AN ANTIVIRAL SUBSTANCE FROM PENICILLIUM CYANEO-FULVUM

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AN ANTIVIRAL SUBSTANCE FROM PENICILLIUM CYANEO-FULVUM (Further Studies)

by

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DEDICATION

With the kind approval of Professor R.W. Reed, Chairman of the Department of Microbiology and Immunology, McGill University, this thesis is dedicated to the memory of my mother who contributed so much to my education, but who died while the thesis was being written.

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TABLE OF CONTENTS

I. INTRODUCTION AND PURPOSE 1

II. HISTORICAL REVIEW 3

1. Introduction 3

2. Antiviral Agents and the Phases of Virus Multiplication 4

a) Phases of Adsorption and Penetration 4

b) Eclipse and Latent Phases and Phase of Maturation 13

i. Discussion of "Eclipse", "Latent", and "Maturation" 13
ii. Antagonists of nucleic acid synthesis 14
iii. Antagonists of protein synthesis 31
iv. Inhibitors of energy-yielding mechanisms 35

c) Phase of Release of Newly Formed Virus 36

3. Miscellaneous Substances Including the Antiviral Substance from Penicillium cyaneo-fulvum (McGill strain) 37

4. General Comments on the Problems of Antiviral Chemotherapy 52

III. MATERIALS AND METHODS 54

A. Production of the Antiviral Substance from P. cyaneo-fulvum 54

i. Maintenance of the mould 54

ii. Preparation of batches of culture filtrates of the mould 54

B. Extraction and Partial Purification of the Antiviral Substance 56

i. Ammoniumsulphate - Ethyl alcohol method of fractionation 56

a. Culture filtrates from McGill glucose beef heart infusion broth 56
b. Mycelial felt from McGill glucose beef heart infusion broth 58

ii. Acetone method of fractionation 59

a. Culture filtrates of the mould grown in Czapek-Dox broth .. 59
b. Mycelial felt (Czapek-Dox broth) 60
c. Culture filtrates and mycelial felt from McGill glucose beef heart infusion broth 60

iii. Processing of uninoculated Czapek-Dox broth for antiviral activity control 61

iv. Sterilization of the antiviral extracts 61

v. Dry weight determinations..... 61

C. Maintenance, Passage and Titration of Influenza A Virus (PR8 strain).. 62

i. Maintenance and passage of influenza A virus 62

ii. Determintation of the haemagglutinin titre 63

D. Screening of Culture Filtrate Extracts for Antiviral Activity 64

i. Preparation of Modified Maitland cultures 64

ii. Determination of the profile of activity of the extract 67

iii. Toxicity tests 68

iv. Testing the extracts for haemagglutinating and haemagglutination-inhibition activities 69

E. Preparation of Tissue Cultures 70

i. Chick fibroblast cultures 70

ii. Monkey kidney cells 72

F. Techniques for Bacteriophage Studies 72

i. Titration of phage 72

ii. Preparation of standard phage pool 73

G. Chromatographic Techniques 74

i. Preparation of standard silica gel plates 74

ii. Hydrolysis of the samples..... 77

IV. EXPERIMENTAL PROCEDURES AND RESULTS 79

A. Biological Studies with the Culture Filtrates of Penicillium cyaneo-fulvum 79

i. Antiviral activities 79

a. The effect of the antiviral substance on the infectivity of influenza virus in Modified Maitland Cultures 79
b. The effect of the antiviral substance on the infectivity of Newcastle Disease Virus and Influenza B Virus in Modified Maitland Cultures 80
c. In ovo studies with Influenza, Newcastle Disease, Mumps, Vaccinia and Herpes Simplex Viruses 81
d. In vivo studies with Influenza and Vaccinia Viruses 84
e. Tissue culture studies with vaccinia virus and poliovirus 90
f. The effect of the antiviral substance on bacteriophage 92

ii. Haemagglutination and haemagglutination-inhibition studies with the antiviral substance 94

iii. Mode of action of the antiviral substance 95

a. The effect of the antiviral substance on the growth curve of influenza virus (PR8) 97
b. Minimal time for effective adsorption of influenza virus to chorioallantoic membrane 98
c. The effect of the antiviral substance on the adsorption and the penetration of influenza virus 98
d. The effect of the antiviral substance on virus release 99
e. The effect of the antiviral substance on the susceptibility of chorioallantoic membrane to influenza virus infection ... 100
f. The study of viricidal effect of the antiviral substance 101

iv. The dose-response curve with the antiviral substance 102

B. Chemical Studies on the Antiviral Substance 104

i. Chemical composition of the antiviral extract 104

a. Determination of carbohydrate 104
b. Determination of protein 105
c. Determination of ribonucleic acid 106
d. Determination of deoxyribonucleic acid 106

ii. Enzyme and chemical inactivation studies 107

a. Trypsin 107
b. Lipase 107
c. Ribonuclease 108
d. Deoxyribonuclease 108
e. Periodate 108

iii. Chromatographic studies 109

a. Amino acids 110
b. Sugars 112

iv. Colorimetric detection of tryptophan 113

v. Trichloroacetic acid precipitation 114

vi. Lipid analysis 114

C. Physical Treatments of the Antiviral Substance 115

D. Ultraviolet Absorption Spectrum of the Antiviral Substance 116

E. Comparison of Czapek-Dox broth and McGill Glucose Beef Heart Infusion Broth for the Production of the Antiviral Substance 117

F. Growth characteristics of Penicillium cyaneo-fulvum in Czapek-Dox broth and Temporal Elaboration of the Antiviral Substance 119

G. Comparison of the Antiviral Substance of Penicillium cyaneo-fulvum with Other Antiviral Substances of Penicillium Origin 124

V. GENERAL DISCUSSION 125

VI. SUMMARY 143

VII. CONTRIBUTION TO KNOWLEDGE OR CLAIMS TO ORIGINALITY ... 145

APPENDIX i

BIBLIOGRAPHY xi

I. INTRODUCTION AND PURPOSE

Interest in the study of antiviral substances stems from the fact that classical chemotherapy as it is known in bacterial diseases has thus far not been successful in the field of viral diseases. However, any proven antiviral agent is a potential drug against virus infection. Although the ultimate goal of effective chemotherapy of virus infections may or may not be attained, information concerning such substances may provide clues to the nature of host-virus interactions and help to elucidate the patterns of the pathogenesis of virus diseases (Zimmermann and Schäfer, 1960; Melnick and Rapp, 1965) and also to afford tools for the classification of viruses (Tamm and Eggers, 1962; Barry et al., 1962).

Penicillium cyaneo-fulvum was isolated in the Department of Microbiology and Immunology, McGill University, in 1947, as a contaminant of a Lowenstein's slope which was inoculated with a sputum specimen from a tuberculosis patient. The mould was later found to produce a toxin-neutralizing substance, "Noxiversin" (Diena, 1954, 1956; Tanner, 1956, 1957; Murray et al., 1958) and, although some preliminary studies with influenza virus were reported by Diena (1956), detailed virus studies with culture filtrates of the mould were started by Cooke (1958, 1960) who showed that the mould elaborated an antiviral factor different and distinct from noxiversin. The studies on the antiviral substance were continued by Syeklocha (1962, 1964).

The present investigation was designed to explore more fully the antiviral factor of the culture filtrates. The mould has been grown on a simple synthetic medium. This has facilitated the production and the partial purification of the anti-

viral substance, as well as making possible a more definitive study on the nature of the antiviral extract. The spectrum of activity of the substance has been extended and more detailed studies on the mode of action have been conducted. A new and simpler method of extraction and partial purification of the antiviral substance has also been introduced.

