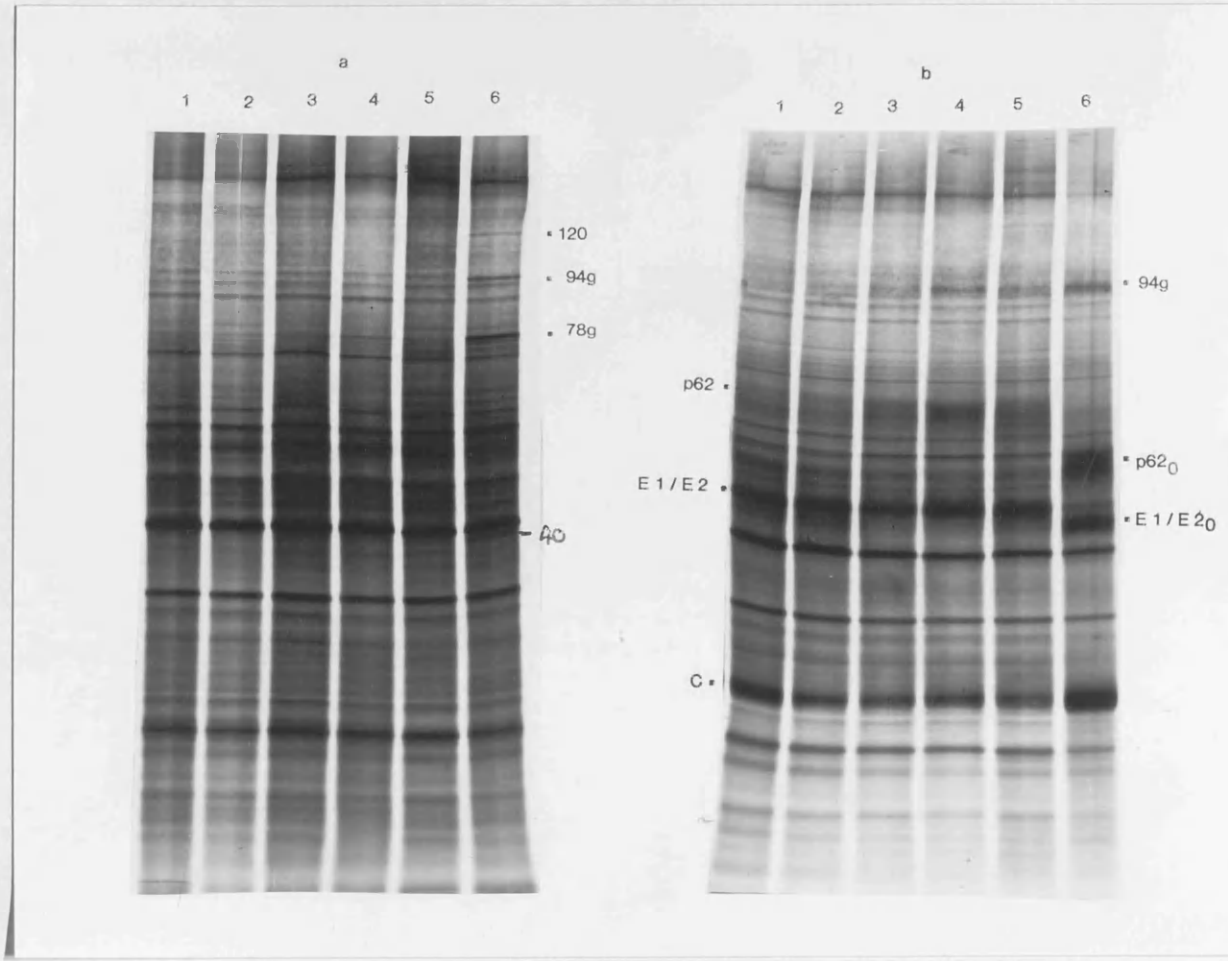

a
1
2
3
4
5
6
120
94g
78g
40
b
1
2
3
4
5
6
94g
p62
E1/E2
C
p62o
E1/E2o

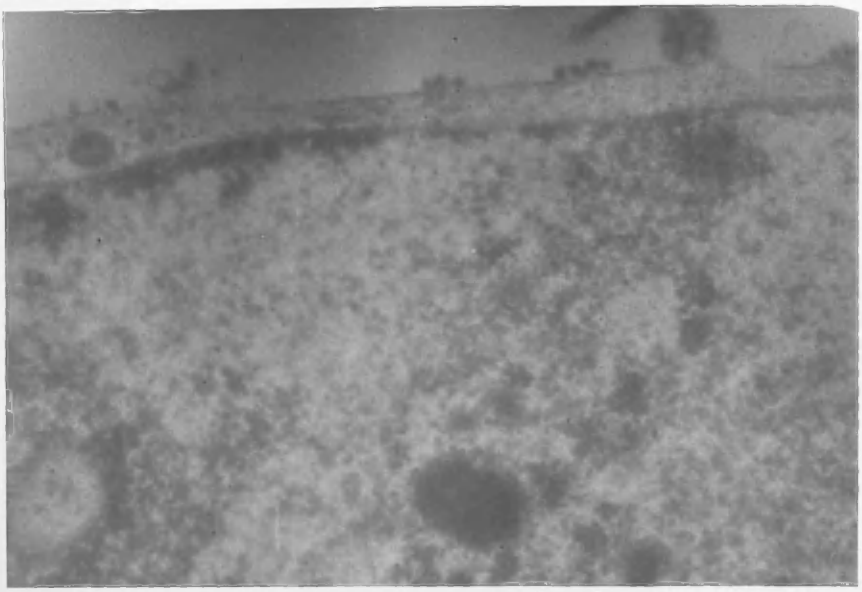
Figure 46

SDS PAGE profile on a 10% gel of the infected cell polypeptides induced in BS-C-1 cells treated with 0,100,200 or 300uM CCX (lanes 1-4), 10uM monensin (lane 5) or 2ug/ml tunicamycin (lane 6) from 1-24h p.i.

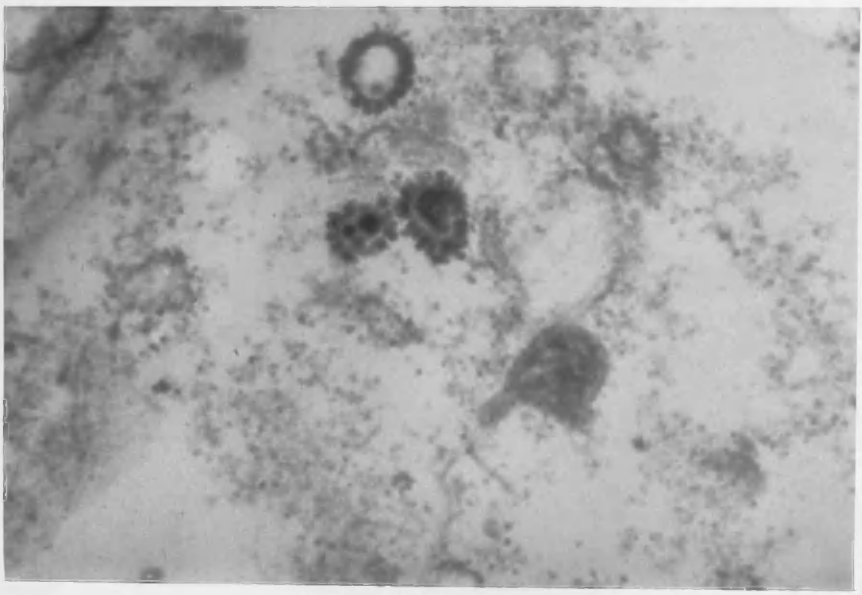
Cells were either mock-infected (a) or infected with SFV at a m.o.i. of 50 p.f.u./cell (b).
^{35}Smet was added from 1-24h p.i. in the presence of ACT. D. (5ug/ml).
Equal volumes of sample were loaded onto each track.

3.4.5. EM ANALYSIS OF MONENSIN AND CCX TREATED SFV-INFECTED BS-C-1 CELLS

In the absence of any drug, SFV was observed to bud exclusively from the PM of BS-C-1 cells (Fig 47a). In CCX treated cells, SFV nucleocapsids were found associated with intracellular vacuoles (Fig 47b). Closer examination of these vacuoles (Fig 47c), revealed that nucleocapsids had in fact budded into the vacuoles. Intravacuole budding was also observed in monensin treated SFV infected BS-C-1 cells, although aggregates of nucleocapsids were also found at the PM (Fig 47d). Budding of SFV into intracellular vacuoles, has previously been reported in a variety of monensin treated SFV-infected cells, including BHK and CEF (Griffiths, Quinn and Warren, 1983; Pesonen and Kaariainen, 1982). In these cases, staining with specific cytochemical markers and lectins, revealed that these vacuoles were Golgi derived. Given the similarities observed between monensin and CCX, it seems likely that the vacuoles observed in CCX treated cells are also derived from Golgi membrane.



a

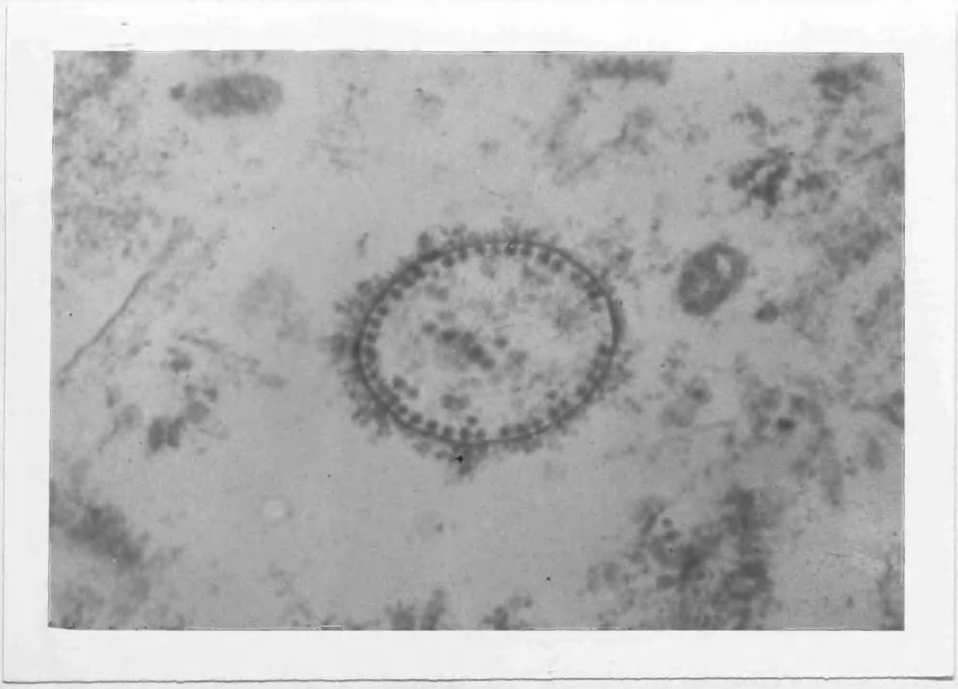


b

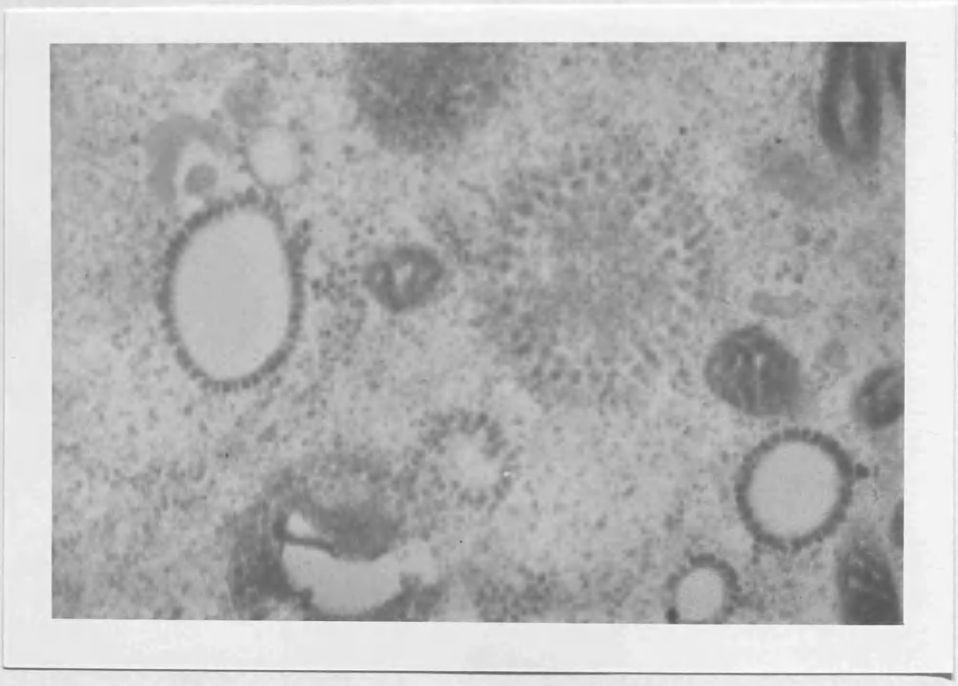
Figure 47 (a and b)

Monolayers of BS-C-1 cells (4 x 10^6 cells/50mm plate), were infected with SFV at m.o.i. of 50 p.f.u./cell. Following virus adsorption for 1h at 37°C, infected monolayers were washed 3 times with PBS and overlaid with drug-free medium (a) and EFC_2 containing 300uM CCX (b).

At 24h p.i. infected cell monolayers were embedded in epon and thin sections examined by transmission EM. (as described in 2.2.6.2.)



c



d

Figure 47 (c and d)

Monolayers of BS-C-1 cells (4×10^6 cells/50mm plate), were infected with SFV at m.o.i. of 50 p.f.u./cell. Following virus adsorption for 1h at 37°C, infected monolayers were washed 3 times with PBS and overlaid with EFC2 containing 300uM CCX (c) and 10uM monensin (d).

At 24h p.i. infected cell monolayers were embedded in epon and thin sections examined by transmission EM. (as described in 2.2.6.2.)

3.5. THE EFFECT OF CCX, MONENSIN AND TUNICAMYCIN ON CLASS 2 VIRUSES

3.5.1. THE EFFECT OF CCX, MONENSIN AND TUNICAMYCIN ON INFECTIOUS YIELDS FROM GERMISTON AND BUNYAMWERA VIRUS INFECTED BS-C-1 CELLS

The effect of CCX, monensin and tunicamycin on infectious virus yields from both Germiston and Bunyamwera-infected BS-C-1 cells was determined (Fig 48). Mock-infected BS-C-1 cells were treated in parallel with drug and cell viabilty determined by trypan blue dye exclusion at the time of harvest (24h). There was no significant effect on cell viability with CCX, monensin or tunicamycin (viabilities remaining above 80%), allowing uncoupling of antiviral and cytotoxic effects. Virus yields were separated into cell-associated (CA) and cell-released (CR) fractions, revealing no inhibition of virus release from the cell in the presence of any drug. In fact, decreased CA/CR ratios indicate a slight increase in virus release.

Infectious yields of both fractions were progressively reduced with increasing monensin concentrations achieving a 100 fold reduction with 10uM monensin (Fig 48b). Treatment with tunicamycin (to 2ug/ml) (Fig 48b) also reduced both fractions by 100 fold. This reveals that processing of glycoprotein in the Golgi is important for virus infectivity, disruption of this step reducing infectivity by the same magnitude as that achieved by completely blocking sugar addition.

Treatment with 50uM CCX had little significant effect on virus infectivity in either fraction (Fig 48a).This was followed by a progressive reduction in both fractions with increasing concentrations of CCX to 200uM, with little further effect at 300uM, achieving a 100 fold reduction in infectivity (Fig 48a). This reduction is the same as that obtained with monensin, therefore, disruption of sugar processing in the Golgi could totally account for the anti-Bunyamwera and anti-Germiston effect of CCX.

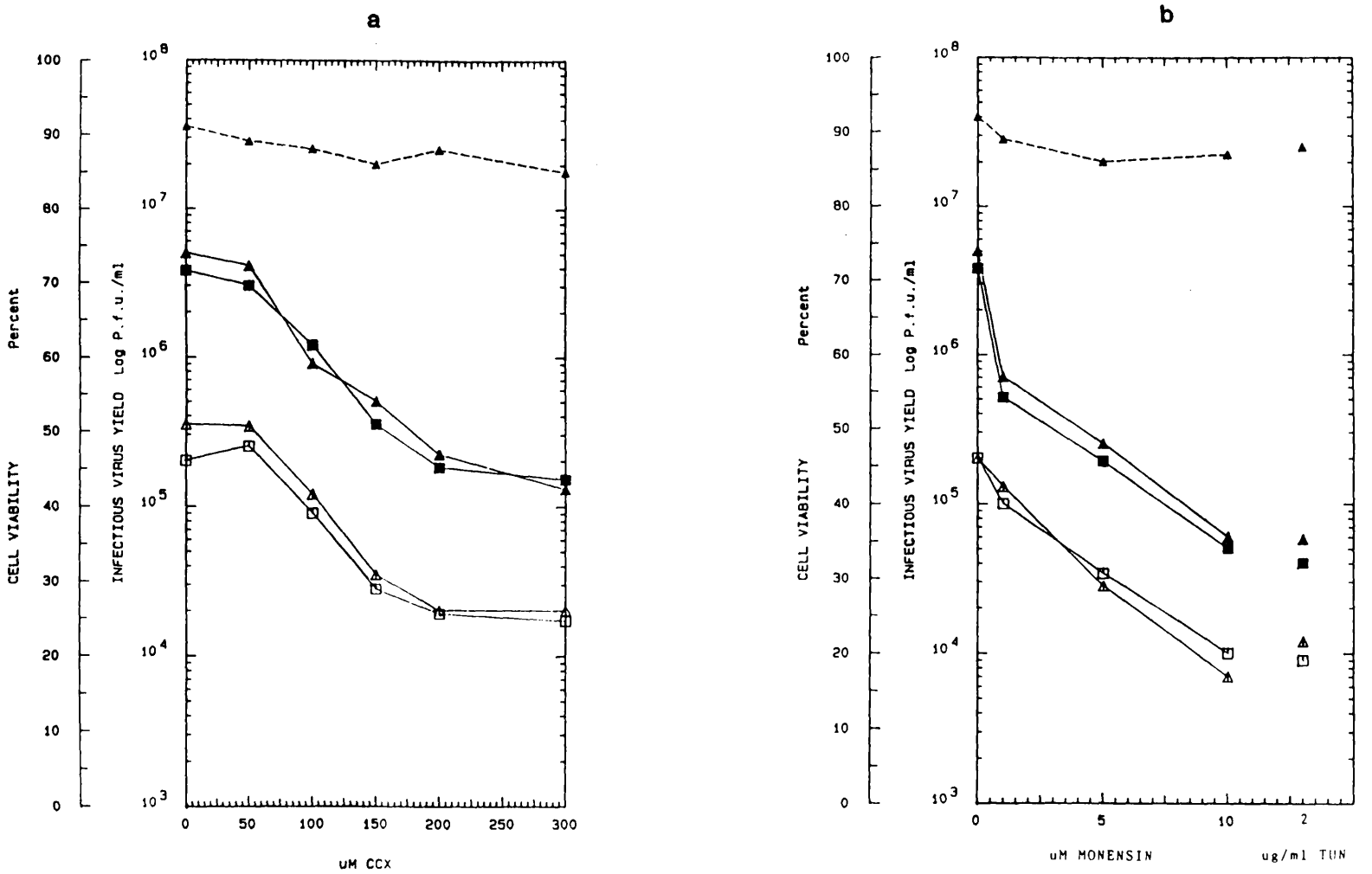

a
b
CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD Log P.f.u./ml
uM CCX
uM MONENSIN
ug/ml TUN

Figure 48

The effect of CCX (a), monensin or tunicamycin (b) on infectious yields from Germiston (▲ △) and Bunyamwera (■ □) infected BS-C-1 cells.

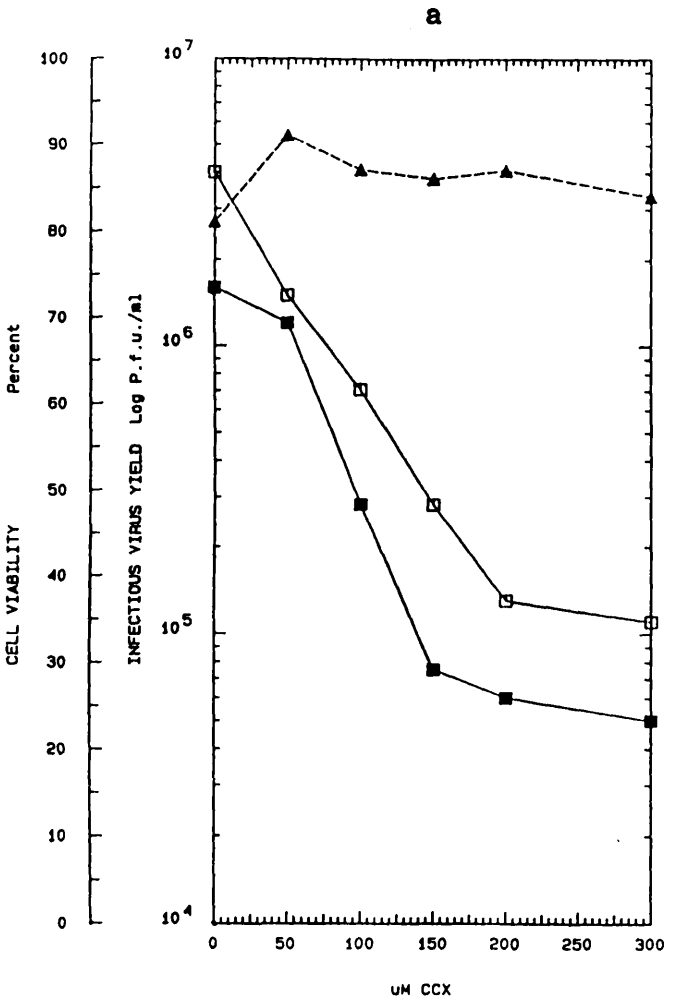
The m.o.i. was 5 p.f.u./cell and virus yields were harvested at 24h p.i. and divided into cell-associated (CA) (▲ ■) and cell-released (CR) (△ □) fractions.

Viability of uninfected BS-C-1 cells from control cultures treated in parallel with CCX, monensin or tunicamycin (—△—).

3.5.2. THE EFFECT OF CCX, MONENSIN AND TUNICAMYCIN ON INFECTIOUS VIRUS YIELDS FROM POLIOVIRUS-1 INFECTED BS-C-1 CELLS

The effect of CCX, monensin and tunicamycin on infectious yields from Poliovirus infected BS-C-1 cells was determined (Fig 49). Mock-infected controls show that cell viability remained high at all drug concentrations. Yields were divided into cell-associated (CA) and cell-released (CR) fractions: CA/CR ratios were not significantly altered by CCX, monensin or tunicamycin indicating no effect on virus release from the cell.

There was a 30 fold reduction in virus infectivity, with CCX in both the cell-associated and cell-released fractions (Fig 49a). The reduction was progressive with increasing concentrations of CCX to 150uM CCX, with little further effect at 200 and 300uM CCX. In contrast, neither monensin or tunicamycin had any effect on virus infectivity (Fig 49b). This is the expected result as poliovirus contains no glycoprotein. Therefore, disruption of glycoprotein processing can play no role in the anti-Poliovirus effect of CCX. Poliovirus infectivity may be reduced by inhibition of protein synthesis and/or binding of CCX directly to virus particles, perhaps resulting in direct inactivation


a
CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD Log P.f.u./ml
uM CCX
0
50
100
150
200
250
300

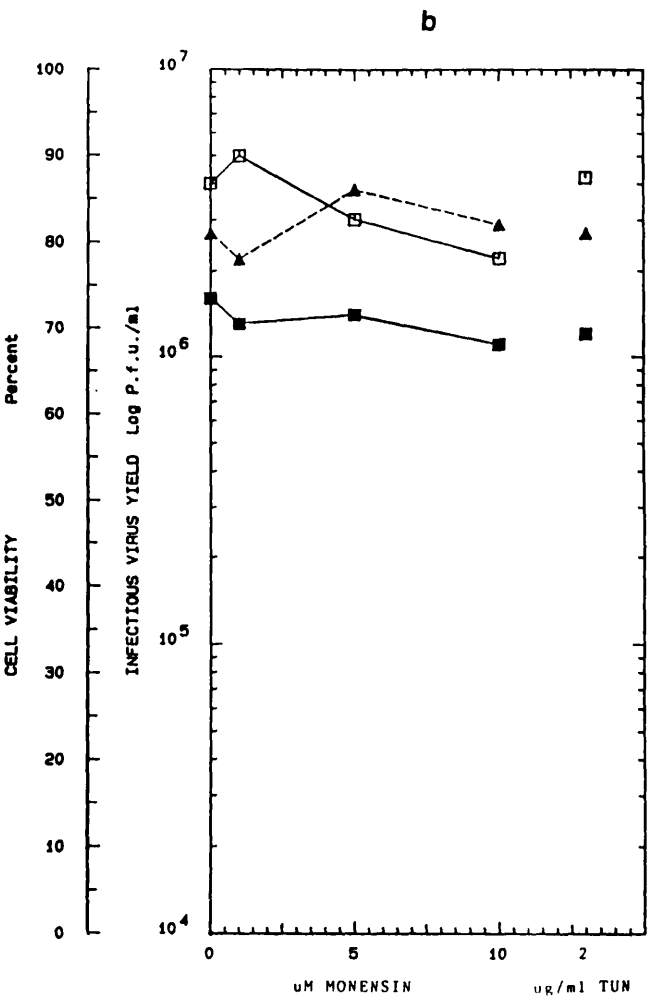

b
CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD Log P.f.u./ml
uM MONENSIN
ug/ml TUN
0
5
10
2

Figure 49

The effect of CCX (a), monensin or tunicamycin (b) on infectious yields from Poliovirus-1 infected BS-C-1 cells.

The m.o.i. was 5 p.f.u./cell and virus yields were harvested and divided into cell-associated (CA) (■) and cell-released (CR) (□) fractions.

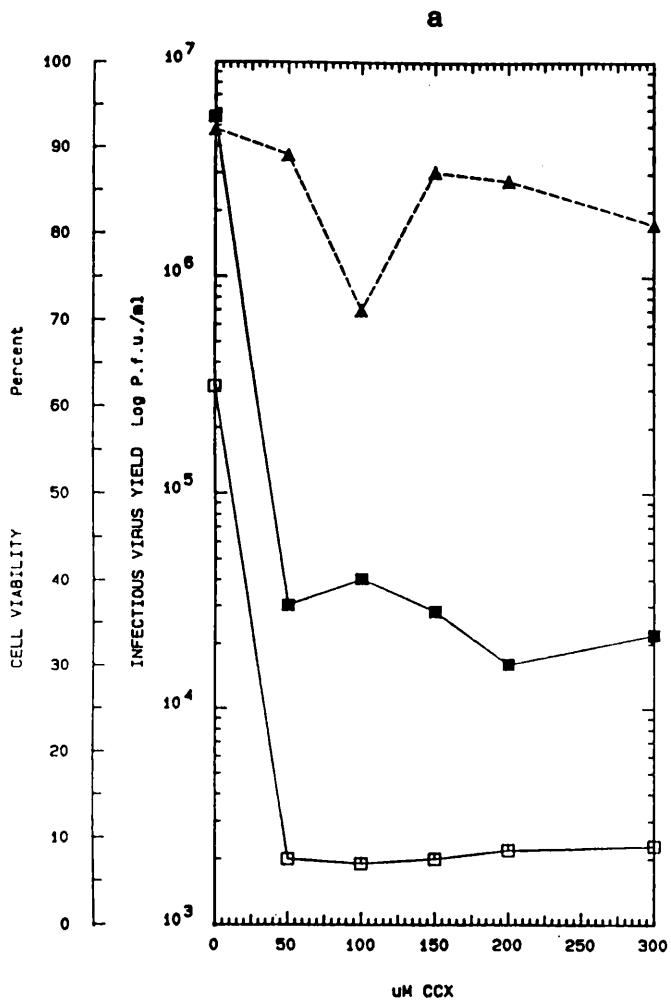
Viability of uninfected BS-C-1 cells from control cultures treated in parallel with CCX, monensin or tunicamycin (▲).

3.5.3. THE EFFECT OF CCX, MONENSIN AND TUNICAMYCIN ON INFECTIOUS YIELDS FROM ADENOVIRUS-5 INFECTED HELA CELLS

The effect of CCX, monensin and tunicamycin on infectious yields from Adenovirus infected cells was determined (Fig 50a). Mock-infected controls show that viability was not significantly affected at any drug concentration. Virus yields were divided into cell associated (CA) and cell-released (CR) fractions revealing no effect on virus release from the cell.

The CCX dose-response curve obtained for Adeno-5, was as previously observed (Fig 50a). Cell-associated and cell-released virus yields were reduced 150 fold with only 50uM CCX, with little further effect at higher CCX concentrations.

In contrast, neither monensin or tunicamycin had any effect on Adenovirus infectivity (Fig 50b). Unlike Poliovirus-1, Adenovirus-5 does contain glycoproteins. However, these results suggest that neither the addition of core sugar or subsequent processing in the Golgi are important for Adenovirus infectivity. Therefore, disruption of glycosylation can play no role in the anti-Adenovirus effect of CCX. The effect of CCX on Adenovirus replication was investigated further and will be discussed more fully later.


a
CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD Log P.f.u./ml
uM CCX

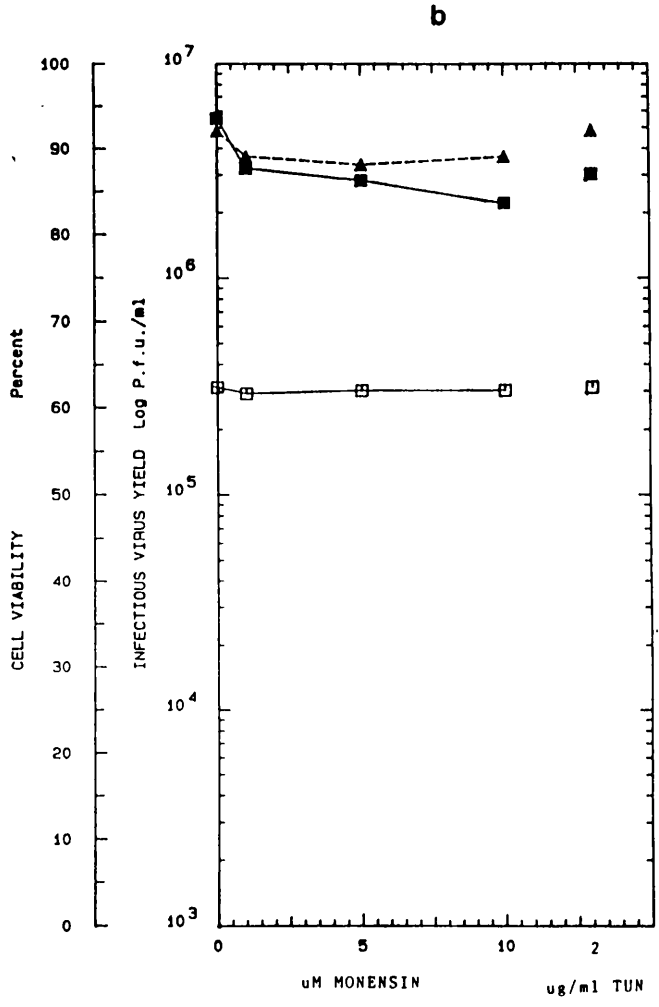

b
CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD Log P.f.u./ml
uM MONENSIN
ug/ml TUN

Figure 50

The effect of CCX (a), monensin or tunicamycin (b) on infectious virus yields from Adenovirus-5 infected HeLa cells.

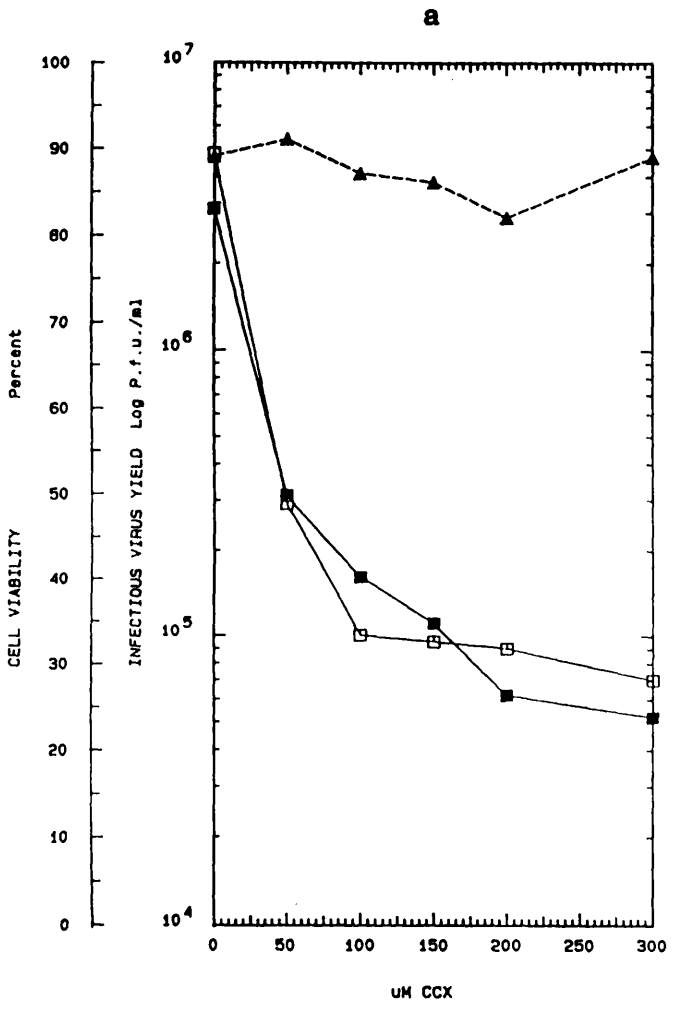
The m.o.i. was 5 p.f.u./cell and virus yields were harvested at 48h p.i. and divided into cell-associated (CA) (■) and cell-released (CR) (□) fractions.