II. HISTORICAL REVIEW

1. Introduction

Numerous reports of antiviral agents have appeared in the literature, especially in the last few years, making a comprehensive review almost an impossible task. Excellent and elaborate reviews have also been written on the subject by many authors (Matthews and Smith, 1955; Hurst and Hull, 1956; Tamm, 1956a; Cutting and Furst, 1958; Staehelin, 1959; Sadler, 1963; Wagner, 1963a; Thompson, 1964; Pienta and Groupé, 1964; Kaufman, 1965; Eggers and Tamm, 1966) and in the Department of Microbiology and Immunology, McGill University, by Cooke (1960) and Syeklocha (1964). The present review will therefore not be a catalogue of compounds which have so far been reported to exhibit antiviral activity; rather, selected data pertinent to the problem of host-virus interactions will be discussed in so far as they bear on the concepts of viral replication. Attention will be focused on those antiviral substances that have been shown to be active against the smaller animal viruses, conventionally termed "true" viruses. The agents of the Psittacosis-Lymphogranuloma-Venereum-Trachoma group will thus be excluded, as the majority of workers no longer consider these agents as true viruses (Weiss, 1955; Matthews and Smith, 1955; Wenner, 1958; Andrews, 1964; Moulder, 1964; Weiss et al., 1964) but rather to consider them as more akin to bacteria.

The term "antiviral" has been used in various contexts in the literature so as to make its meaning a bit confusing. The term is used, for instance, to cover a wide spectrum of substances ranging from purely virucidal agents to substances that exhibit their antiviral activity by interfering with the host cell metabolic

mechanisms. A working definition seems therefore to be desirable for the purposes of this review. Antiviral substances are here defined as substances, either synthesized or naturally occurring biological products, which when introduced into a particular host-virus system have the ability to block or suppress virus multiplication, or to cure or ameliorate virus infection. Passively administered specific serum or immunologically active serum fractions and substances that exert a direct virucidal effect on the inert virus particle do not come within the scope of this definition. Moreover, the last group of substances hold little promise in virus chemotherapy (Kaufman, 1965).

Investigation of antiviral agents has followed two major lines of approach, viz., the academic approach and the empirical approach. The former is based on the reasonable supposition that analogues of precursors of essential building blocks of nucleic acids and proteins could interfere with viral replication by their antagonistic action against the corresponding normal precursors; the latter method of approach is based on empirical screening of a vast number of agents for possible antiviral activity. Both approaches have yielded interesting antiviral agents that are directed at different targets in the phases of the replicative cycle of viruses.

2. Antiviral Agents and the Phases of Virus Multiplication

a) Phases of Adsorption and Penetration

Viruses multiply exclusively within the living host cell and, in order to gain access into the interior of the host cell, the virus particles must first penetrate the host cell membranes. Adsorption to the host cell is mediated through electrostatic forces, van der Waals forces, hydrogen-bonding and receptor sites. For stable adsorption to take place there should be both electrostatic and geometric

complementarity between the adsorbing surfaces. The lack of the above complementarity has been advanced as a possible explanation for the insusceptibility of certain lines of cells to virus infection (McLaren et al., 1959a, 1959b) since naked nucleic acid extracted from such virus particles infects both susceptible as well as insusceptible cells (Holland et al., 1959; Mountain and Alexander, 1959) by circumventing the requirements of specific receptor sites. The progeny virus resulting from such an infection is serologically indistinguishable from the parent virus supplying the nucleic acid.

Unlike the well-documented syringe mechanism by which bacteriophages inject their nucleic acid into the host cell, relatively little is known about the details of how animal viruses get their genome into the host cells. It is also possible that the mechanism may vary from one animal virus to another (Dales, 1965). However, for the influenza-type receptors, one of the best studied sites, Fazekas de St. Groth (1948a) has proposed that the virus particles penetrate the cell by a process of "viropexis", a process analogous to "pinocytosis" (Lewis, 1931) by which cells ingest colloidal particles. The concept of viropexis seems to be widely accepted; the strongest evidence has come from electron microscopy (Dales and Choppin, 1962; Dales, 1965).

Because the phases of adsorption and penetration are very crucial in any successful virus infection, Cohen (1963) has suggested that attempts to block viral replication at these early stages may, in fact, be more advantageous than at the later stages of the replicative cycle. Several antiviral agents have been reported to affect these early stages of virus infection with varying degrees of success.

Receptor Destroying Enzyme

Myxoviruses possess an hydrolytic enzyme, neuraminidase, on their surfaces. The enzyme splits N-acetylneuraminic acid from myxovirus receptor sites. The action of the viral enzyme is mimicked by an exo-enzyme originally isolated from Vibrio cholerae and termed Receptor Destroying Enzyme (RDE) (Burnet, 1948). The enzyme has also been isolated from Clostridium welchii (McCrea, 1947) and Lee and Howe (1966)have recently presented substantive evidence for its presence in cultures of D. pneumoniae. Substrate destruction by the enzyme rendered the receptor sites no longer able to adsorb virus particles, at least for some time.

Treatment of susceptible host cells with RDE completely or partially inhibited infection of such cells by myxoviruses (Stone, 1948a; Cairns, 1951). However, such cells are not permanently impaired since the receptor sites are regenerated with time (Stone, 1948b; Fazekas de St. Groth, 1948b; Finter et al., 1954).

Carbohydrate Substances

Certain carbohydrate substances inhibit virus infection because they act as analogues of the receptor substances of cells which are susceptible to infection. Their success as inhibitors therefore depends on how firmly they bind the virus receptor sites (Woolley,1953). Such soluble carbohydrate inhibitors are usually, in nature, found complexed with other macromolecules such as muco-protein, muco-polysaccharides, glyco-proteins, or lipo-protein-polysaccharides (Burnet et al., 1947; Ginsberg, 1960). Among the myxoviruses such substances usually, but not always,both inhibit haemagglutination without exhibiting neutralizing activity (Ginsberg et al., 1948a); and the

active component is sometimes associated with N-acetylneuraminic acid. However, the possession of the acid does not seem to , ipso facto, make a substance an inhibitor: Orosomucoid (Mayron et al., 1961) is attacked by neuraminidase, but it is not an inhibitor. On the other hand, the ganglioside of brain (Bogoch, 1957) is an inhibitor, but not a substrate for the enzyme.

Cohen (1960) showed that an inhibitor from horse serum protected mice from the lethal action of influenza virus. The inhibitor was most effective when given prior to virus infection; the intranasal route of administration seemed to be superior to the other routes tried. By this route the substance was active even when given 48 hours before virus and acted by blocking the virus receptor sites.

Hollos (1961) coupled aniline derivatives by diazonium linkage to purified alpha-inhibitor preparation and showed that the modified inhibitor combined with influenza virus more firmly than did the original alpha-inhibitor. The aniline-inhibitor complex was able to reduce the multiplication of influenza virus in the embryonated chick egg, even when injected 30 minutes after virus infection.

A sulphated polysaccharide extracted from agar inhibited the cytopathogenic effect (CPE) caused by type 2 dengue virus on KB cell cultures (Schulze and Schlesinger, 1963). It also inhibited plaque formation by the virus as well as its haemagglutinating activity. Mice inoculated intracerebrally with the virus were also protected. A similar polysaccharide was also shown to interfere with the cycles of infection of encephalomyocarditis virus in vitro (Liebhaber and Takemoto, 1963). The mode of action of the polysaccharide seemed to be by a direct combination with the virus particles, thereby interfering with virus adsorption. Schulze (1964) has suggested that this may be due to the interaction of charged groups on the virus protein coat with complementary ionic groups on the polysaccharide.