Viability of uninfected HeLa cells from control cultures treated in parallel with CCX, monensin or tunicamycin (▲).

3.5.4. THE EFFECT OF CCX, MONENSIN AND TUNICAMYCIN ON INFECTIOUS YIELDS FROM REOVIRUS-3 INFECTED BS-C-1 CELLS

The effect of CCX, monensin and tunicamycin on infectious yields from Reovirus-3 infected BS-C-1 cells was determined (Fig 51). Mock-infected controls show that cell viability remained high at all drug concentrations. Virus yields were separated into cell associated (CA) and cell released (CR). CA/CR ratios were slightly increased in the presence of CCX, monensin and tunicamycin indicating some block in virus release.

CCX reduced cell-associated and cell-released Reovirus-3 infectious yields 100 fold (Fig 51a). Reovirus-3 was very sensitive to low concentrations of CCX, the curve reaching a plateau between 100 and 200uM CCX. In contrast, infectious yields were not significantly affected by either monensin or tunicamycin treatment (Fig 51b). Therefore, although Reovirus-3 contains glycoproteins, disruption of glycosylation does not affect virus infectivity and the reduction in infectious virus yields obtained with CCX cannot therefore be due to inhibition of sugar addition or transport. The effect of CCX on Reovirus-3 replication was investigated further and will be discussed more fully in the next section.


a
CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD Log P.f.u./ml
uM CCX
0
50
100
150
200
250
300
10
20
30
40
50
60
70
80
90
100
10^4
10^5
10^6
10^7

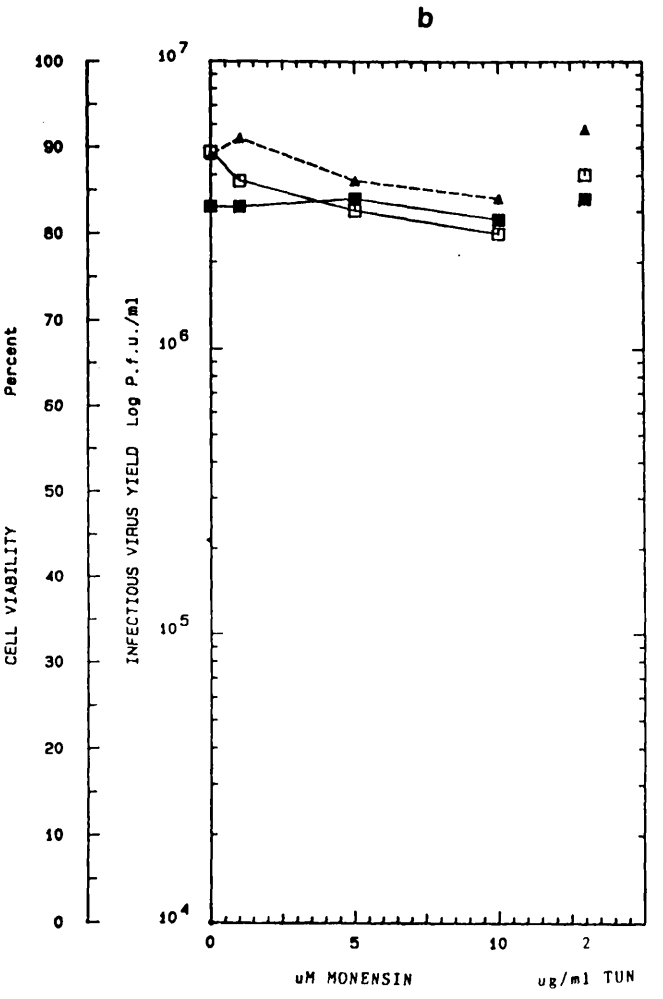

b
CELL VIABILITY
Percent
INFECTIOUS VIRUS YIELD Log P.f.u./ml
uM MONENSIN
ug/ml TUN
0
5
10
2
10
20
30
40
50
60
70
80
90
100
10^4
10^5
10^6
10^7

Figure 51

The effect of CCX (a), monensin or tunicamycin (b) on infectious yields from Reovirus-3 infected BS-C-1 cells.

The m.o.i. was 5 p.f.u./cell and virus yields were harvested at 24h p.i. and divided into cell-associated (CA) (■) and cell-released (CR) (□) fractions.

Viability of uninfected BS-C-1 cells from control cultures treated in parallel with CCX, monensin or tunicamycin (▲).

3.6. THE EFFECT OF CCX ON THE REPLICATION OF REOVIRUS-3; REPRESENTING THE CLASS 2 RESPONSE

3.6.1. ANALYSIS OF REOVIRUS-3 PRODUCED IN CCX TREATED BS-C-1 CELLS

THERMOSTABILITY OF REOVIRUS-3 PROGENY VIRUS PRODUCED IN BS-C-1 CELLS TREATED WITH CCX

Although the CCX dose-response curve obtained for Reovirus-3, plateaued at higher CCX concentrations, indicating no further effect on virus infectivity, it was important to determine the quality of progeny virus produced in the presence of CCX. The thermostability of infectious virus produced from BS-C-1 cells treated with 0, 50, 150 or 300uM CCX was determined by incubating the virus at 42^{o}C for various times and then immediately titrated. The curves obtained (Fig 52), suggests no evidence of difference in the thermostability of virus produced in the presence and absence of CCX.

ANALYSIS OF REOVIRUS-3 SYNTHESISED IN BS-C-1 CELLS : SUSCEPTIBILITY TO REPEATED TREATMENT WITH CCX (DOSE-RESPONSE CURVES)

Reovirus-3 was very sensitive to low concentrations of CCX, with the dose-response curve reaching a plateau between 100 and 200uM CCX. To determine if the plateau resulted from the selection of a sub-population of resistant virus, virus produced in CCX treated and drug-free cells, was used in repeat dose-response experiments. The resulting curves (Fig 53), indicate that this was not the case, with virus produced in CCX treated cells responding to further CCX treatment in the same way as virus produced in drug free cells.

3.6.2. ADSORPTION OF REOVIRUS-3 TO BS-C-1 CELLS TREATED WITH VARYING CONCENTRATIONS OF CCX

Adsorption experiments (as described in 2.2.5.6.) were performed with Reovirus-3 and BS-C-1 cells pre-treated with varying concentrations of CCX (Fig 54).There was only a slight effect on virus adsorption (reduction of about 10% for all three tested concentrations of CCX), with Reovirus-3 still able to bind to cell receptors at concentrations as

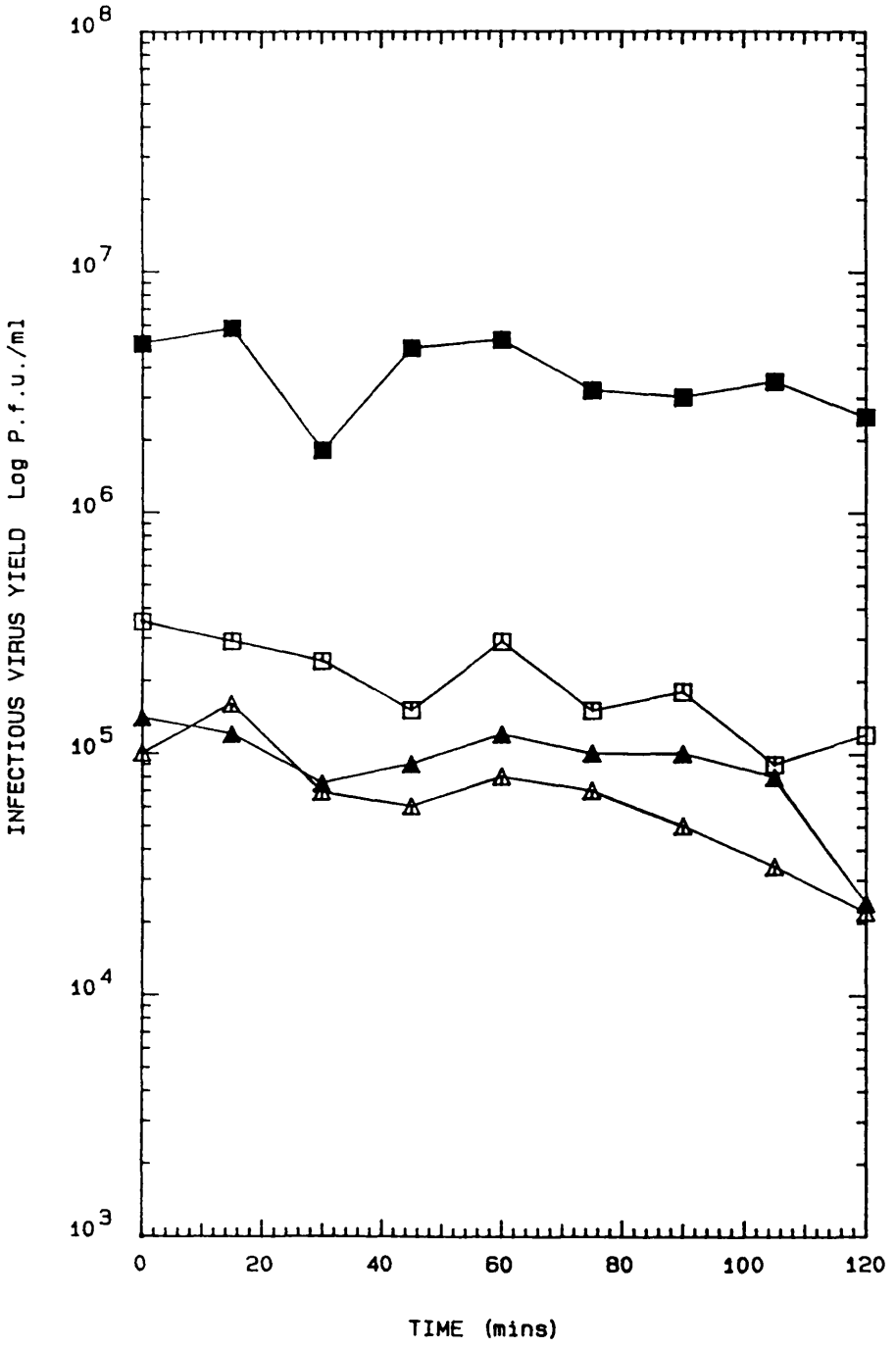

INFECTIOUS VIRUS YIELD Log P.f.u./ml
10^8
10^7
10^6
10^5
10^4
10^3
0
20
40
60
80
100
120
TIME (mins)

Figure 52

Thermostability of Reovirus-3 progeny virus made in BS-C-1 cells treated with CCX : 0 (■), 50uM CCX (□), 150uM CCX (▲), 300uM CCX (△).

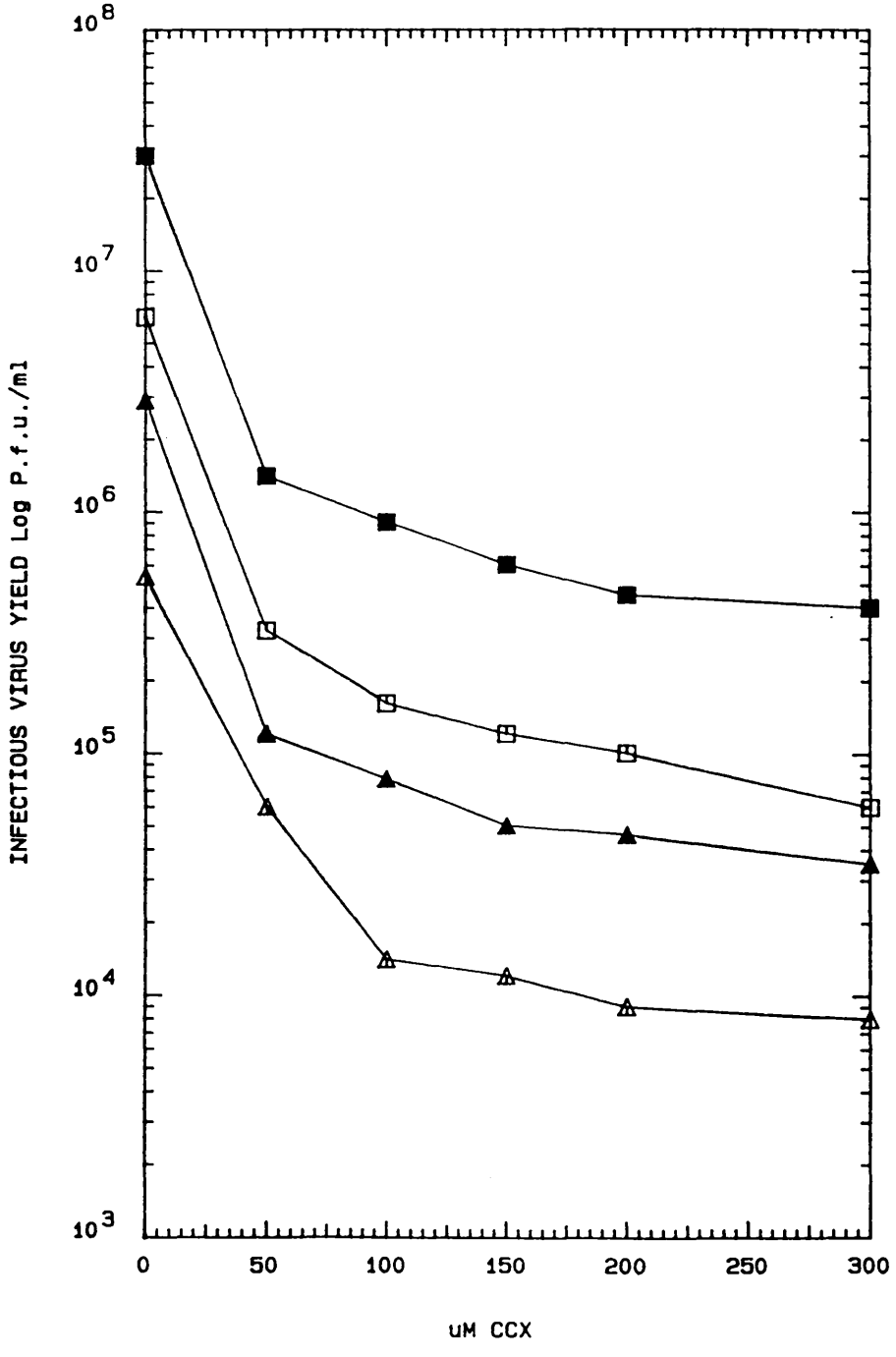

INFECTIOUS VIRUS YIELD Log P.f.u./ml
10^8
10^7
10^6
10^5
10^4
10^3
0
50
100
150
200
250
300
uM CCX

Figure 53

The effect of increasing concentrations of CCX on infectious virus yields from BS-C-1 cells infected with

(■) virus produced from drug-free cells
(□) virus produced in cells maintained in 50uM CCX
(▲) virus produced in cells maintained in 150uM CCX
(△) virus produced in cells maintained in 300uM CCX.

Dose-response curves have been transposed down by 0.5 log (□), 1 log (▲) or 1.5 log (△), to allow clear presentation.