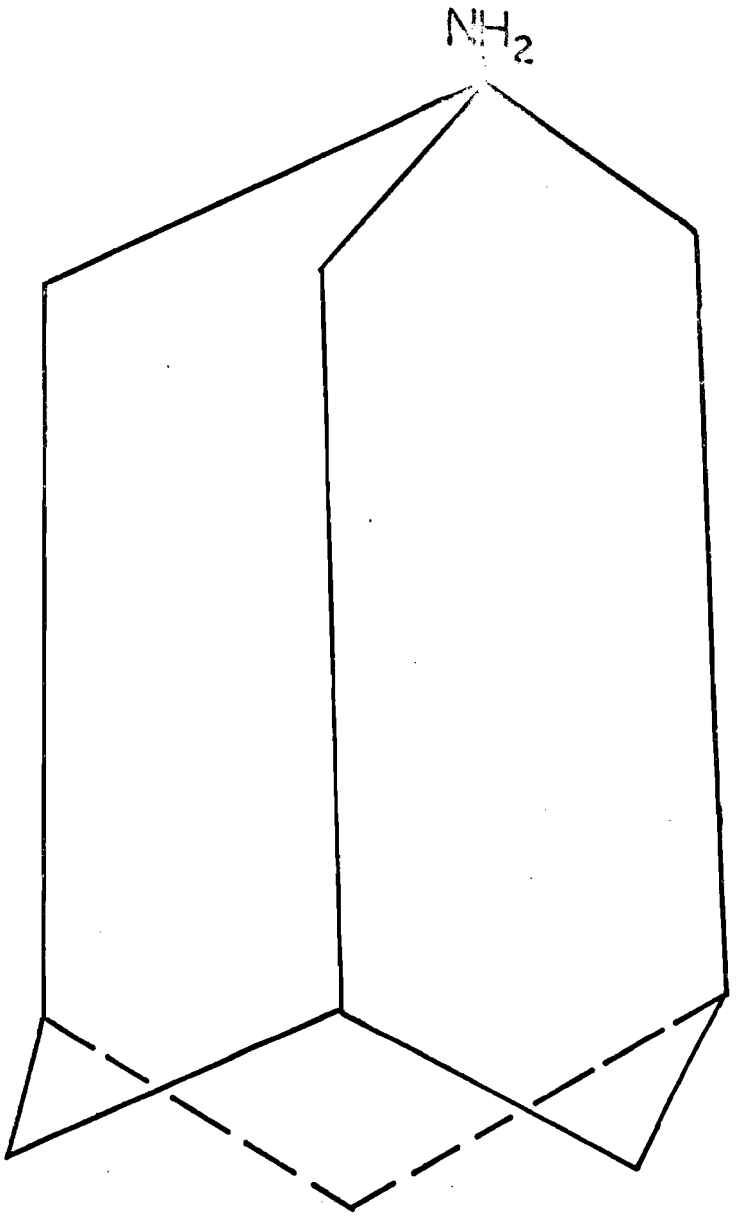
An extract from kelp containing both carbohydrate and protein components has been shown to be a noncompetitive inhibitor of neuraminidase activity (Kathan, 1965) and it inhibited the multiplication of influenza virus in embryonated eggs, probably by preventing the penetration of the virus into the cells.

The development of resistant virus strains has been reported following the use of certain polysaccharides. Ginsberg and Horsfall (1949) established that serial passage of mumps virus, sensitive to inhibition by capsular polysaccharide of Friedländer bacilli in the presence of the inhibitor, resulted in the emergence of a resistant variant. Back mutation to the sensitive strain was demonstrated upon passing the resistant variant in the absence of the polysaccharide.

Synthetic Substances

Ackermann and Maassab (1954a and 1954b) demonstrated that the processes of adsorption and penetration of chick embryo cells by influenza virus were inhibited by α-amino-p-methoxyphenylmethane sulphonic acid (AMPS). Working with pieces of chorioallantoic membrane (CAM), these workers showed that AMPS was effective when added within 30 minutes of virus inoculation; when added after this time AMPS was no longer active in inhibiting virus multiplication but the final yield of virus was reduced. In another series of experiments Ackermann et al. (1955) showed that the presence of AMPS or the pretreatment of the CAM with RDE did not completely inhibit the ability of the CAM to bind some virus particles. However, when the CAM was first treated with RDE and then exposed to AMPS effective binding was completely prevented. From this information two types of receptor sites for the virus were suggested for the CAM, one sensitive to RDE and the other sensitive to AMPS. Since these two substances are

Figure 1



1- ADAMANTANAMINE (Crystal)

individually capable of inhibiting virus multiplication, it was proposed that adsorption to both sites was necessary in the infective process. But Cohen (1963) has proposed an alternative explanation, which obviates the necessity for introducing a second type of receptor site. In his hypothesis Cohen has suggested that the results with RDE may be only a quantitative effect of the enzyme, and that the AMPS may be acting by blocking virus penetration.

One-adamantanamine-HCl (Amantadine), a synthetic organic compound of molecular weight 187, is a stable, colourless, crystalline primary amine of an unusual symmetrical structure (Fig. 1), developed by the scientists of E.I. du Pont de Nemours & Company of Wilmington, Delaware, as a new anti-influenza drug. Because of its many interesting properties and prospects in viral chemotherapy, amantadine has become one of the foci of greatest interest in the field. Amantadine has been shown to possess significant and reproducible antiviral activity under a variety of cultural conditions. Its activity appeared to be specific for strains of influenza A viruses (Davies et al., 1964) with considerable variation in the degree of sensitivity (Schild and Sutton, 1965), although a strain of influenza C, parainfluenza 1/Sendai and pseudorabies, were also found to be sensitive (Neumayer et al., 1965; Hoffmann, 1964). Cochran and Maassab (1964) showed that the growth of rubella virus in tissue culture was also inhibited by amantadine. In mice inoculated intranasally with sensitive strains of influenza viruses, amantadine has been shown (Grunert et al., 1964; 1965) to exhibit both a delaying effect in the time of death and a sparing effect in the number of survivors of the infection. It was equally effective when given by oral or intraperitoneal routes and generally effective when given simultaneously with the virus, although it could also protect when given as

late as 72 hours post-infection.

The greatest antiviral activity of amantadine was demonstrated when the compound was present throughout the incubation period of the infection. Neumayer et al. (1965) made the striking observation that the addition of as much as 40 ug/ml of the compound shortly after infection of chick embryo cells with influenza A/WSN only reduced plaque formation whereas pretreatment with a dose as small as 0.8 to 1.6 µg/ml resulted in complete plaque suppression.

Amantadine treatment did not cause complete inhibition of virus multiplication. Mice surviving infection as a result of amantadine treatment were immune to a challenge infection with the original infecting virus (Davies et al., 1964). Davies and his colleagues also showed that the activity of amantadine appeared to be enhanced when the compound was administered in conjunction with specific antiserum. This observation resulted in the speculation that antibody may also play some part in the antiviral activity of amantadine demonstrated in vivo. Davies et al. (1966) studying the antibody response in influenza-infected, amantadine-treated mice found that when the primary infecting virus dose was high, treated survivors were generally resistant to challenge virus due to antibody production elicited by those virus particles that escaped the inhibitory effects of the compound. However, the picture was different when the primary infecting virus inoculum was small. In this group, because of a complete prevention of infection by the compound, some of the treated mice escaped an immunizing infection. Cochran et al. (1965) showed, by impairing early antibody synthesis in mice by X-ray irradiation, that specific antibody was not a necessary complement to the antiviral activity of amantadine in vivo. They

also showed that the locus of activity of amantadine was identical with that of AMPS.

Amantadine has been shown to act by blocking virus penetration into the host cell (Davies et al., 1964; Hoffmann et al., 1965; Schild and Sutton, 1965). It did not have any effect on influenza virus neuraminidase activity on red blood cells (Hoffmann et al., 1965) and did not modify the receptors on the red cells for myxovirus attachment (Schild and Sutton, 1965). Schild and Sutton also showed that recently-isolated influenza A strains of virus were generally more sensitive to amantadine than those that have undergone some laboratory passages.

Clinical trials with the drug have been reported by Wendel (1964); a group of 850 prisoner volunteers were fed the drug in doses of 200 mg for 10 to 13 days. In prophylactic trials only 1.1% of the drug-treated subjects became ill. There were no obvious side effects. The drug was rapidly absorbed by the tissues and in man up to 90% of the amount administered orally could be recovered from the urine unchanged (Bleidner and Hermann, 1964).

Influenza virus readily developed resistance to amantadine in vitro (Cochran et al., 1965). However, resistant variants have not been demonstrated in in vivo experiments in mice (Grunert et al., 1965; Cochran et al., 1965).

A second compound, 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate, has also been shown by Fletcher et al. (1966) to be similar to amantadine in both mode of action and spectrum of antiviral activity.

Nishmi (1966) showed that a compound termed "cephaloridine" interfered with the adsorption of vaccinia virus in tissue culture; at near toxic dose

cephaloridine produced complete suppression of plaque formation by the virus.

Caprochlorone has been shown to be highly effective against influenza virus multiplication in the de-embryonated egg (Liu et al., 1957a) and in mice infected intranasally with the virus (Liu et al., 1957b). In both test systems the compound was most effective when present during the early phases of virus replication, and had to be present throughout the entire replicative cycle. The compound had to be used in relatively large doses. Its efficacy in mice was greatest when a virus dose of 10 Lethal Doses Fifty (10 LD_{50}) or less was used. A synergistic effect of caprochlorone was demonstrated with specific antiserum. The compound suppressed, but never completely blocked, virus multiplication in the mouse lungs. It was also found to be more effective in the deembryonated egg than in mice. It was not virucidal and did not inhibit virus adsorption to the host cell. Liu and colleagues therefore suggested that its action was directed at an intracellular stage of the replicative cycle of the virus. More recent evidence by Stanfield et al. (1965) has more specifically delineated the site of action of caprochlorone. The greatest activity of the compound was achieved when it was added during the first 15 minutes of infection, at 37°C. Caprochlorone thus has a reaction time similar to that of antiserum. Based on this and other evidence it was concluded that the compound acts by stopping virus penetration. A comparative study of caprochlorone, 1-Adamantanamine and ammonium ions has suggested an identical locus of action for all three substances (Stanfield et al., 1966).

Synthetic lysine polypeptides have been reported to inhibit the multiplication of influenza, mumps and Newcastle disease viruses in the chick embryo

(Rubini et al., 1951; Green and Stahmann, 1953; Tsuyuki et al., 1956; Green et al., 1953). The multiplication of infectious bronchitis virus was also inhibited (Green et al., 1953). The mode of action is by a direct interaction of the polypeptide with virus receptor sites, thereby inhibiting infection. The greatest effect was demonstrated when polypeptide and virus were given simultaneously or the peptide injected prior to virus inoculation.

b) Eclipse and Latent Phases and Phase of Maturation

i. Discussion of "Eclipse", "Latent" and "Maturation"

The concept of a latent or lag phase in the multiplication cycle of viruses is now universally accepted. But there seems to be confusion in the literature in the use of the terms "ECLIPSE", "LAG", and "LATENT". The following distinctions are, therefore, made to facilitate discussion:

Eclipse

The first phase after infection in which the virus undergoes a conversion into a state that is not readily detectable but forms a necessary step in the subsequent events of the replicative cycle (Burnet, 1955; Isaacs, 1959; Waterson, 1961). This may represent the stage when the viral nucleic acid becomes separated from the protecting protein coat (Isaacs, 1963). Joklik (1965) has limited the eclipse phase to the period between adsorption and penetration of the virus particle and the subsequent accumulation of viral structural proteins, the major event during the period being the uncoating of the viral protein and the loss of identity of the virus particle as such.

Latent or Lag

This represents the time between the initiation of infection and the

emergence of the newly formed virus particles (Isaacs, 1959; Horsfall, 1959; Hollinshed and Smith, 1958). Thus, from its limitations the latent phase also includes the earlier eclipse phase and is a period characterized by active biosynthetic processes and synthesis of virus precursors probably by a diversion of the host's metabolic processes.

Maturation

The different viral subunits synthesized during the previous phases are assembled into intact virus particles and prepared for release from the host cells.

ii. Antagonists of Nucleic Acid Synthesis

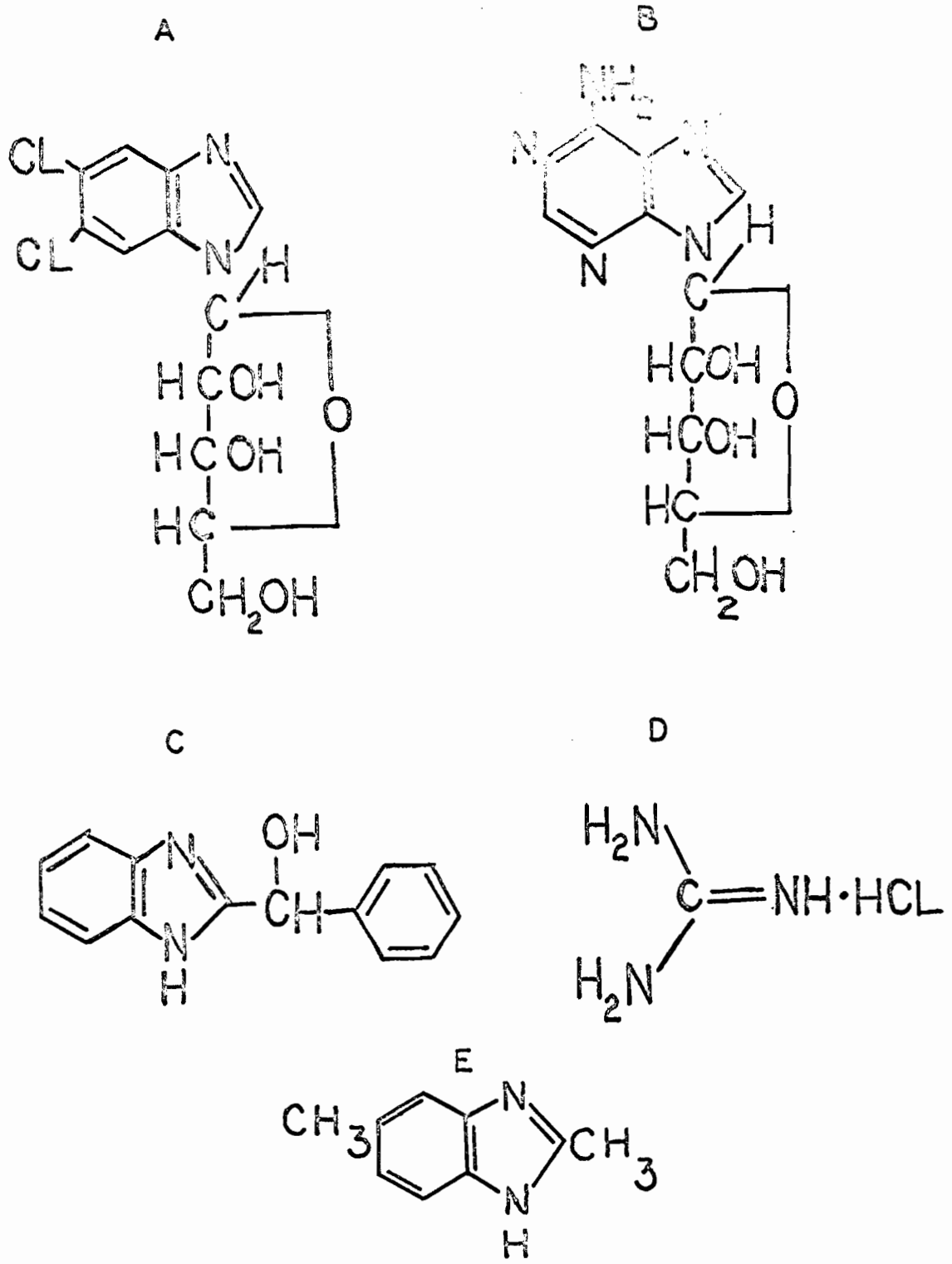
One of the central concepts of modern virology is that the infectivity of any virus particle is a direct function of its nucleic acid component. The so-called "rational" approach to viral chemotherapy is therefore directed towards finding means of modifying the nucleic acid selectively.