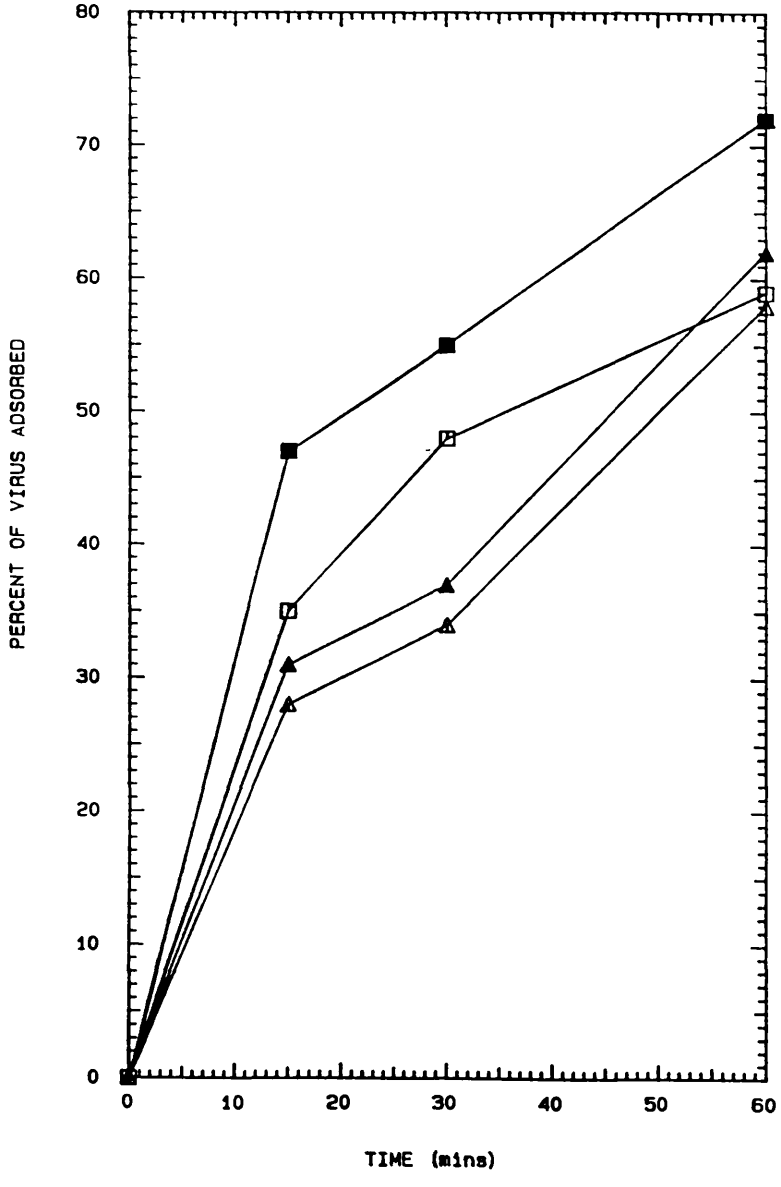

80
70
60
50
40
30
20
10
0
PERCENT OF VIRUS ADSORBED
0
10
20
30
40
50
60
TIME (mins)

Figure 54

Adsorption of Reovirus-3 particles to BS-C-1 cells pre-treated for 1h at 37°C with 0 (■), 300uM CCX (□), 600uM CCX (▲) or 900uM CCX (△).

high as 900uM. It is therefore unlikely that inhibition of adsorption of virus to pre-treated cells would play a significant role in limiting the spread of infection at least in vitro.

3.6.3. THE EFFECT OF INCREASING CONCENTRATIONS OF CCX ON PARTICLE NUMBERS, INFECTIOUS VIRUS YIELDS (P.F.U.) AND PARTICLE/P.F.U. RATIOS

Virus particle counts were performed by electron microscopy (2.2.6.1.) on total virus yields from dose-response experiments. Table 38 shows the results obtained from two independant experiments. The data show that both particle numbers and infectious virus yields were progressively reduced with increasing concentrations of CCX. However, at 10 and 50uM CCX, the reduction in infectivity was consistently greater than the reduction in particle numbers, resulting in elevated particle/p.f.u. ratios. Surprisingly, at 150 and 300uM CCX, the particle/p.f.u. ratios were similar to those produced under drug free conditions. This suggests that at low CCX concentrations there was some block in virus assembly accompanied by a lowering in the quality of at least some of the virus which was produced. At higher CCX concentrations, the reduction in infectivity and particle numbers were similar, with progeny virus produced in the presence of CCX being as infectious as virus produced from drug free infected cells. This suggests that the lower quality particles could no longer be assembled. Therefore, the anti-Reovirus-3 effect of CCX was essentially of two types: a) reduction in the number of particles produced b) production of low quality particles only at low (up to 50uM CCX) concentrations of CCX.

3.6.4. DIRECT INACTIVATION OF REOVIRUS-3 PARTICLES

To determine whether CCX directly inactivated Reovirus particles, suspensions of virus were incubated with either mock drug or 300uM CCX (2.2.5.7) at $4^{o}C$ or $37^{o}C$ for 24h and infectious yields titrated before and after drug addition. The experiment was performed twice and the results are shown in Table 39. There was no significant reduction in virus titre in either experiment suggesting that direct inactivation of particles does not play a major part in the

CCX (uM)	PARTICLE NUMBER	P.F.U.	PARTICLE/P.F.U. RATIO
0	$7.15*10^{10}$	$3.4*10^{8}$	210
10	$6.44*10^{10}$	$1.4*10^{8}$	495
(1) 50	$2.57*10^{10}$	$1.8*10^{7}$	1,427
150	$1.43*10^{9}$	$8.0*10^{6}$	178
300	$1.43*10^{9}$	$6.0*10^{6}$	238
0	$5.6*10^{10}$	$2.23*10^{8}$	251
10	$2.9*10^{10}$	$8.0*10^{7}$	362.5
(2) 50	$1.4*10^{10}$	$1.3*10^{7}$	1,076
150	$2.8*10^{9}$	$9.0*10^{6}$	311
300	$1.4*10^{9}$	$7.0*10^{6}$	200

Table 38

The effect of increasing concentrations of CCX on Reovirus particle numbers, infectious virus yields (p.f.u.) and particle/p.f.u. ratios.

1 and 2 represent data from 2 independent experiments.

a

TIME(hr)	TEMP (C)	DRUG	TITRE	%AGE	REDUCTION
0	-	ORIGINAL STOCK	$4.1*10^6$		
24	4	MOCK DRUG	$3.7*10^6$	100	21.7
24	4	300 uM CCX	$2.9*10^6$	78.3	
24	37	MOCK DRUG	$5.8*10^6$	100	0
24	37	300 uM CCX	$5.8*10^6$	100	

b

TIME(hr)	TEMP (C)	DRUG	TITRE	%AGE	REDUCTION
0	-	ORIGINAL STOCK	$6.0*10^6$		
24	4	MOCK DRUG	$5.2*10^6$	100	15.4
24	4	300 uM CCX	$4.4*10^6$	84.6	
24	37	MOCK DRUG	$5.7*10^6$	100	7.9
24	37	300 uM CCX	$5.25*10^6$	92.1	

Table 39

Direct inactivation of Reovirus particles by CCX.

a) and b) represent data from 2 independent experiments.

anti-Reovirus-3 action of CCX.

3.6.5. THE EFFECT OF CCX ON POLYPEPTIDE SYNTHESIS IN MOCK-INFECTED AND REOVIRUS-3 INFECTED BS-C-1 CELLS

MOCK-INFECTED CELL EXTRACTS (Fig 55a)

Host cell protein synthesis was not affected by CCX treatment in this experiment. In addition, no stress response was induced in CCX treated mock-infected BS-C-1 cells.

REOVIRUS-3 INFECTED CELL EXTRACTS (Fig 55b)

Treatment with increasing concentrations of CCX resulted in a progressive decrease in all identified viral bands. Several low m.wt. bands appeared in virus-infected but not mock-infected tracks; these were designated UK_1, UK_2, UK_3, UK_4 and UK_5. Protein bands UK_1, UK_2 and UK_3 increased with increasing CCX, levels of band UK_4 remained constant and levels of band UK_5 decreased.

3.6.6. THE EFFECT OF CCX ON THE PHOSPHORYLATION OF POLYPEPTIDES INDUCED IN REOVIRUS-3 INFECTED BS-C-1 CELLS

Dargan and Subak-Sharpe (1986a and b), reported that phosphorylation was not significantly affected in either mock-infected or HSV-1 and HSV-2 infected Flow 2002 and BHK-21 cells. In CCX treated, Reovirus-3 infected BS-C-1 cells, there was no demonstrable reduction in the phosphorylation of protein bands. If anything, phosphorylation of some bands actually appeared to be slightly increased.

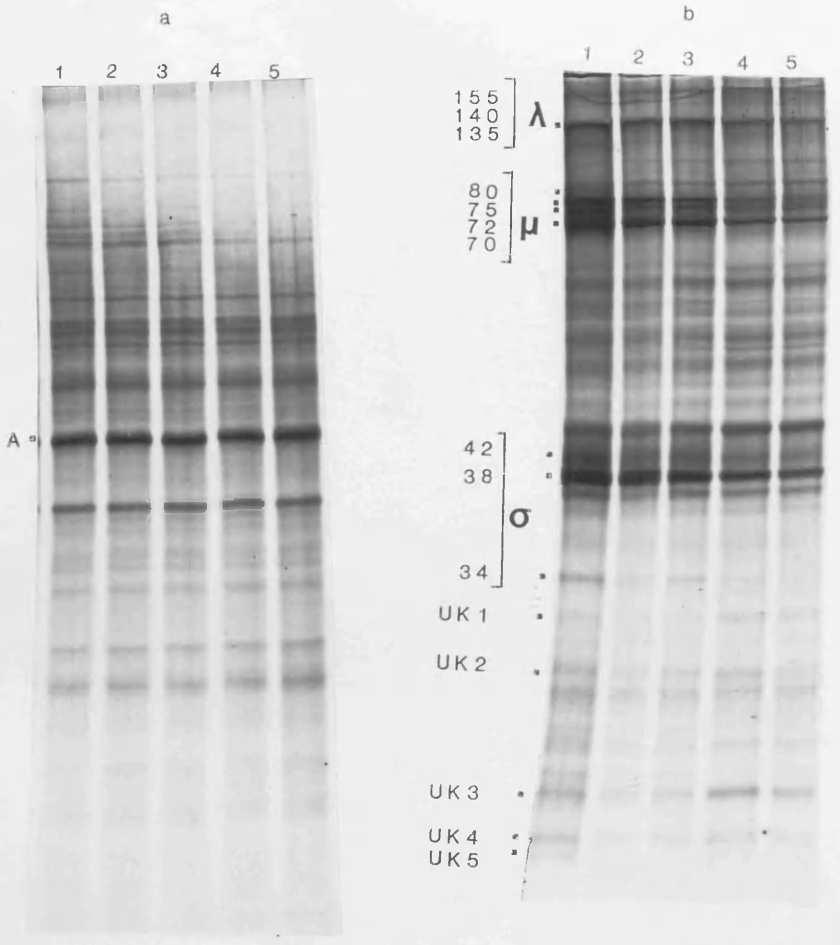

a
1
2
3
4
5
A
b
1
2
3
4
5
155
140
135
λ
80
75
72
70
μ
42
38
σ
34
UK 1
UK 2
UK 3
UK 4
UK 5

Figure 55

SDS PAGE profile on a 10% gel of the infected cell polypeptides induced in BS-C-1 cells treated with 0 ,50, 100, 200 or 300uM CCX (lanes 1-5), from 1-24h p.i.

Cells were either mock-infected (a) or infected with Reovirus-3 (b) at m.o.i. 10 p.f.u./cell.
35Smet was added from 1-24h p.i. and equal volumes of sample were loaded onto each track.

The positions and m.wt. of known Reovirus-3 coded proteins are indicated on the left :

1 (155K), 2 (140K), 3 (135K);
1 (80K), NS (75K), 1c (72K), 2 (70K);
1 (42K), 2 (38K), NS (36K), 3 (34K);

The positions of 5 low m.wt. bands of unknown origin present in Reovirus-3 infected but not mock-infected cell extracts are also indicated (UK1, UK2, UK3, UK4 and UK5).

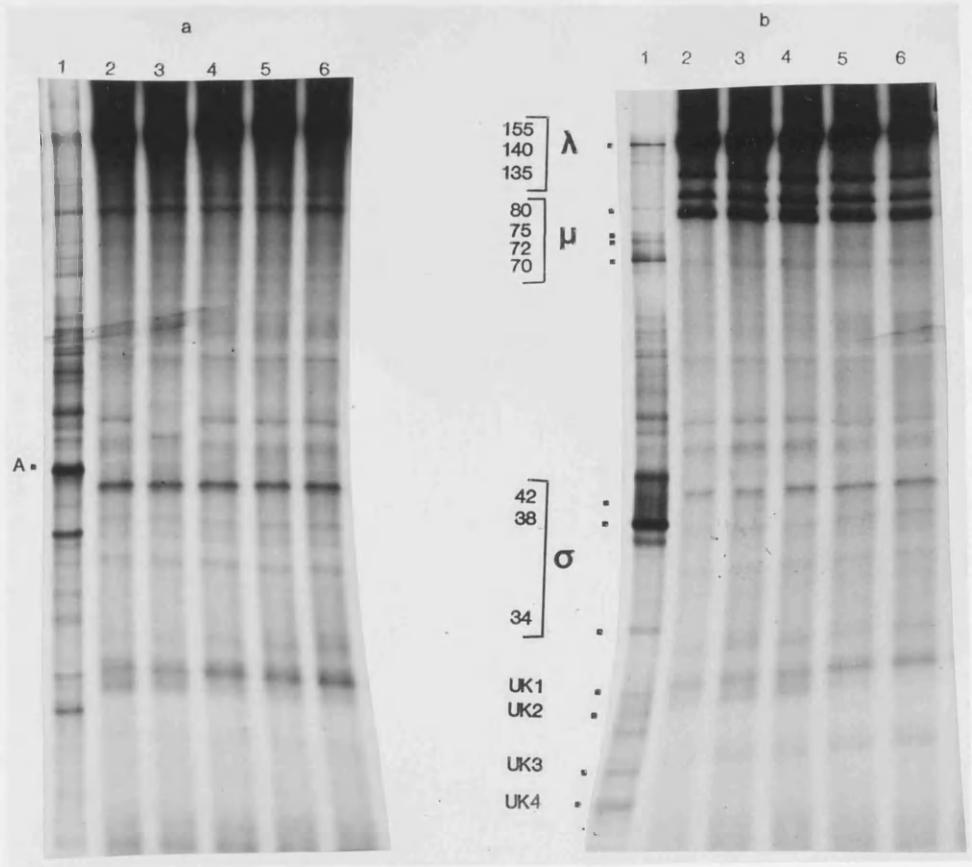

a
1 2 3 4 5 6
A
b
1 2 3 4 5 6
155
140
135
λ
80
75
72
70
μ
42
38
σ
34
UK1
UK2
UK3
UK4

Figure 56

SDS PAGE phosphorylated polypeptide profile on a 10% gel from BS-C-1 cells treated with 0, 50, 100, 200 or 300uM CCX (lanes 2-6).

Cells were either mock-infected (a) or infected with Reovirus-3 at m.o.i of 10 p.f.u./cell.
^{32}P-orthphosphate was added from 1-24h p.i. in parallel with ^{35}Smet (shown in Figure 55).
Lane 1 shows ^{35}Smet-labelled polypeptides induced in mock-infected (a) and Reovirus-3 infected (b) drug-free BS-C-1 cells.
Equal volumes of sample were loaded onto each track.

3.7. THE EFFECT OF CCX ON THE REPLICATION OF ADENOVIRUS-5; REPRESENTING THE CLASS 2 RESPONSE

3.7.1. ANALYSIS OF ADENOVIRUS-5 PRODUCED IN CCX TREATED BS-C-1 CELLS

THERMOSTABILITY OF ADENOVIRUS-5 PROGENY VIRUS MADE IN HELA CELLS TREATED WITH CCX

The thermostability of infectious virus made in cells treated with 0, 50, 150 or 300uM CCX was studied to assess the quality of Adenovirus-5 progeny synthesised during drug treatment. Virus was incubated for various times at 42^{o}C and then immediately titrated. The curves show that there was no significant difference in the thermostability of Adenovirus produced in CCX treated and untreated HeLa cells (Fig 57). In all cases, progeny virus was sensitive to high temperature, reducing infectivity by greater than a log. Therefore, the quality of virus produced in the presence and absence of CCX, was approximately the same.

SUSCEPTIBIITY OF ADENOVIRUS-5 PRODUCED IN HELA CELLS TO REPEATED TREATMENT WITH CCX (DOSE-RESPONSE CURVES)

Like Reovirus-3, Adenovirus-5 was very sensitive to low concentrations of CCX, the dose-response curve reaching a plateau between 100 and 200uM CCX. To determine if the plateau resulted from the selection of a sub-population of resistant virus, virus produced in CCX treated and drug free cells was used in repeat dose-response experiments. The resulting curves (Fig 58), indicate that this was not the case with virus produced in CCX treated cells responding to further CCX treatment in the same way as virus produced in drug free cells.