The antagonists chosen for discussion are substances that have undergone some modifications in either their purine or pyrimidine moiety and consequently have acquired some antiviral properties. In addition, the effects of actinomycin D will be discussed, since it has a direct bearing on nucleic acid synthesis.

Benzimidazoles

Alterations in the purine bases have been shown (Tamm et al., 1952) to exert some selective inhibitory activity on virus multiplication. A number of benzimidazoles were synthesized and tested against influenza virus cultivated on pieces of chorioallantoic membrane of the embryonated egg maintained in a simple glucosol medium (Tamm et al., 1953a; 1953b). The following benzimidazoles turned out to be

Figure 2



Chemical structures of adenosine and four specific virus inhibitors: 5, 6-Dichloro-1- β -D-ribofuranosyl-benzimidazole (A), Adenosine (B), 2-(α -Hydroxybenzyl)-benzimidazole (C), Guanidine Hydrochloride (D), 2,5-Dimethyl-benzimidazole (E).

of special interest: 5, 6-dichloro-1-B-D-ribofuranosyl-benzimidazole (DRB), 2-(∝-hydroxybenzyl)-benzimidazole (HBB), and 2,5-dimethyl-benzimidazole (Fig. 2).

There seems to be a relationship between the structure of the benzimidazoles and their inhibitory activity (Tamm et al., 1953c; Tamm and Overman, 1957; Tamm et al., 1961; Tamm and Eggers, 1963a). The position, nature and number of the substituent groups seem to be of decisive importance. For the pentose moiety, the highest inhibition was reported when the sugar was ribose in the form of a beta linked, ribofuranose ring. Tamm and Overman (1957) suggested that the substituted benzimidazoles exerted their inhibitory activity by directly affecting nucleic acid synthesis.

The alkylated and halogenated benzimidazoles were originally considered to be antagonists of vitamin B_{12}, which also contains a benzimidazole nucleoside moiety, 5, 6-dimethyl-1- ∝ -ribofuranosyl-benzimidazole. However, none of the results of the studies on the activity of the benzimidazole derivatives link the virus inhibitory activity with the function of the vitamin (Tamm et al., 1952; Tamm and Eggers, 1963b) and it failed to reverse the inhibitory action of the compound.

DRB prolonged the latent period of the multiplication of influenza virus and also reduced the final virus yield. It was found to be 35 times more active than 2, 5-dimethyl-benzimidazole. Exposure of the tissue for three hours before the inoculation of virus did not cause a greater inhibitory activity than when virus and drug were added simultaneously. This indicated that the speed of the penetration of the compound into the tissue was perhaps not important in the final action of the drug

(Tamm and Tyrrel, 1954). In contrast with 2, 5-dimethyl-benzimidazole, the inhibitory action of DRB persisted after the transfer of the infected and treated tissue to fresh medium (Tamm et al., 1954). Structurally, DRB may be considered an analogue of adenosine and it has been shown to inhibit the incorporation of adenosine into RNA (Tamm et al., 1960; Tamm and Eggers, 1963a). Because DRB inhibited both the RNA-containing influenza virus and the DNA-containing adenovirus and vaccinia virus, Tamm and colleagues suggested the participation of RNA synthesis in the replication of DNA-containing viruses. This has been confirmed by Ikegami et al., (1960).

HBB has been shown to be a very selective inhibitor of the picornaviruses (Eggers and Tamm, 1961; Tamm et al., 1961; Eggers and Tamm, 1962). It was found to be much more selective in its antiviral activity than DRB. It inhibited both the multiplication and the cytopathogenic effect (CPE) of the picornaviruses in tissue culture. HBB is not a metabolic antagonist and its inhibitory action was reversed by removing it from the culture medium (Tamm et al., 1961).

The action of HBB is similar, but not identical, to that of another selective inhibitor of picornaviruses, guanidine (Tamm and Eggers, 1962; Crowther and Melnick, 1961). A grouping of these viruses has been suggested based on their sensitivity to HBB and guanidine (Tamm and Eggers, 1962). Both compounds have been shown to act by inhibiting the synthesis of RNA-polymerase in virus-infected cells (Baltimore et al., 1963; Tamm and Eggers, 1963b). The result of such an inhibition would be the prevention of the synthesis of new viral RNA, and consequently no viral coat protein would be formed owing to the lack of the appropriate messenger

RNA. Viral RNA is unique in this aspect since it serves as it own genetic determinant.

Because HBB and guanidine are structurally very different (Fig. 2) the similarity in their mode of action has stimulated interest and it has been shown that both compounds may have different loci of activity (Tamm and Eggers, 1962; Eggers and Tamm, 1963a).

HBB has been shown to be effective in mice inoculated with a very small dose of poliovirus (Hollinshed and Smith, 1958). The effects in mice and monkeys, however, have been minimal and in certain cases enhancement of the infection has been reported (Brown et al., 1953). O' Sullivan et al., (1964a) showed that the D-isomer of HBB is superior to the DL-isomer in protecting tissue culture cells from the CPE of any one of the three types of polioviruses. On the basis of inhibition by guanidine and resistance to HBB foot-and-mouth disease virus has been shown to be similar to the Coxsackie A viruses (Pringle, 1964).

Some benzimidazoles for example 5-methyl-2-d-ribobenzimidazole, enhanced instead of inhibited the growth of influenza virus in the chorioallantoic membrane but inhibited the multiplication of vaccinia virus in the same test system (Tamm, 1956b).

Frequent emergence of drug-resistant and drug-dependent variants have been reported following the exposure of sensitive viruses to HBB or guanidine. Eggers and Tamm (1961) demonstrated that serial passage of picornaviruses in the presence of HBB resulted in the appearance of resistant variants. It was suggested that the virus inoculum was heterogeneous with respect to sensitivity to the drug and

that the CPE often observed in drug-treated cultures after long incubation may be due to the action of the resistant variants of the virus population.

In addition to drug-resistance among the picornaviruses it was also discovered that some of the viruses were dependent upon HBB for their replication (Eggers and Tamm, 1963b). Infectious viral RNA extracted from these drug dependent strains, showed a dependence on the drug for replication; the progeny virus was also drug-dependent. Thus drug-dependence as well as drug-resistance was genetically determined. Moreover, back mutations among the drug-dependent variants was either to drug-resistance or to drug-sensitivity.

Melnick et al. (1961) showed that poliovirus passaged in the presence of guanidine hydrochloride rapidly develops resistance to the drug and may become as much as 10,000 times more resistant to the drug. However, among the picornaviruses it was shown that there are differences in the guanidine "marker" (Crowther and Melnick, 1961). The attenuated types 1 and 2 vaccine strains of poliovirus were more sensitive to the drug than the virulent strains but the attenuated type 3 oral vaccine strain was as sensitive as the corresponding virulent strain.

Guanidine-dependent polioviruses have also been reported (Nakano et al., 1963; Ledinko, 1963) with a corresponding drug-dependence exhibited by the extracted infectious RNA (Nakano et al. 1963).

The emergence of drug-dependence is correlated with the loss of neurovirulence (Loddo et al., 1964). A guanidine-dependent poliovirus failed to produce the characteristic paralysis in monkeys after either intramuscular or intracerebral inoculation. Evidence for the multiplication of the virus in the monkey was afforded

by the fact that specific antibodies developed in the inoculated animals and they were resistant to a subsequent challenge with a virulent poliovirus.

It is of clinical importance that there is very little cross resistance among the picornaviruses with respect to HBB and guanidine (Tamm and Eggers, 1962). Moreover, synergism has been demonstrated between the two drugs (Eggers and Tamm, 1963a). This would mean that the sites of action of the two drugs are different.