3.7.2. ADSORPTION OF ADENOVIRUS TO PRE-TREATED HELA CELLS

Adsorption experiments were performed with Adenovirus-5 and HeLa cells (as described in 2.2.5.6.) The adsorption curves obtained (Fig 59) are very similar and clearly show that even at 900uM CCX, Adenovirus was still able to bind to cell receptors. It is therefore unlikely that inhibition of adsorption of virus to pre-treated cells would play any part in limiting the spread of Adenovirus-5 infection at least *in vitro.*

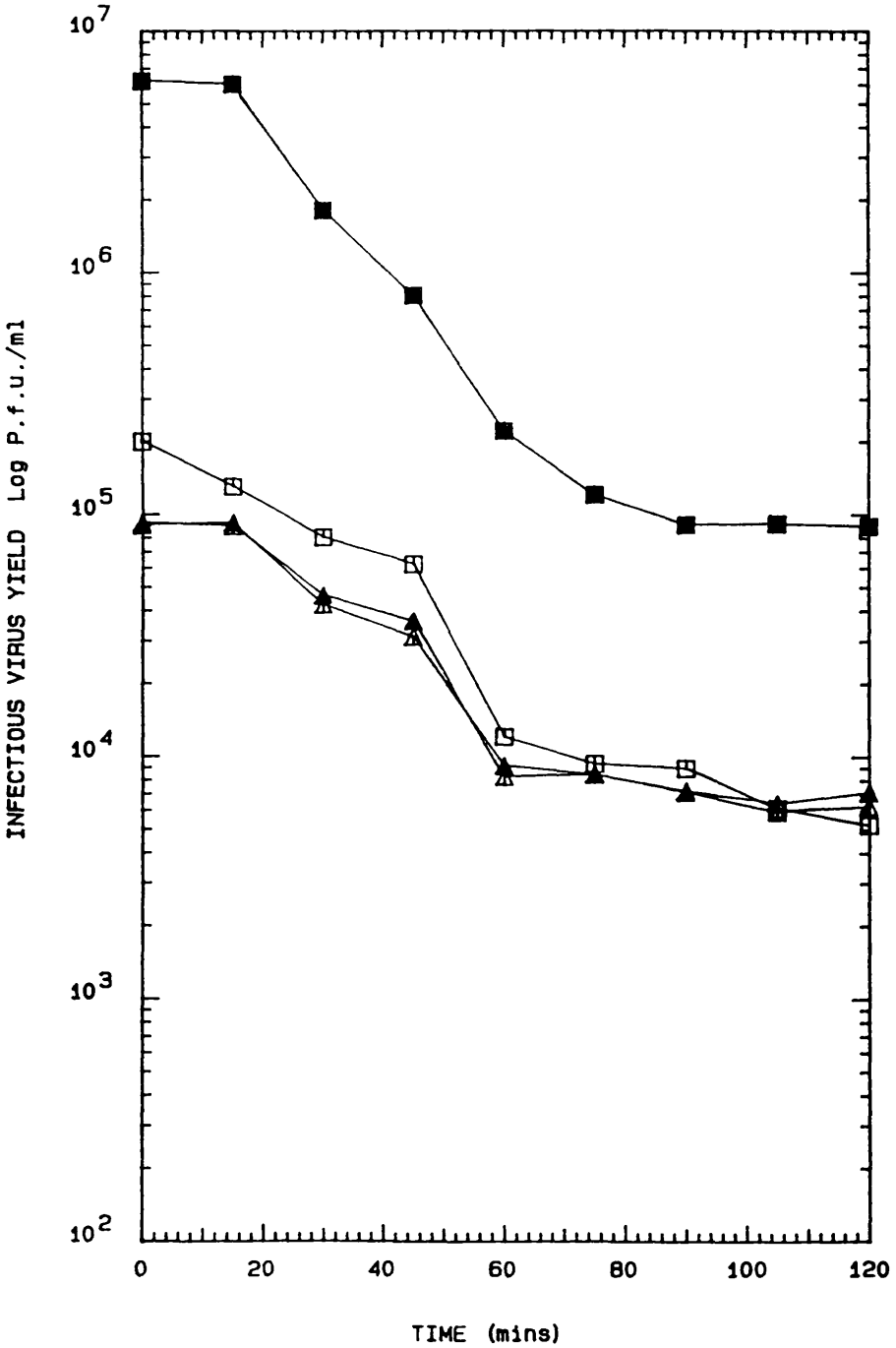

INFECTIOUS VIRUS YIELD Log P.f.u./ml
10^7
10^6
10^5
10^4
10^3
10^2
0
20
40
60
80
100
120
TIME (mins)

Figure 57

Thermostability of Adenovirus-5 progeny virus made in HeLa cells treated with CCX : 0 (■), 50uM CCX (□), 150uM CCX (▲), 300uM CCX (△).

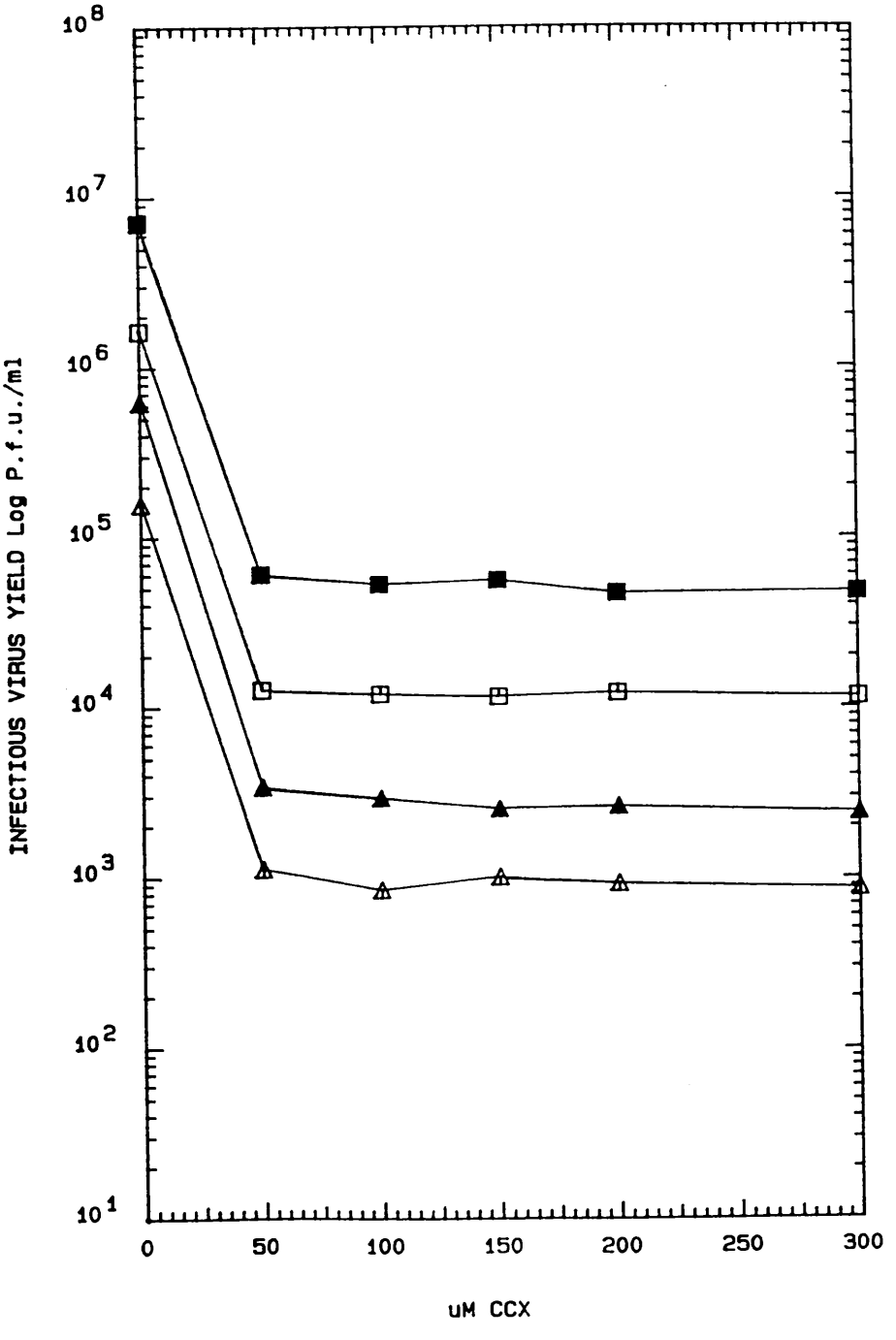

INFECTIOUS VIRUS YIELD Log P.f.u./ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
50
100
150
200
250
300
uM CCX

Figure 58

The effect of increasing concentrations of CCX on infectious Adenovirus yields from HeLa cells infected with

(■) virus produced in drug-free cells

(□) virus produced in cells maintained in 50uM CCX

(▲) virus produced in cells maintained in 150uM CCX

(△) virus produced in cells maintained in 300uM CCX

Dose-response curves have been transposed down by 0.5 log (□), 1 log (▲), or 1.5 log (△) to allow clear presentation.

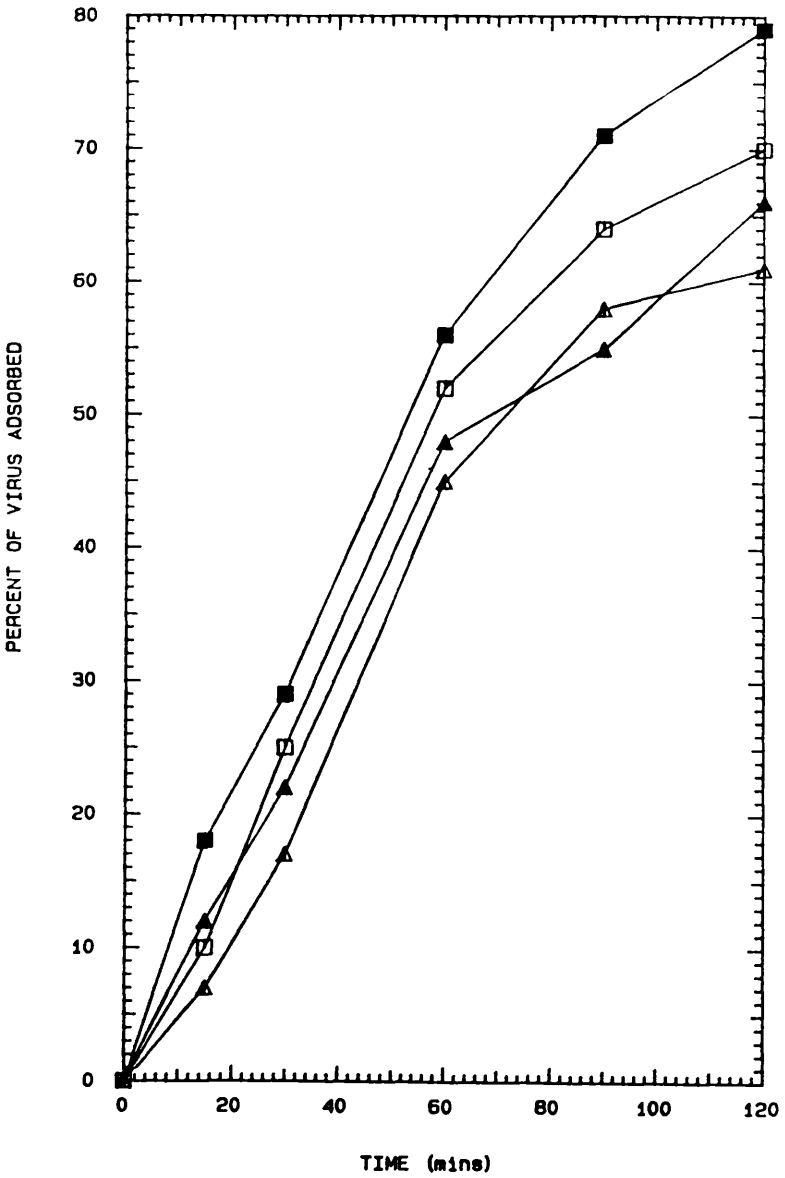

80
70
60
50
40
30
20
10
0
PERCENT OF VIRUS ADSORBED
0
20
40
60
80
100
120
TIME (mins)

Figure 59

Adsorption of Adenovirus-5 to HeLa cells pre-treated with 0 (■), 300uM CCX (□), 600uM CCX (▲) or 900uM CCX (△).

3.7.3. THE EFFECT OF INCREASING CONCENTRATIONS OF CCX ON PARTICLE NUMBERS, INFECTIOUS VIRUS YIELDS (P.F.U.) AND PARTICLE/P.F.U. RATIOS.

Virus particle counts were performed by electron microscopy (2.2.6.1.) on total virus yields from dose-response experiments (Table 40). The data show that treatment with CCX had no effect on particle/p.f.u. ratios, with particle numbers and Adenovirus-5 infectivity affected to the same extent at each CCX concentration. Therefore, CCX acts by blocking the assembly of Adenovirus-5 at relatively low concentrations, with no further effect at higher concentrations of CCX.

3.7.4. DIRECT INACTIVATION OF ADENOVIRUS-5 PARTICLES

Suspensions of Adenovirus-5 particles were incubated with either mock drug or 300uM CCX (2.2.5.7.) at 4^{o}C or 37^{o}Cfor 48h, and infectious yields titrated before and after drug addition.

This experiment was performed twice and the results are shown in Table 41. The data show that CCX was able to directly inactivate virus particles, with the extent of the effect varying between 10% and 50% . This reduction in infectivity however was not great enough to account for the total anti-Adenovirus-5 effect of CCX.

CCX (uM)	PARTICLE NUMBER	P.F.U.	PARTICLE/P.F.U. RATIO
0	$1.43*10^{10}$	$4.0*10^{7}$	357
10	$1.43*10^{10}$	$3.0*10^{7}$	476
30	$2.8*10^{9}$	$6.0*10^{6}$	466
50	$2.8*10^{8}$	$9.0*10^{5}$	311
150	$1.43*10^{8}$	$8.0*10^{5}$	178
300	$1.43*10^{8}$	$7.0*10^{5}$	204

Table 40

The effect of increasing concentrations of CCX on Adenovirus-5 particle numbers, infectious virus yields (p.f.u.) and particle/p.f.u. ratios.

a

TIME(hr)	TEMP (C)	DRUG	TITRE	%AGE	REDUCTION
0	-	ORIGINAL STOCK	$1.6*10^{7}$		
48	4	MOCK DRUG	$1.38*10^{7}$	100	51.5
48	4	300 uM CCX	$6.7*10^{6}$	48.5	
48	37	MOCK DRUG	$5.8*10^{6}$	100	10.4
48	37	300 uM CCX	$5.2*10^{6}$	89.6	

b

TIME(hr)	TEMP (C)	DRUG	TITRE	%AGE	REDUCTION
0	-	ORIGINAL STOCK	$1.9*10^{7}$		
48	4	MOCK DRUG	$1.4*10^{7}$	100	35.8
48	4	300 uM CCX	$9.0*10^{6}$	64.2	
48	37	MOCK DRUG	$4.7*10^{6}$	100	53.2
48	37	300 uM CCX	$2.2*10^{6}$	46.8	

Table 41

Direct inactivation of Adenovirus-5 particles by CCX.

a) and b) represent data from 2 independent experiments.

3.7.5. THE EFFECT OF CCX ON POLYPEPTIDES INDUCED IN MOCK-INFECTED AND ADENOVIRUS-5 INFECTED HELA CELLS FROM 8-48h P.I.

MOCK-INFECTED CELL EXTRACTS

There was no significant effect on host cell protein synthesis with 10, 20, 30, 40 or 50uM CCX (Fig 60a, lanes 2-6). Treatment with 100, 200 or 300uM CCX resulted in a reduction in most cellular bands, accompanied by the induction of proteins previously identified as stress proteins; grp 94 and 78 and also a lower m.wt. band (Fig 60a, lanes 7-9).

ADENOVIRUS-INFECTED CELL EXTRACTS

Treatment with increasing concentrations of CCX, resulted in a progressive reduction in most viral bands, with the exception of the low m.wt. bands 14K and 13K, which were actually increased in the presence of CCX (the increase in band 13K, becoming marked with 100, 200 and 300uM CCX). Levels of the fibre band (62K) and the non-structural DNA binding protein band (72K), were markedly reduced at 30 and 20 uM CCX respectively. In addition, at concentrations of 20uM CCX and above the 72K band becomes less diffuse and separates into 2 distinct bands, becoming barely detectable with 100, 200 or 300uM CCX (Fig 60b, lanes 3-9). This protein is normally phosphorylated, a variety of phosphorylated forms existing in infected cells. The appearance of distinct bands with CCX, may indicate some effect on phosphorylation. It is interesting that the structural protein band, m.wt. 66K, which is also phosphorylated, also becomes less diffuse with increasing concentrations, separating into 2 distinct bands with 100, 200 or 300uM CCX (Fig 60b, lanes 3-9).