Thiosemicarbazones

The antiviral activity of thiosemicarbazone was first discovered by Hamre and colleagues (1950). They demonstrated that benzaldehyde thiosemicarbazone, administered orally or intraperitoneally, protected mice infected by the intranasal route with vaccinia virus. These observations were later confirmed by Thompson et al., (1951), who also showed that benzaldehyde thiosemicarbazone when fed in the diet protected mice infected intracerebrally with vaccinia virus and prevented the multiplication of the virus in chick embryo tissues. The effects of the compound on neurovaccinia were further studied by Thompson et al.(1953a; 1953b), who, for the first time, demonstrated marked anti-vaccinia activity with heterocyclic derivatives of the parent compound. They showed that isatin thiosemicarbazone (ITSC) possessed a potent anti-vaccinia activity and that the $= N - NH - CS - NH_2$ group appeared essential for the activity of the compound. Although ITSC was very active in mice it failed to prevent vaccinial skin lesions in the rabbit.

Despite the fact that ITSC acts as a potent selective inhibitor of the pox group of viruses it showed only a very moderate antiviral activity against ectromelia virus, which is antigenically very closely related to vaccinia virus (Sheffield et al., 1960). However, by modifying the parent molecule a number of derivatives were

produced which did exhibit anti-ectromelia activity (Bauer and Sadler, 1961) as for example 4' : 4' - dialkythiosemicarbazones of isatin, N-methylisatin, or N-ethylisatin. The best results were obtained when the virus was inoculated intracerebrally; intraperitoneal inoculation produced rather irregular results. The exact mode of action of ITSC is not yet known although some information is available in this respect. Electron microscopic studies have revealed that, in infected cells treated with ITSC, there was an accumulation of immature and abnormal forms of virus particles (Easterbrook, 1962; O'Sullivan et al., 1964b; Sadler, 1965). The compound did not prevent adsorption of virus to the cells and was not virucidal. This evidence also points to an intracellular locus of action. Although ITSC-treated cells formed no infective virus particles, such cells did form some soluble viral antigens and viral DNA (Easterbrook, 1962; Bach and Magee, 1962). Appleyard et al. (1965) also demonstrated the synthesis of these virus-specific macromolecules in HeLa (ERK) cell cultures which were infected with ectromelia virus and treated with ITSC. They demonstrated the presence of viral DNA with acridine orange staining and the soluble antigens by immunodiffusion. Both Easterbrook (1962) and Bach and Magee (1962) used the incorporation of tritiated thymidine into DNA as evidence of DNA synthesis in ITSC treated cells. These findings indicate that ITSC may be exerting its inhibitory action at a late stage in the replicative cycle of the virus. Appleyard et al. (1965) showed that the inhibitory action of ITSC was prevented by both DL-p-fluorophenylalanine and actinomycin. It was hypothesized that ITSC induced the formation of a new RNA which, in turn, directed the formation of a new protein which might be the actual inhibitor of virus multiplication. Pollikoff et al. (1965) failed to demonstrate any interferon or antibody in the brains of ITSC treated mice prior to 10 days after infection.

Other possible modes of action of ITSC have been suggested; these include its intercalation into the DNA helix (Sadler, 1965) or the chelation of copper ions by virtue of the sulphur in the side chain of ITSC (Bauer and Sadler, 1960); in viruses like vaccinia virus where copper is a natural constituent such a process of chelation would result in the uptake of more ITSC.

Some cells are refractory to the action of ITSC and, of all the cells tested, HeLa (ERK) cells were most sensitive (Appleyard et al., 1965). Prevention of CPE in tissue culture may be due to the localization of the infection to the cells initially infected (Sheffield, 1962; Sheffield et al., 1960).

The action of ITSC contrasts with that of HBB and guanidine, in that with the latter compounds the synthesis of almost all viral components are suppressed (Tamm, 1965).

ITSC has had a very encouraging clinical trial in Madras, India (Bauer et al., 1963); among 1126 vaccinated but untreated contacts there were 78 cases of smallpox with 12 fatal cases, while among 1101 vaccinated and treated contacts there were only 3 mild cases of smallpox. A dramatic improvement in a case of eczema vaccinatum has also been reported following ITSC therapy (Turner et al., 1962).

Until recently no report appeared in the literature about any ITSC-resistant poxvirus. However, Appleyard and Way (1966) have shown that rabbitpox virus can develop resistance to the drug. Resistant variants developed after passing the sensitive virus 16 times in HeLa (ERK) cells or 3 times in mice in the presence of the drug. The resistance appeared stable since two subcultures in the absence of the drug

showed no reversion to the sensitive strain. A cross resistance between isatin β-thiosemicarbazone, N-methyl-isatin β-thiosemicarbazone (Marboran) and 4-bromo-3-methylisothiazole-5-carboxaldehyde thiosemicarbazone (M and B 7714) was also demonstrated. This would mean that the mode of action of these three thiosemicarbazones is similar.

Halogenated Nucleosides

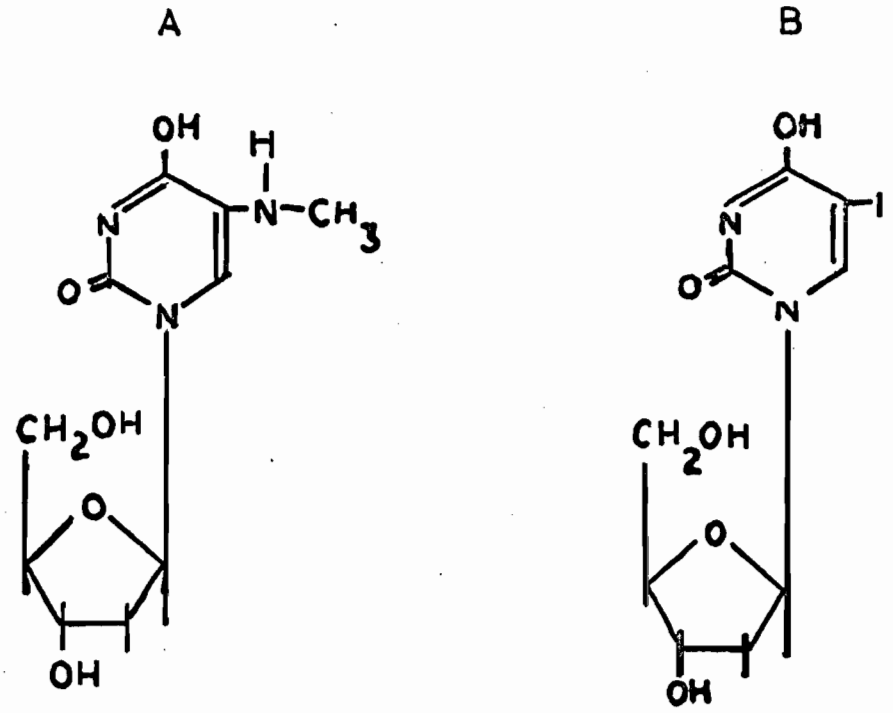
Virus infection of a host cell sets off a train of biochemical events which, among other things, includes the syntheses of enzymes and virus precursor components and the assembly of these components into intact virus particles culminating either in the death of the cell or impairment of its function. The syntheses of these new virus-induced enzymes have been reported for many systems (Kit et al., 1962; McAuslan and Joklik, 1962; Franklin and Baltimore, 1962; Baltimore et al., 1963). A significant observation, especially in the light of possible viral chemotherapy, was made by McAuslan (1963) who found that the thymidine kinase synthesized as a result of pox virus infection possessed certain distinctive characteristics as compared to the same enzyme in normal tissues. The virus-induced enzyme was more thermostable and had a lower Michaelis constant. The possibility therefore exists that certain antiviral agents could preferentially inhibit the virus-induced enzymes without concomitantly affecting the host's normal enzyme systems thereby effecting selective inhibition of virus replication.