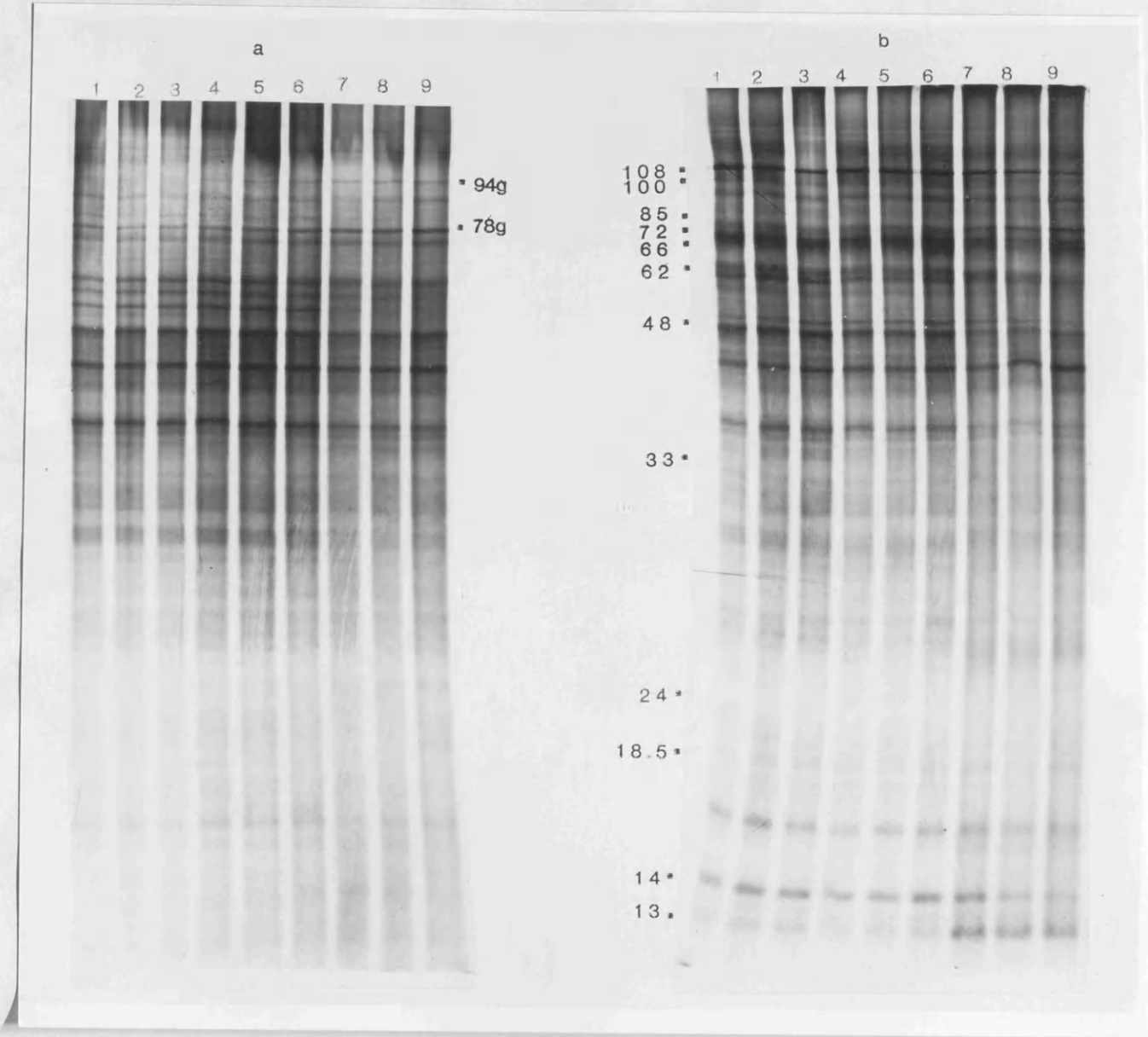

a
1 2 3 4 5 6 7 8 9
94g
78g
b
1 2 3 4 5 6 7 8 9
108
100
85
72
66
62
48
33
24
18.5
14
13

Figure 60

SDS PAGE profile obtained on a 12.5% gel of the infected cell polypeptides induced in HeLa cells treated with 0, 10, 20, 30, 40, 50, 100, 200 or 300uM CCX (lanes 1-9)

Cells were either mock-infected (a) or infected with Adenovirus-5 at 100 p.f.u./cell (b).
^{35}Smet was added from 8-48h p.i.
Equal volumes of sample were loaded onto each track.
The positions and m.wt. of known Adenovirus-coded proteins are indicated.

4. DISCUSSION

4.1. THE EFFECT OF CICLOXOLONE SODIUM (CCX) ON A RANGE OF CELL LINES

Prior to the investigation of the antiviral action of CCX, it was necessary to identify cell lines resistant to the drug and permissive for virus growth, to allow separation of antiviral and cytotoxic effects. Dargan and Subak-Sharpe (1985) had previously reported that the three cell lines they examined (each from a different species), differed in their sensitivity to CCX : Flow 2002 (human) cells were resistant, Vero (monkey) cells were sensitive, while BHK-21 (rodent) cells displayed intermediate sensitivity. Therefore twenty cell lines were screened for sensitivity to CCX treatment, not only to identify cell lines resistant to the drug and thus suitable hosts for virus CCX dose-response experiments, but also to establish how different cell lines tolerated CCX and to check whether any consistent pattern emerged i.e. would all human cells be more resistant than all monkey and rodent cells ?

The survey revealed that the cell lines differed in their tolerance to the drug and could be designated as Resistant, Intermediate or Sensitive on the basis of cell viability counts. It should be noted that the viability of cells in the Resistant class was not significantly affected, but the replication of these cells was inhibited at high CCX concentrations (200uM and 300uM CCX). However, this should not contribute to any antiviral effect as all virus CCX dose-response experiments were performed in confluent monolayers. Most importantly, the effect of CCX on the cell lines used in the virus dose-response experiments was shown to be fully reversible.

Cell lines did not clearly separate into classes depending upon their species of origin. For example the Resistant class contains human, rodent, monkey and canine cells (Table 1). However all human cell lines except one, fell into the Resistant or Intermediate class, while all of the rodent cells fell into the Intermediate or Sensitive class, with the exception of RK-13 cells which proved resistant. It is interesting that the sensitive human cell line, Fg 293, is an artificially transformed cell line (transformed with Adenovirus Ela and Elb), and that other artificially transformed cell lines also fall into this

group i.e. Reα, Re99 and HOOD cells. However, Hela cells which are naturally transformed with human papillomavirus and have also been grown extensively in culture belongs to the Resistant class. It may be that differences between artificially transformed and naturally transformed cell lines contribute to their sensitivity / tolerance to CCX. This inference could be tested indirectly by screening a wider range of naturally and artificially transformed cell lines. If the hypothesis is correct, then it should be possible to change the phenotype of a Resistant cell line to a Sensitive cell line by transforming the cell line in vitro.

Why cell lines should differ in their tolerance to CCX is not yet clear. There are however several possible explanations. Dargan and Subak-Sharpe (1986a and b), reported that CCX affected glycosylation in both HSV-infected and mock-infected Flow 2002 and BHK-21 cells. It may be that some cells are able to tolerate inhibition of glycosylation better than others. This could be tested by performing comparative cytotoxicity studies with CCX and specific inhibitors of glycosylation e.g. tunicamycin which blocks addition of N-linked core sugar in the ER; deoxymannojirimycin (DMJ), which specifically inhibits the cleavage of 1,2 mannose residues by inhibiting mannosidase 1 located in the cis stack of the Golgi apparatus and swainsonine which inhibits - mannosidase 11, located in the medial stack of the Golgi (Montefron, Robinson and Mitchell, 1987).

The triterpenoid compounds are all known to have mineralcorticoid activity (Epstein et al., 1977; Baron and Green, 1986; Stewart et al., 1987). In humans this presents as hypokalemia (loss of serum potassium) and serum sodium retention (pseudoaldostereoism). At the cellular level, the related compound carbenoxolone seems to stimulate the activity of the Na+ K+ ATP"ase (i.e.the sodium pump), thus pumping K+ into and Na+ out of the cell. Cells may differ in their tolerance to changes in ion levels, or indeed resist gross changes in ion levels by having fewer sodium pumps, less active sodium pumps or altered sodium pumps with less affinity for triterpenoid compounds. To determine if there is any correlation with the mineralcorticoid activity and

CCX tolerance, intracellular ion levels in drug-free and drug-treated cells could be measured using ^{86}rubidium (a model for potassium) and ^{22}sodium (Baron and Green, 1986) and directly compared with cell viability. Cicloxolone treatment has been observed to cause cells to shrink. This may be a consequence of the effect of CCX on the sodium pump thought to regulate cell volume.

CCX treatment was shown to induce a stress response in cell lines belonging to the Resistant class (Hela, Flow 2002 and BS-C-1), the Intermediate class (BHK-21) and the Sensitive class (Fg 293),although the number, m.wt. and intensity of stress protein bands differed between cell lines. The significance of this is not clear and may simply be a consequence of differential cell cycle inducement (all samples were harvested after 18h treatment with CCX). The CCX-induced cellular stress response is unusual in that both recognised classes of stress protein are induced i.e. heat shock proteins (hsp) and glucose related proteins (grp). The latter are probably induced by the effect of CCX on glycosylation as other glycosylation inhibitors also induce this set of proteins. However, Kozutumi *et al*. (1988) have shown that malfolding of proteins, rather than inhibition of glycosylation *per se*, induces the synthesis of grps. CCX is known to have a high affinity for proteins and may cause malfolding and so trigger the synthesis of grps. The mineralcorticoid activity of CCX, may also contribute to the inducement of the stress response as other compounds which disrupt ion fluxes such as ouabain which inhibits the sodium pump, also induces a stress response.

Induction of stress proteins involves a vigorous activation of transcription, as well as selective translation of the stress protein mRNA.This is accompanied by a reduction in the transcription and translation of other cellular mRNAs.(Ashburner and Bonner,1979; Schlesinger *et al*., 1982). It seems likely therefore that the stress response contributes to the observed inhibition of cell growth mediated by CCX. The functions of stress proteins have long been a matter for speculation; it is widely assumed that these proteins protect cells from the effects of stress, although the mechanisms are not entirely clear. Sorger and Pelham (1987), have suggested that all of the

major stress proteins, grp 94, grp 78, hsp 90, and hsp 70 have essentially the same function, which is to solubilise aggregates of malfolded or denatured proteins, but do so at different locations within the cell. All of the major stress proteins are present in the lumen of the ER and it is possible that all may function within that particular enviroment. However, in addition hsp 70 is found within the nucleus (Sorger and Pelham, 1987), while grp 94 has been localised within the Golgi apparatus (Welch et al., 1983) and hsp 90 is also abundant within the cytoplasm (Sorger and Pelham, 1987). Disruptive vacuolation of CCX-treated cells has been observed, and several lines of evidence suggest that the vacuoles are derived from the Golgi apparatus. It is tempting to speculate that the low levels of grp 94 observed is related to this vacuolation.

The fact that different classes of cell line induce the stress response at different concentrations e.g. Fg 293 (Sensitive) at 100uM CCX, BHK-21 (Intermediate) at 200uM CCX and BS-C-1 (Resistant) at 300uM CCX may simply reflect differences in the efficiency of the uptake of the drug. Sensitive cell lines may take up the drug more efficiently and so become susceptible at lower concentrations. Another possible explanation is that sensitive cell lines do not have the the capacity to metabolize or store high concentrations of CCX.

In summary, cell lines clearly exhibit differential sensitivity to CCX. The drug affects glycosylation, exhibits mineralcorticoid activity and can induce a stress response in all classes of cell line. Whether these contribute to the cytotoxic effect of CCX is not yet clear, although it seems likely that they play some role. Dargan and Subak-Sharpe (1985, 1986a and b), from their investigations of CCX-treated HSV-infected and mock-infected Flow 2002 and BHK-21 cells have postulated that the drug acts by disturbing the normal functioning of host cell membranes. Elucidation of the anti-cellular effect of CCX would then also provide valuable information regarding the antiviral effect of the drug.

4.2. THE EFFECT OF CICLOXOLONE SODIUM (CCX) ON A RANGE OF VIRUSES

4.2.1. THE ANTIVIRAL ACTIVITY SPECTRUM OF CCX

A range of viruses was examined for their sensitivity to CCX, and placed into three general groupings depending upon their response (Table 23).This spectrum of CCX sensitivities cannot be correlated with greater sensitivty of DNA viruses compared to RNA viruses; nor double-stranded compared to single-stranded genomes; nor positive compared to negative-stranded RNA genomes; or segmented compared to non-segmented genomes.

Dargan and Subak-Sharpe (1988), have shown that CCX reduced the overall percentage of enveloped HSV particles in the yield from drug-treated cells. Of the viruses now studied, only Polio-1, Reovirus-3 and Adenovirus-5 are non-enveloped. There was no evidence to suggest that the growth of enveloped viruses per se was more affected by CCX than that of non-enveloped viruses. Moreover the dose-response curves obtained both with the enveloped (Bunyaviruses) and non-enveloped (Reovirus, Adenovirus and Polivirus) viruses reach well defined plateaus. This could be because virus stocks are composed of genetically sensitive and resistant virus, with the plateau reflecting the surviving fraction. However, this was shown not to be the case for both Reovirus-3 and Adenovirus-5. Alternatively, CCX may affect a host or virus-specified function which enhances but is not essential for virus infectivity. Until CCX resistant mutants are isolated the latter explanation is favoured.

Corrobarative evidence that the antiviral activity of CCX is linked to its effects on host cells is supplied by the diastereoisomer study, where the antiviral activity of the compounds paralleled their cytotoxic activity (690 < CCX <688). This is in agreement with the proposed mode of action of CCX i.e. by disturbance of the normal functions of host cell membranes (Dargan and Subak-Sharpe 1985, 1986a and b). Investigation of the anti-HSV effect of CCX has indicated that the mechanism of action is complex and that with HSV at least, the virus replication cycle provides multiple targets for CCX inhibition (Dargan and Subak-Sharpe,1986a and b, 1988). Clearly irrespective of whether viruses are enveloped

or not, to varying extents,they must utilise during their replication cycle, either pre- existing or virally modified membrane systems of the cell. The effect of CCX on the replication of viruses from each group was investigated, with the purpose of more precisely pinpointing the particular antiviral targets that block production of infectious virus yield and identifying the mechanism of action in these very diverse cases.

4.2.2. THE EFFECT OF CCX ON THE REPLICATION OF VSV (INDIANA) : REPRESENTING THE CLASS 1 RESPONSE

CCX exhibited potent activity against the rhabdovirus, vesicular stomatitis virus (VSV) (Indiana strain), progressively reducing infectious virus yields with increasing concentrations of CCX, 300uM achieving a 10,000 fold reduction. VSV was the most extensively studied virus in this survey, as apart from the herpesviruses (CCX-treated HSV-1 and HSV-2 infected Flow 2002 and BHK-21 cells, were investigated by Dargan and Subak-Sharpe, 1985, 1986a and b, 1988), this was most drastically affected by the drug. Growth curves performed in the absence and presence of CCX, revealed that CCX inhibited VSV replication. That the reduction in infectious yields was not due to delay in virus growth was established, as inhibition was sustained throughout a 36h period.

The effect of increasing concentrations of CCX on infectious virus yields (p.f.u.), particle numbers and particle/p.f.u. ratios was determined.The results indicated that the anti-VSV effect of CCX, operates both by lowering the quality of progeny virions and through strong inhibition of virus particle production. This is in contrast to the anti- HSV effect of CCX, where there is only a slight reduction in particle numbers, the predominant effect being the lowering of potential infectivity of progeny virus particles (Dargan and Subak-Sharpe, 1985). The dramatic alteration in the particle/p.f.u. ratio in HSV yields from CCX treated cells, was deduced to be a consequence of abnormal virus assembly as evidenced by the polypeptide composition of virus produced in the presence of CCX: some proteins are under-represented and others are over-represented. (Dargan and Subak-Sharpe, 1986a and b). This was not the case for VSV, where the virion polypeptide composition appeared to be unaffected by CCX. However, there was an increase in the mobility of the G band, indicative of an effect on glycosylation processing, possibly including sialic acid addition. Schloemer and Wagner (1975), demonstrated that removal of sialic acid by neuraminidase inhibits adsorption of VSV particles to L cells (Schloemer and Wagner, 1975). CCX however is able to inactivate VSV particles directly causing up to a 10 fold reduction in

infectivity, which also furnishes an explanation for the observed rise in particle/p.f.u. ratio. CCX treatment directly reduces HSV particle infectivity, but this only makes a minor contribution to the much greater rise in particle/p.f.u. ratio.

How direct inactivation is achieved is not clear. CCX is a highly lipophilic compound and has strong affinity for proteins (Symons and Parke, 1980). The drug may bind to virus particles by hydrophobic interaction with the virus envelope membrane and/or by interaction with the glycoprotein protruding from the virus envelope. Binding in turn may inhibit adsorption to cellular receptors. This however has not been investigated and must remain speculation.

To determine how CCX reduces the production of VSV particles, several stages within the life cycle of the virus were investigated for susceptibility to CCX. Drug addition experiments, where CCX was added at various times post-adsorption, revealed that CCX did not block any specific stage in the VSV life cycle. CCX had a progressive effect operating throughout the life cycle, with greater yield reductions achieved, the earlier the drug was added. This is in agreement with the progressive effect of CCX on HSV-1/2 replication (Dargan and Subak-Sharpe, 1985). VSV enters cells mainly by receptor-mediated endocytosis (Schlegel et al., 1982), viral nucleocapsid being released into the cell cytoplasm following fusion of viral and endosomal membranes. In all CCX dose-response experiments, CCX was added after virus absorption had been allowed to occur (i.e. 1h p.i.). Therefore, although CCX may be capable of affecting virus absorption, inhibition of this step can play no role in the reduction of infectious virus yields achieved in these one step growth curve experiments.

The first synthetic event in the infectious cycle of any negative strand RNA virus, including VSV, is mRNA synthesis. RNA synthesis was shown to be affected in CCX-treated mock-infected and VSV-infected BS-C-1 cells. However, there was a differential effect on VSV transcription: primary transcription was not thought to be affected (as protein synthesis from 1-8h p.i. was not significantly affected by CCX treatment). In contrast, there

was a progressive reduction with increasing concentrations of CCX, in levels of all secondary VSV transcripts. It is not known how CCX affects transcription. However, there are several possible explanations for the reduction in levels of both cellular cytoplasmic RNA VSV secondary transcripts, based on other known actions of CCX (Table 42). Each of these will be considered in turn and should not be regarded as mutually exclusive.