The halogenated nucleosides seem to have met some of these conditions. In general these substances act either by inhibiting the function of the enzymes involved in the synthesis and polymerization of virus precursor macromolecules or by

being incorporated into DNA or RNA which results in the formation of "fraudulent", non-functional nucleic acid. They are able to do this by virtue of the similarity of the Van der Waals' radii of the halogen substituent in the 5-position of the benzene ring and the corresponding groups in the normal nucleoside (Prusoff et al., 1965).

An analogue of thymidine, 5-iodo-2'-deoxyuridine (IUDR), first synthesized by Prusoff (1959), exerts its action by inhibiting the kinases of thymidine and thymidylic acid as well as DNA polymerase (Delamore and Prusoff, 1962). The antiviral activity of IUDR has attracted a lot of interest since its chemotherapeutic potentialities were reported by Kaufman (1962, 1963) and Kaufman et al. (1962) against herpes simplex and vaccinia infections. The drug was found effective in curing localised virus infections such as herpetic keratitis and ocular lesions produced by vaccinia virus. Animals in which treatment was begun before virus inoculation or up to 12 hours post-infection failed to develop definite herpetic infection. When treatment was delayed up to 24 hours after virus infection keratitis was inhibited and the lesions, if present, were cleared except for tiny non-progressive dentritic foci which also began to clear up after 1 to 2 days of therapy. A delay of therapy for 48 hours resulted in a very severe keratitis in all animals and irititis in most of them. But these, however, also clear up after 48 hours of therapy. The drug is usually administered as a saturated solution at near neutral pH and is given every two hours both day and night. The 5-bromo-, but not the 5-fluoro- compound was also active. The 5-chloro-2'-deoxyuridine was also found to be active. However, the bromo- and the chloro-derivatives were not as suitable for clinical use as was the iodo-derivative partly

Figure 3



Chemical structures of 5-methylamino-2'-deoxyuridine (MADU) (A) and 5-iodo-2'-deoxyuridine (IUDR) (B).

because of their irritant nature. IUDR could replace as much as 18% of vaccinia viral DNA-thymidine by being incorporated into the viral DNA (Prusoff et al., 1963).

A recently synthesized compound, 5-methylamino-2'-deoxyuridine (MADU) has been used to treat herpes simplex keratitis in the rabbit (Nemes and Hilleman, 1965). This compound eliminates the introduction of a halogen atom (Fig. 3). It is reported to have a low toxicity and is minimally incorporated into host cell DNA.

In addition to herpes simplex and vaccinial infections, IUDR has been shown to suppress subcutaneous tumors induced by adenovirus type 12 in hamsters (Huebner et al., 1963). However, the compound had to be given at the same subcutaneous site as the virus and in relatively large doses.

Studies of herpes-virus cell interactions in the presence of IUDR were conducted by Smith (1963) and Smith and Dukes (1964) and Siminoff (1964). The picture that emerged from these studies is that the principal antiviral activity of the drug is due to an interruption in the assembly of intact virus particles and not in the production of viral components. IUDR inhibited the replication of virus already underway but was ineffective against infectious virus already formed. In tissue culture, although the production of infectious virus was arrested, the CPE caused by the virus was not prevented. It was also shown that most of the activity of the drug was exerted during the eclipse and latent phases; there was no complete suppression of virus replication since some stable resistant variants escaped suppression. A complete eradication of virus was not demonstrated in the rabbit cornea infected

with herpes simplex virus and treated with maximum concentrations of IUDR (Jawetz et al., 1965). Upon the termination of treatment the virus content of the treated corneas rose and may have contained even more viable virus than the untreated control corneas. Some strains of the virus, although susceptible to the drug in other test systems, were resistant in human infections.

Recent studies by Prusoff et al. (1965) have elucidated some of the molecular bases for the antiviral activity of IUDR. IUDR is converted to the corresponding mono-, di- and tri-phosphate. The triphosphate, along with the normal host cell deoxythymidine triphosphate, is polymerized into DNA by DNA polymerase. The same sequence of events apply to the 5-bromo-2'-deoxyuridine (BUDR). The picture seems to be different for 5-fluoro-2'-deoxyuridine (FUDR), the corresponding monophosphate of which, like the normal deoxyuridylic acid, has great affinity for the enzyme thymidylic synthetase (Delamore and Prusoff, 1962; Kaufman, 1965). However, after conversion to the free fluorouracil and subsequent conversion to the corresponding triphosphate, FUDR can also be incorporated into RNA (Prusoff et al. 1965).

With pseudorabies virus Kaplan et al. (1965) have shown that although both IUDR and BUDR were incorporated into the virus DNA, the synthesis of viral DNA progressed at the same rate in both treated and untreated cells and viral antigens were also synthesized in the presence of the drugs. No infectious virus particles were formed under these conditions; in contrast with the BUDR-treated cells little assembly of viral components occurred in the IUDR-treated cells. It has been suggested that this was due to the transmission of incorrect information from the sub-

stituted DNA and not directly to its structure.

BUDR does not appear to exhibit uniform inhibition among the DNA-containing viruses and, even among the poxviruses, there are variations in susceptibility to the compound (Easterbrook and Davern, 1963).

Kjellén (1965) showed that the concentration of BUDR that inhibited the production of infectious adenovirus type 5 in a line of human cells was the same as the concentration of the compound that prevented cell growth. Thus the cell type also seems to be an essential factor in the activity of BUDR.

Kjellén (1962) also found that with FUDR the concentration required to inhibit adenovirus multiplication was about 100 times smaller than the amount that would prevent cell proliferation. With FUDR both the CPE caused by the virus and the formation of virus antigens were inhibited. The situation appears to be different with BUDR where, although the production of infectious virus was inhibited, virus antigens were formed and the CPE was not prevented. However, even with FUDR there appears to be a number of variations in the efficacy of the compound in relation to adenoviruses. Green (1962) and Flanagan and Ginsberg (1962) reported complete inhibition of adenovirus types 2 or 4 in the presence of FUDR; it was effective even when added to cultures as late as 10 hours post-infection (Flanagan and Ginsberg, 1962). The inhibition was completely reversed by thymidine. Seto et al. (1964) failed to obtain a complete inhibition of adenovirus type 1 with the compound. It was suggested that FUDR is inhibitory to adenovirus when low virus doses are used and when the antiviral effect is assayed within the first growth cycle of virus replication. It was also suggested that the effect is through interruption of virus maturation since increase in the virus yield was demonstrated after the compound was removed from the

culture. With vaccinia infection Loh and Payne (1965a) showed that although FUDR prevented the formation of infectious virus as well as the haemagglutinin, the formation of both the nucleoprotein and the heat-labile-heat-stable (LS) antigens was not prevented.

Zalmanzon and Liapunova (1963) showed that 5-fluorouracil (FU) inhibited the multiplication of adenovirus type 5 and that the effect was exclusively through the inhibition of viral DNA synthesis. Removal of the compound from the culture medium after 20 hours contact with the cells failed to restore the ability of the cells to produce virus. However, the effect was nullified by equimolar amounts of thymidine but not of uracil or uridine. FU was also reported to inhibit the production of infectious canine hepatitis virus in dog kidney cultures (Altera and Moulton, 1966). However, the formation of viral haemagglutinins and other viral antigens was not inhibited.

Eight-azaguanine seems to differ rather markedly from any of the analogues previously discussed. It appears to be specific for only one of the DNA-containing animal viruses , vaccinia virus. This analogue was shown to be incorporated into cellular RNA exclusively (Clerc, 1962), and so appears to function by interfering with RNA-dependent DNA synthesis. The inhibitory effect was reversed by equimolar concentrations of guanine or guanosine.

The activity of cytosine arabinoside (1- β -D-arabinofuranosylcytosine . HCl) is similar to that of the halogenated nucleosides except that it would appear more toxic (Renis and Johnson, 1962; Underwood, 1962).