Dargan and Subak-Sharpe (1986a and b),have previously reported that in CCX treated HSV-1/2 -infected or mock-infected Flow 2002 and BHK-21 cells, the nuclear/cytoplasmic distribution of certain proteins is disturbed. This disturbance may be due to the alteration of the nuclear membrane by CCX, thereby affecting the transport of proteins between the nucleus and the cytoplasm. Reduced levels of cellular cytoplasmic RNA, may therefore be a consequence of impaired transport of RNA, from the nucleus to the cytoplasm. This however cannot affect levels of VSV transcripts, as VSV replication is completely cytoplasmic.

CCX induces a stress response in both mock-infected and VSV-infected BS-C-1 cells. Induction of stress proteins involves a vigorous activation of stress protein mRNA transcription as well as their selective translation, accompanied by the inhibition of the transcription and translation of other mRNAs (Ashburner and Bonner, 1979; Schlesinger et al., 1982). It is likely that this at least contributes to the reduction in cellular cytoplasmic RNA. However, it can play no role in reducing the levels of VSV secondary transcripts, as labelling of these transcripts was performed in the presence of actinomycin D (Act. D) which abolishes the cellular stress response.

The phosphorylation of VSV NS protein has been shown to regulate transcription in vitro (Kingsford and Emerson, 1980; Hsu, Morgan and Kinsbury, 1982). The xanthate compound, D609, inhibits VSV secondary but not primary transcription, by inhibiting the phosphorylation of VSV NS protein (Muller-Decker, Amtmann and Sauer, 1977). However CCX has no effect on phosphorylation in VSV-infected or mock-infected BS-C-1 cells. Since phosphorylation is not inhibited by CCX, this cannot contribute to reduced cellular cytoplasmic or VSV secondary transcripts (this also confirms

ACTION OF CCX	POSSIBLE CONSEQUENCE	REDUCED LEVELS OF CELLULAR CYTOPLASMIC RNA	REDUCED LEVELS OF VSV SECONDARY TRANSCRIPTS
ALTERATION OF MEMBRANE FUNCTION	INHIBITION OF NUCLEAR/CYTOPLASMIC TRANSPORT OF TRANSCRIPTS	P	NP
STRESS RESPONSE	INHIBITION OF TRANSCRIPTION	P	NP
PHOSPHORLYATION ?	AFFECTING ACTIVITY OF TRANSCRIPTASE	NP	NP
DIRECT BINDING OF CCX TO ENZYMES	ACTIVATION/ INHIBITION OF +/- - FACTORS INVOLVED IN TRANSCRIPTION	P	P
	ACTIVATION OF RN' ase → DEGRADES RNA	P	P
	ACTIVATION OF PROTEASE → DEGRADES TRANSCRIPTION COMPLEX		
	DIRECT INHIBITION/ MODIFICATION OF TRANSCRIPTASE COMPLEX; REDUCED LEVELS OF RNA AND/OR UNSTABLE RNA	P	P

Table 42

Inhibition of transcription by CCX: proposed mode of action.

P : Possible

NP : Not Possible

that the observed reduction in transcripts is real, and not a consequence of reduced uptake of radioactive phosphate).

Carbenoxolone can bind to various enzymes causing either activation or inhibition (Roberts and Taylor, 1980). Given that cicloxolone and carbenoxolone are closely related, it seems likely that CCX can similarly inhibit or activate enzymes. One of the enzymes inhibited by carbenoxolone, is gastric 15-OH dehydrogenase. Pre-treatment of cells with indomethacin, which also alters the biosynthesis of prostaglandins, renders cells reversibly non-permissive for productive infections of VSV, at the level of viral RNA and protein synthesis (Mukherjee and Simpson, 1987). These authors suggested that indomethacin may act by modifying a cellular factor required for the transcriptase activity of the VSV nucleocapsids. As both CCX and indomethacin alter the biosynthesis of prostaglandins, CCX may also inhibit transcription in this way. The effect of CCX and indomethacin on prostaglandin biosynthesis may be directly responsible for the modification of this cellular membrane-associated factor, as prostaglandins are continuously synthesised from membrane phospholipids. However, while indomethacin inhibited primary VSV transcription, CCX inhibited only secondary transcription. This may be explained by the fact that cells were pre-treated and maintained in indomethacin, while CCX was added only after virus absorption had occurred. Therefore CCX uptake at the time of primary transcription may not be sufficient to saturate target molecules. If the mode of action of these compounds is indeed similar, then pre-treatment of cells with CCX should result in the inhibition of primary transcription.

Of course, other possibilities exist, which include the activation of RN"ases, resulting in degradation of RNA, activation of proteases resulting in the degradation of the transcriptase complex or direct binding to the transcriptase complex itself, thereby modifying or inhibiting its function to produce reduced amounts of RNA or unstable RNA. These general mechanisms involving binding to enymes are equally applicable to viral or cellular transcription.

In conclusion, although there are several possible explanations for the reduction in cellular cytoplasmic RNA,

and VSV secondary transcription, until further investigations of the effect of CCX on transcription are performed, the various possibilities outlined here must remain speculation.

Dargan and Subak-Sharpe (1986a and b), reported that polypeptide synthesis and post-translational modifications, particularly glycosylation and sulphation were all affected in CCX-treated mock-infected and HSV-1/2 -infected Flow 2002 and BHK-21 cells. In my experiments protein synthesis in CCX-treated mock-infected BS-C-1 cells was also inhibited although the extent of the inhibition varied between experiments. It is notable that inducement of a stress response correlated with the degree of inhibition of protein synthesis; the stress response being induced strongly in cells where protein synthesis inhibition was marked, and not detectably induced when protein synthesis was only slightly affected. This would seem to indicate the involvement of the stress response in the inhibition of protein synthesis mediated by CCX. Howver, this cannot furnish the entire explanation, as protein synthesis is inhibited with increasing concentrations of CCX in the presence of Actinomycin D, which inhibits the stress response. As CCX is a highly lipophilic compound with great affinity for membranes (Symons and Parke, 1980), protein synthesis may also be inhibited by a direct effect on the ER.

CCX also affected protein synthesis in VSV infected cells. However the result was unexpected, with some bands decreasing with increasing CCX (L, N and NS), while protein bands G, M and UK_3 increased with increasing CCX, despite reduced levels of transcript. There are several possible explanations for this result including differential secretion of proteins (as CCX affects the PM, causing it to become progressively leaky with increasing CCX concentrations; Symons and Parke, 1980), differential stability of mRNAs and/or differential translatibility of mRNAs. However investigations of the effect of CCX on glycosylation in CCX-treated VSV-infected BS-C-1 cells provided the most likely explanation.

Dargan and Subak-Sharpe (1986a and b), showed that glycosylation in both mock-infected and HSV-1 and HSV-2 infected BHK-21 cells, was inhibited by CCX, as evidenced by

the reduction in mannose incorporation with increasing concentrations of CCX. It seemed likely that glycosylation would also be affected in CCX-treated VSV-infected and mock-infected BS-C-1 cells. Comparative studies were performed using compounds known to disrupt this process : tunicamycin (Tzack and Lampen, 1975), which blocks addition of the core sugar occurring in the ER, and monensin (Ledger and Tanzer, 1984; Boss et al., 1984), which disrupts the Golgi apparatus where sugar processing occurs. ^{35}Smet-labelled polypeptide profiles obtained from VSV-infected BS-C-1 cells treated with CCX, monensin and tunicamycin revealed that 1) CCX did not affect glycosylation as dramatically as tunicamycin as evidenced by the relative mobility shifts; there was a marked downward shift in the G band with tunicamycin, concomitant with the significant change in m.wt., due to the absence of the sugar moiety. 2) monensin and CCX were similar in that both these tracks contained elevated levels of G and M. In addition both caused only a slight shift in the mobility of the G band. As expected, tunicamycin completely blocked glucosamine incorporation. In contrast, monensin had no effect, while CCX only had a slight effect at 300uM CCX. Glucosamine is added to protein both as part of the core sugar in the ER, and then during processing in the medial stack of the Golgi apparatus. These results therefore indicate that monensin and CCX (at concentrations < 300uM) had no effect on the medial stack of the Golgi. However further modifications occurring in the trans stack of the Golgi e.g. galactose or sialic acid addition, may be inhibited by CCX and monensin. This however must remain speculation as galactose labelling of the VSV G protein in BS-C-1 cells was not successful. Thus, unlike CCX-treated HSV-1 and HSV-2 BHK-21 cells (Dargan and Subak-Sharpe, 1986a and b), glycosylation was not strongly inhibited in CCX-treated VSV-infected BS-C-1 cells. Moreover, these studies revealed similarities between CCX and monensin treated VSV-infected BS-C-1 cells.

Monensin-treated VSV-infected cells have been extensively studied elsewhere. Johnson and Schlesinger (1980), showed that monensin caused dilation of Golgi membrane, and that the transport of the VSV G protein from

the Golgi apparatus to the cell surface was blocked in monensin-treated VSV-infected BHK and CEF cells. VSV assembly normally occurs at the PM, at the site of insertion of the G protein (Simons and Garoff, 1980). Retention of the G protein at the Golgi membrane therefore effectively inhibits VSV assembly. The VSV matrix protein (M) is thought to play a pivotal role in assembly mediating the interaction between virus nucleocapsid and glycoprotein (Simons and Garoff, 1980). Monensin also blocks the assembly of the paramyxovirus Newcastle Disease virus, in BHK cells (Yoshida et al., 1986). The M protein of paramyxovirus also performs a similar function i.e. also interacts with nucleocapsid and glycoprotein, thereby facilitating assembly (Simons and Garoff, 1980). These studies revealed that the transport of NDV glycoprotein from the Golgi apparatus to the cell surface is inhibited and furthermore that the M protein cannot associate with the glycoprotein retained at the Golgi membrane or with the cell surface membrane. This may also be the case in monensin treated VSV-infected BS-C-1 cells with M protein accumulating at another site within the cell. However it is possible that the M protein can associate with the G protein at the Golgi membrane, but not in the correct manner to facilitate budding (whereas the other proteins may be less stable if assembly is prevented).

It is not entirely clear how monensin blocks glycoprotein transport to the cell surface. It does not seem likely to be due to the effect on glycosylation, as monensin is not a specific drug blocking at different stages in different virus/cell systems. For example, monensin blocks glycoprotein transport between the medial and trans stacks of the Golgi in SFV-infected BHK cells, but blocks post-trans Golgi in SFV-infected CEF cells (Griffiths, Quinn and Warren, 1983; Pesonen and Kaariainen, 1982). Therefore in SFV-infected BHK cells, the processing steps normally occurring in the trans stack are blocked, while the glycoprotein is presumably fully processed in the SFV-infected cells. However, the transport of both to the cell surface PM is inhibited. This block therefore cannot be due to disruption of glycosylation.

This block in glycoprotein transport seems more likely to be linked to the disturbance of ion fluxes in the cell,

mediated by monensin. Monensin is a linear polyether which has affinity for Na+ (Pressman, 1976). A comparative study was performed with monensin and A23187, which is also an ionophore, depleting Ca^{2+} inside cells. Both these compounds were shown to inhibit the transport of VSV and Sindbis glycoprotein to the cell surface in BHK and CEF infected cells (Johnson and Schlesinger, 1980). CCX also disrupts ion transport by enhancing the activity of the sodium pump (Epstein et al., 1977; Baron and Green, 1986; Stewart et al., 1987). In addition CCX has also been shown to induce vacuolation in treated cells (Dargan and Subak-Sharpe, 1988). Monensin and CCX most probably block VSV G transport from the Golgi membrane to the cell surface, by disrupting ion fluxes, the consequence in each case being inhibition of VSV assembly.

It has recently been proposed that monensin elicits its effects on sorting, processing and movement of proteins through the Golgi region by neutralising the trans portion of the organelle (Anderson and Orci, 1988). This mode of action would also explain the differential effects on glycosylation observed in CCX-treated HSV-1 and HSV-2 BHK cells (fibroblasts) and CCX-treated VSV-infected BS-C-1 cells (epithelial). While all cells have acidic trans Golgi vesicles, only fibroblasts have acidic trans stacks (Anderson and Orci, 1988). This therefore means that monensin and presumably CCX would neutralize the trans stack in fibroblastic cells, resulting in disruption of glycosylation, whereas only trans-Golgi vesicles would be affected in epithelial cells. This proposition could be further tested then by comparing the effects of CCX and monensin on glycosylation in VSV infected BS-C-1 cells and BHK-21 cells and HSV infected BS-C-1 cells and BHK-21 cells.

In conclusion, CCX perturbs protein synthesis in VSV-infected BS-C-1 cells, resulting in decreased levels of L, N and NS and increased levels of G and M (and UK_3). The decrease in levels of L, N and NS could be due to differential effects on transcription (reduced levels of transcript were obtained with CCX), translation, mRNA stability and/or polypeptide secretion. Like CCX, monensin causes an increase in G and M. It seems most likely that the ionophoric activity of these compounds, results in the

vacuolation of the Golgi and inhibition of transport of glycoprotein to the cell surface PM. As VSV assembly normally occurs at the PM at the site of insertion of glycoprotein, the production of VSV particles is reduced in both CCX and monensin-treated infected cells. The greater reduction in particle number, achieved by CCX is probably due to its additional effect on protein synthesis. A summary of the anti-VSV effect of CCX is shown in Figure 61.

THE ANTI-VSV EFFECT OF CCX

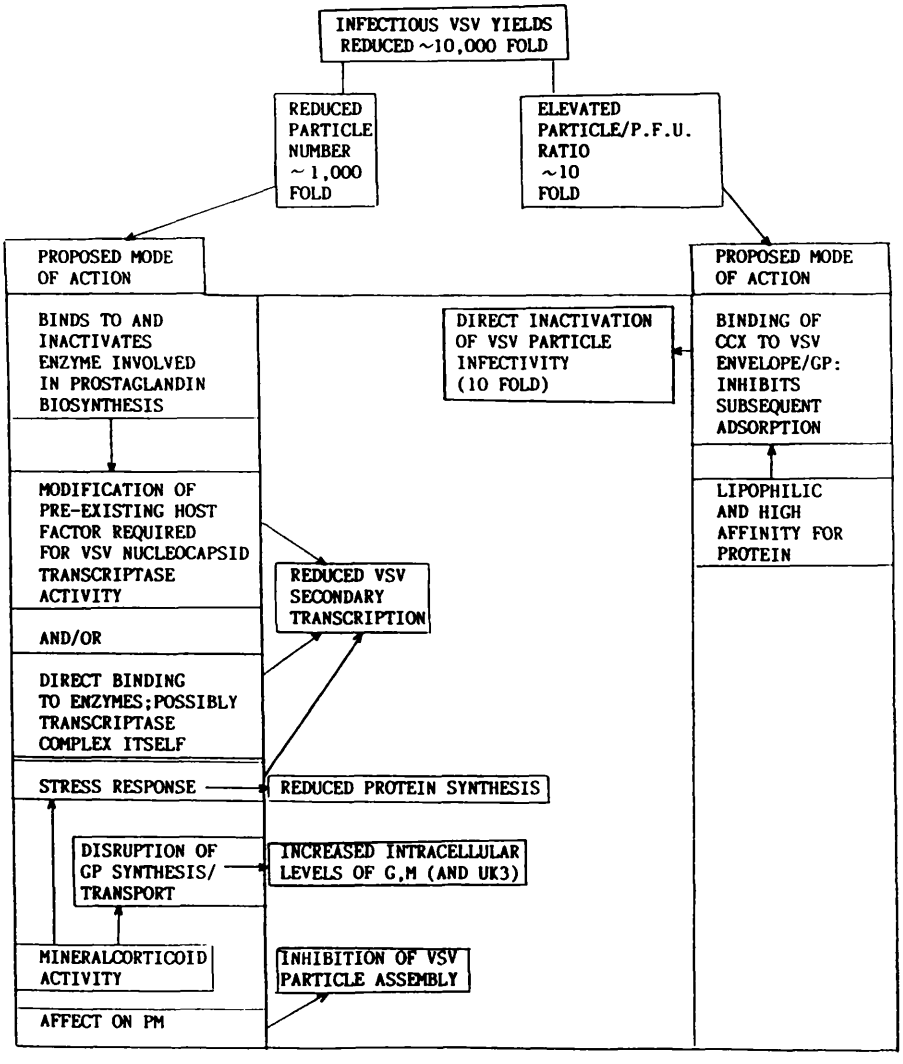


Figure 61

The anti-VSV effect of CCX.

4.2.3. THE EFFECT OF CCX ON THE REPLICATION OF SFV : REPRESENTING THE CLASS 3 RESPONSE

Total infectious yields were not affected by CCX treatment, the virus thus being classified as resistant to drug treatment. However, when infectious yields were divided into cell-associated (CA) and cell-released (CR) fractions, the CA fraction was seen to increase with increasing CCX concentrations with a concomitant decrease in the CR fraction. Therefore, although total virus yields are not reduced, virus maturation is affected by CCX treatment. Monensin dose-response experiments performed in parallel, produced similar results, with the CA fraction increasing and the CR fraction decreasing. In addition, both monensin and CCX induced similar changes in SFV polypeptide synthesis in treated BS-C-1 cells, resulting in the accumulation of viral glycoproteins. Monensin treated SFV-infected BHK cells were investigated by Griffiths, Quinn and Warren (1983), who showed that monensin caused vacuolation of Golgi cisternae, and furthermore that SFV nucleocapsids associated with and bud into these vacuoles. The EM analysis described here revealed that this also occurred in both monensin and CCX-treated SFV-infected BS-C-1 cells.

More recently, McDowell et al. (1987), have investigated the effect of 1-deoxymannojirimycin (dMM), a mannosidase 1 inhibitor (cis stack of Golgi), on SFV-infected BHK cells. While appearance of virus in the medium was inhibited, when the yield of virus from the cells and medium was combined, there was no difference between untreated and dMM treated cultures. When examined by EM, in dMM treated cultures, nucleocapsids were found lining intracellular membranes, and budding of virus was seen to occur at these membranes. These results therefore indicate, that although initial stages of mannose removal are important for the transport of SFV glycoprotein, it is not required for the infectivity of the virus.

In conclusion, total SFV infectious yields are not affected by CCX treatment, due to the ability of this virus to bud into intracellular vacuoles, the virus produced in these vacuoles being as infectious as those normally produced at the PM. It is postulated therefore, that the differential sensitivity of VSV and SFV to CCX treatment is

a consequence of the different mode of assembly of these viruses. While SFV assembly requires only a simple interaction between nucleocapsid and glycoprotein, VSV assembly is more complex, requiring the matrix protein M to mediate the interaction between nucleocapsid and glycoprotein, thus facilitating virus assembly and budding.

4.2.4. THE EFFECT OF CCX ON CLASS 2 VIRUSES : PARTICULARLY REOVIRUS-3 AND ADENOVIRUS-5

Similarities between the effects of monensin and CCX emerged in both VSV-infected and SFV-infected BS-C-1 cells. These studies suggested that CCX, like monensin, also disrupted Golgi membranes, affecting processing and transport of glycoprotein to the surface; in CCX treated VSV-infected BS-C-1 cells this contributed to the anti-VSV effect of CCX while in CCX treated SFV-infected BS-C-1 cells this was responsible for the apparent resistance of SFV to CCX treatment when whole cell yields were measured. It was important to establish how monensin affected other viruses and of interest whether and to what extent this effect on the Golgi contributed to the antiviral action of CCX in each case.

All earlier tested class 2 viruses were included in this comparative study. Monensin and CCX both reduced cell-associated and cell-released yields from Germiston and Bunyamwera infected cells by 100 fold. In this case therefore, the effect on the Golgi membrane, could at least in theory, account for the total anti- Bunyavirus effect of CCX. However, monensin had no effect on the infectivity of the other three viruses in this class: Poliovirus-1, Adenovirus-5 and Reovirus-3. In these cases therefore, disruption of the Golgi apparatus cannot contribute to the antiviral effect of CCX. Reovirus-3 and Adenovirus-5 were of particular interest as these two viruses exhibited great sensitivity to low concentrations of CCX. Indeed, the reduction in infectivity with low concentrations was even greater than that achieved for class 1 viruses with similar concentrations, with the exception of BHV-1. More detailed studies were therefore performed with these two viruses.

With both Reovirus-3 and Adenovirus-5, thermostability experiments revealed that the virus produced in CCX-treated cells, was similar in quality to that obtained from drug-free cells. This is in contrast to the observed increased thermolability of HSV particles produced from CCX treated Flow 2002 and BHK 21 cells. The virus produced from CCX treated cells was further investigated to determine whether the plateau obtained in the CCX dose-response curves was a consequence of the selection of a sub-population of

resistant virus. Progeny virus from CCX dose-response experiments, was grown up to high titre and then employed in second generation CCX dose-response experiments. Similar dose-response curves were obtained for virus produced from drug-free and CCX treated cells, indicating that the plateau was not a consequence of survival of genetically resistant virus. It seems likely therefore, that CCX affects a function (either host or virus-specified) which enhances but is not essential for virus replication.

The effect of increasing concentrations of CCX on Reovirus-3 and Adenovirus-5 particle numbers, infectivity and particle/p.f.u. ratios was determined. The anti-Reovirus-3 effect of CCX appeared to be of two types. At low CCX concentrations, the drug operated predominantly by lowering the quality of virus particles produced, at higher CCX concentrations by inhibiting virus particle assembly (with the quality of virus unaffected). In contrast, there was no significant effect on particle/p.f.u. ratios of Adenovirus yields from Hela cells at any CCX concentration. The anti-Adenovirus effect of CCX therefore operates totally by inhibiting particle production, with those particles that are produced from CCX treated Adenovirus-infected Hela cells, being equally as infectious as virus produced from drug-free cells.

Reovirus-3 particle infectivity was not significantly affected by direct exposure to CCX. Therefore unlike HSV-1 and HSV-2 (Dargan and Subak-Sharpe, 1985, 1986a and b), where direct inactivation of particles contributed to the elevation in particle/p.f.u. ratio and VSV, where it totally accounts for the rise in particle/p.f.u. ratio, direct inactivation can play no role in the observed lowering in the infectivity of Reovirus-3 particles at low CCX concentrations. One possible explanation, is that CCX has a progressive effect on virus particle assembly; at low concentrations CCX may not be able to effectively block virus particle production, but is able to modify virus assembly resulting in the production of high numbers of non-infectious particles. With regard to this, HSV particles assembled in the presence of CCX, have abnormal polypeptide composition which predominantly contributes to the elevated particle/p.f.u. ratios of these yields. Whether this is also

true for Reovirus-3 produced from cells treated with low concentrations of CCX has not been determined.

Cells infected with reovirus develop characteristic cytoplasmic inclusions referred to as viral "factories" (Gomatos, Tamm, Dales and Franklin, 1962). These begin as phase-dense granular material scattered in the cell cytoplasm and subsequently coalesce and move towards the nucleus. Inclusions have been shown to contain dsRNA (Gomatos, Tamm, Dales and Franklin, 1962; Silverstein and Schur, 1970), virus specific polypeptides (Fields, Raine and Baum, 1971; Spenlove, Lennette, Knight and Chin, 1963)and both complete and incomplete viral particles (Dales, Gomatos and Hsu, 1965; Fields, Raine and Baum, 1971). Viral particles are often found in crystalline arrays. Ribosomes are not part of viral factories; viral mRNA must travel outside the factory and newly synthesised structural proteins must travel back for assembly into virus particles. This process may be perturbed at low drug concentrations, resulting in abnormal virus assembly, and transport of proteins inhibited at higher CCX concentrations resulting in inhibition of particle assembly.

Immunocytochemical studies of intermediate filaments in infected cells show progressive disruption of vimentin filament organization (Sharpe, Chen and Fields, 1982). Inside viral factories masses of vimentin filaments appear to pass between microtubules. Virus particles are intimately associated with these filaments (Sharpe, Chen and Fields, 1982; Dales, 1963). It has been suggested that vimentin plays an important role in the organization of viral factories and therefore, the entire process of replication (Sharpe and Fields, 1983; Sharpe, Chen and Fields, 1982). From their EM studies of CCX treated HSV-infected Flow 2002 and BHK cells, Dargan and Subak-Sharpe have postulated an effect on the cell cytoskeleton (D. Dargan, personal communication). This therefore might furnish an explanation for at least part of the anti-Reovirus activity of CCX.

Investigations of the polypeptides induced in CCX treated Reovirus-3 and mock-infected BS-C-1 cells, revealed a selective progressive reduction in Reovirus-3 proteins with increasing CCX. The basis for this has not been investigated and may be a consequence of reduced

transcription and/or interference with the translation process itself. Regardless of the mechanism this reduction in viral protiens almost certainly contributes to reduced particle numbers in the presence of CCX.

As evidenced by the reductions in particle number and almost unchanged particle/p.f.u. ratio of Adenovirus yields from CCX treated infected Hela cells, the anti-Adenovirus effect of CCX operates totally by inhibiting particle production. Investigations of polypeptides induced in CCX treated Adenovirus infected cells revealed a general reduction with increasing CCX in most Adenovirus bands, with the exception of low m.wt. bands 14 and 13K, which actually increased in the presence of CCX (the increase in 13K becoming marked with high CCX concentrations). It is interesting that both these proteins are hexon-associated (Everitt and Philipson, 1974; Persson et al., 1978). Levels of the fibre protein band (62K and the non-structural DNA binding protein band 72K), were markedly reduced at 30 and 20uM respectively. In addition the diffuse 72K band separated into 2 distinct bands at this low CCX concentration. This protein is normally phosphorylated, a variety of phosphorylated forms existing in infected cells (Russell and Blair, 1977; Linne and Philipson, 1980). The appearance of distinct bands may therefore be indicative of an effect on phosphorylation by CCX. With regard to this it is interesting that the structural protein band (66K), which is also phosphorylated, also becomes less diffuse with increasing CCX concentrations, separating into 2 distinct bands with 100, 200 and 300uM CCX. How any effect on phosphorylation may contribute to the anti-Adenovirus effect of CCX is not clear. However removal of phosphate from 72K protein by alkaline phosphatase, does not affect the DNA binding function of this protein, although other unknown functions of the protein may be affected. Even at low concentrations, CCX clearly perturbs protein synthesis in Adenovirus-infected cells. However, whether this contributes to or is a consequence of disruption in virus assembly is not clear.

Dargan and Subak-Sharpe (1986a and b), have previously reported that the nuclear/cytoplasmic distribution of proteins in CCX treated HSV-1 and HSV-2 infected Flow 2002

and BHK-21 cells is disrupted. The nuclear/cytoplasmic distribution of proteins in CCX treated Adenovirus-infected cells has not been examined. However, if affected this would almost certainly contribute to the reduction in virus particle production from CCX treated cells, as Adenovirus assembly occurs in the nucleus and is dependent on the transport of proteins from the cytoplasm to this site of assembly.

4.3. FUTURE WORK

This study confirmed that CCX displays broad antiviral activity. In some cases only one repesentative of a family has been tested, although where more than one virus from a family has been studied e.g. Herpesviruses and Bunyaviruses, no notable difference in the class of dose-response to CCX has emerged. Whether this will be a general finding remains to be determined. This survey therefore, could be extended by screening additional viruses belonging to families already investigated, and viruses belonging to other families e.g. Poxvirus, Papovavirus, Retrovirus, Arenavirus, Hepadnavirus, Rotavirus etc.

In all CCX dose-response experiments, the drug was added after virus absorption had been allowed to occur (virus absorption = adsorption {binding to receptor on the cell surface} + penetration) Therefore, although CCX may be able to affect the pre-synthetic stages of the virus life cycle, this cannot contribute to the observed reduction in infectivity. Virus adsorption to cells pretreated with CCX has been investigated, revealing some slight effect in all cases. The effect of CCX on virus penetration however has not been examined. Two observations suggest that CCX may have some effect on this step. Firstly, this study revealed similarities between monensin and CCX. It is known that monensin inhibits virus receptor-mediated endocytosis, by raising the pH of endosomes, thereby blocking fusion of viral and endosomal membranes (Marsh et al., 1982). Secondly, CCX enhances the activity of the sodium pump, which pumps sodium out of and potassium into the cell. Presumably, the net result is decreased levels of intracellular sodium and increased levels of potassium. It has been shown that alteration of sodium and potassium levels in the growth medium can affect entry of SFV into cells. Growth medium normally contains high sodium and low potassium levels. If this is reversed i.e. low sodium and high potassium (10mM NaCl and 100mM KCl), SFV entry into cells is inhibited, by blocking release of SFV particles from endosomes (Helenius et al., 1985). Additional dose-response experiments should therefore be performed with all viruses, adding CCX before or at the time of infection.

This antiviral study allowed placement of viruses into

three broad classes, depending upon their dose-related response to the drug. The first class exhibit a continuous drug dose- dependent reduction in virus infectivity, the extent of the drop varying from 100-100,000 fold. The second class exhibit a less extensive total CCX response, infectivity being reduced by only 10-100 fold even with 300uM CCX, and all the dose-response curves reach their plateau level with concentrations between 100 and 200 uM CCX. The third class contains only SFV, whose total infectious progeny yields were not affected by CCX treatment. VSV was the virus most extensively studied as with the exception of the Herpesviruses, this was most dramatically affected by CCX treatment. Various stages in the VSV life cycle were examined for sensitivity to CCX. CCX was shown to reduce levels of both VSV secondary transcripts and cellular cytoplasmic RNA. However this experiment only allows tentative conclusions to be drawn as it was only performed once and therefore must be repeated and the results confirmed. In addition, in vitro transcriptase assays could be employed to determine how CCX may affect transcription. Products of in vitro transcriptase assays could then be used as templates in in vitro translation systems to uncouple effects of CCX on the processes of transcription and translation.

Both CCX and monensin were shown to induce vacuolation in treated cells. In monensin trated cells these vacuoles have been shown to be derived from Golgi membrane, using cytochemical markers e.g. thiamine pyrophosphatase, and phosphatase (specific for trans Golgi cisternae) and lectin, Riccinus communis agglutinin 1 (RCA) (specific for the middle and trans stack of the Golgi cisternae). Similar experiments should be performed to determine if vacuoles in CCX treated cells are also Golgi derived, as postulated.

Polypeptide analysis revealed that both CCX and monensin treatment of infected cells, resulted in elevated intracellular levels of VSV G and M proteins. Investigations of VSV-infected cells revealed that glycoprotein transport from the Golgi to the cell surface was inhibited (Griffiths, Quinn and Warren, 1983). Immunofluorescent experiments were performed using monoclonal antibody to VSV G protein, to determine if glycoprotein transport was also inhibited in

CCX treated cells. However, these experiments were not successful due to poor quality antibody. These experiments should therefore be repeated and the location of G and M in CCX treated cells more precisely determined by EM studies (immunogold labelling). Another approach would be to use radiolabelled CCX and monensin to follow the entry of these drugs into the cell and reveal any specific target organelles. It has also been shown that CCX can directly inactivate virus infectivity; radiolabelled drug could be useful in determining if this is due to binding of CCX to virus particles.

Class 2 viruses, Adenovirus-5 and Reovirus-3, were shown to be extremely sensitive to relatively low concentrations of CCX. Comparative infectious yield experiments, performed with CCX and monensin, revealed that disruption of glycoprotein transport and processing could play no role in the anti-Adenovirus or anti-Reovirus activity of CCX. Effects of CCX on the cell cytoskeleton and nuclear membrane may play a role in the anti-Reovirus and anti-Adenovirus activity of CCX, respectively. However, more detailed investigations of the effect of CCX on these viruses is required to determine why their replication is so sensitive to low CCX concentrations with no further effect at higher CCX concentrations.

The mode of action of CCX therefore is clearly complex. Although this study has revealed that CCX affects different stages in the VSV life cycle (transcription, protein synthesis and distribution), how CCX affects these steps is not clear. Dargan and Subak-Sharpe (1986a and b), from their investigations of CCX treated HSV-1 and HSV-2 infected Flow 2002 and BHK cells postulated that CCX operated by disturbing the normal functions of host cell membrane. This study revealed that CCX had a differential effect on both cells and viruses, and that the cytotoxic and antiviral activities of CCX were linked (demonstrated by the diastereoisomer study where the cytotoxic and antiviral activities of the compound correlated : 690 < CCX < 680). CCX has multiple effects on cells, including enhancement of the sodium pump, induction of the stress reponse, binding to proteins and vacuolation of membranes, all of which have been implicated in the anti-cellular and antiviral activity

of the compound. To more clearly elucidate the mechanism of action, it is clearly necessary to investigate more fully the effect of CCX on cells and the consequence of particular effects on the cell on the replication of various virus groups.

This study has investigated the action of CCX *in vitro* against a large number of cells and viruses. While CCX and CBX have been shown to have beneficial effects in the treatment of HSV infections in man (Poswillo and Roberts, 1981; Csonka and Tyrrell, 1984; Partridge and Poswillo, 1984), clinical trials have not been performed with any of the other viruses in the survey. If shown to be effective *in vivo*, CCX could be employed in combination with drugs which affect other targets, or to treat cases where resistance to other drugs has developed. Carbenoxolone, which is closely related to CCX, was used for many years in the treatment of duodenal ulcers. This was administered with diuretics as CBX altered sodium and potassium levels, resulting in hypokalemia (loss of serum potassium) and sodium retention (pseudoaldostereoism). Therefore, CCX could conceivably be administered orally, although topical application, where possible would be favoured.

Finally, it is of particular interest, that the parent compound Glycchirizic acid (GA), has been shown to have *in vitro* activity against HIV (Ito *et al*., 1987). This study has shown that CCX has a similar antiviral range as GA, and furthermore that its' activity is more potent, suggesting that the effect of CCX on HIV should be investigated. A compound affecting multiple targets would be useful in the treatment of AIDS patients, particularly in the light of reports of AZT resistance (Larder *et al*., 1989), currently the only licensed anti-HIV drug available.

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