The results with the halogenated nucleosides seem to emphasize one of the difficulties in the screening of antiviral agents since the effect of each of them

varies depending upon the virus, the cell system and the experimental conditions. Even with the same virus in the same cell system there seems to be a rigid specificity in their locus of action. The studies of Rapp et al. (1965) emphasize this point. They showed that with SV40 virus grown in African green monkey kidney cell cultures the use of Actinomycin D, cystosine arabinoside, fluorouracil and iododeoxyuridine revealed sequential steps in the synthesis of the virus.

A single passage of herpes simplex virus in the presence of 500 µg/ml of IUDR resulted in the selection of a resistant variant to the drug (Buthala, 1964). However, these IUDR-resistant variants were sensitive to a much lower dose of cytosine arabinoside. A synergistic effect of the drugs was also reported (Buthala, 1965; Kaufman, 1963); in such conditions no resistant variants emerged. These findings are of great clinical significance in the use of IUDR for vaccinial and herpetic keratitis. Genetically stable IUDR-resistant herpes simplex virus variants have also been reported in human patients treated with the drug (Kaufman, 1965; Jawetz et al. 1965). A further complication of the problem was that some of these resistant strains found in human infections were sensitive to the drug in in vitro studies and in vivo studies in the rabbit cornea (Jawetz et al.,1965). Thus it is difficult to extrapolate even the results of these apparently similar experimental conditions.

Five-trifluoro-methyl-2'-deoxyuridine possesses a potential antiviral activity against herpes simplex infection of the rabbit cornea as well as vaccinial infection of the same organ (Kaufman and Heidelberger, 1964). But by far the most interesting attribute of this drug was the fact that it was inhibitory to strains of the herpes virus which were resistant to IUDR.

Smith (1963) showed that by serial passage of an IUDR-sensitive strain

of herpes simplex virus in tissue culture in the presence of the drug as much as 41 per cent of the virus became resistant to the drug after the third passage.

By infecting a mouse fibroblast cell subline, which lacked both thymidine and deoxyuridine kinase activities, with herpes simplex virus Dubbs and Kit (1964) showed that the cells acquired the ability to synthesize these enzymes after the virus infection. However, some mutants of the virus which were resistant to both BUDR and IUDR were also deficient in the ability to induce the enzyme synthesis. Apart from the implication that the pathways for the synthesis of the resistant virus were different from those of the sensitive strains the data also offer strong support for the concept that upon infection of a cell by virus,virus-induced enzyme synthesis is initiated which is necessary for the replication of the virus.

Drug-dependent variants have not been reported with herpes simplex virus (Renis and Buthala, 1965).

Another phenomenon observed following the use of certain halogenated nucleosides is that of radiosensitization. Djordjevic and Szybalski (1960) showed that as much as 45 per cent of the thymidine of a human cell line could be replaced by BUDR upon continued cultivation of the cell line in the presence of the drug and, although the viability of the cells was not impaired during the process, the cells became sensitized to X-rays and ultraviolet light. The degree of radiosensitization was dependent upon whether the DNA was substituted in one strand or in both strands. A similar situation was also reported for IUDR, but in this case the cells also lost viability with continued subcultures.

Other Inhibitory Bases

A purine derivative, 2, 6-diaminopurine, was shown to inhibit the multiplication of vaccinia virus in chick embryonic tissues but it was ineffective in mice inoculated intracerebrally or intranasally with a neurotropic strain of the virus (Thompson, 1950). The inhibitory effect was reversed by adenylic and guanylic acids; guanine, xanthine and uracil were ineffective. Munyon (1964) also found that the diaminopurine reduced the formation of poliovirus in HeLa cells and the specific infectivity of virus synthesized in the presence of the compound was lower than that of virus formed in the absence of the compound. An equimolar concentration of adenosine was shown to reverse the inhibition. Two, 6-diaminopurine was also shown to be effective against Russian Spring-Summer encephalitis virus in both tissue culture and mice (Friend, 1951).

Borman and Roziman (1965) reported that even the normal purine and pyrimidine nucleosides of nucleic acid of animals could inhibit the multiplication of herpes simplex virus in millimolar concentrations. Although the exact mode of action of these normal nucleosides is not known, their inhibitory action was reversed completely by withdrawing them from the culture.

Actinomycin D

Actinomycin D is a naturally occurring polypeptide antibiotic and is a potent inhibitor of RNA synthesis with only limited inhibition of DNA synthesis. The inhibition of RNA synthesis is mediated through the tenacious binding of the antibiotic to the DNA thereby inhibiting DNA-dependent RNA synthesis (Cavalieri and Nemchin, 1964). In addition actinomycin D has also been reported to inhibit the "migration" of

already incorporated radioactive uridine in acid insoluble form from the nucleus to the cytoplasm (Levy, 1963). Kathan et al. (1963) have suggested that only the guanine moieties of the DNA primers of both RNA polymerase and DNA polymerase bind with the antibiotic. Nevertheless, the exact nature of the binding between Actinomycin D and DNA seems to be obscure since Gellert et al. (1965) have shown that much less actinomycin was tightly bound by DNA than there is guanine in a typical DNA.

Actinomycin D inhibited the multiplication of influenza virus (Barry et al. 1962; Granoff and Kingsbury, 1964). However, the multiplication of Newcastle disease virus was refractory to the action of the antibiotic. It has been suggested (Waterson, 1962) that these two viruses represent two different types of myxoviruses.

The multiplication of vaccinia virus was also inhibited by the antibiotic (Shatkin, 1963).

iii. Antagonists of protein synthesis

A number of amino acid analogues, and even some naturally occurring amino acids have been reported to inhibit the multiplication of various animal viruses. The antibiotic, puromycin, which is an aminonucleoside linked to the amino acid p-methoxyphenylalanine, has been shown to be a potent inhibitor of protein synthesis, and does, by virtue of this inhibition, exert some antiviral effect.

These analogues usually exert their action by competitive inhibition of a reaction involving the corresponding natural amino acids or they may be incorporated into the newly formed virus protein thereby giving rise to an aberrant non-functional protein i.e., a mode of action akin to that of the analogues of purine and pyrimidine bases.

The inhibitory effect of these analogues is usually easily reversed by the appropriate normal amino acids.

One of the most extensively studied amino acid analogues is p-fluorophenylalanine (FPA) which has been shown to inhibit the replication of influenza virus (White et al. 1962), fowl plaque virus (Zimmermann and Schafer, 1960), poliovirus (Ackermann et al., 1954; Levintow et al., 1962; Wecker et al., 1962; Scharff et al., 1965), vaccinia virus (Loh and Payne, 1965b), rabbitpox virus (Appleyard and Zwartouw, 1965), adenovirus (Wilcox and Ginsberg, 1961; 1963), mengovirus (Baltimore and Franklin, 1963) and western equine encephalitis (Wecker et al., 1962). It is apparent from the wide spectrum of viruses which are susceptible to the inhibitory action of the analogue that FPA is not a restricted selective antiviral agent.

With influenza virus FPA has been shown (White et al., 1965) to block two distinct points in the replicative cycle of the virus. High doses of FPA inhibit a process occurring very early in the replicative cycle while lower doses inhibit a process occurring after 2 hours of the cycle; an essentially similar situation has been reported for fowl plaque virus (Zimmermann and Schäfer, 1960) and poliovirus (Ackermann et al., 1954). It was possible to demonstrate the existance of several discrete steps in the replication of fowl plaque virus by adding the FPA at different times of the replicative cycle; the most sensitive stages being within the first three hours after virus infection. It was also shown that the transfer of the S-antigen from the nucleus of the infected host cell is FPA-sensitive. In the case of poliovirus FPA inhibited virus replication but failed to prevent the CPE caused by the infecting virus. Levintow et al.(1962) showed that the synthesis of infectious poliovirus

RNA could proceed in the presence of certain doses of FPA that inhibited the maturation of the virus. The successful inhibition of virus maturation in the presence of continued synthesis of viral RNA was also reported by Wecker et al. (1962) for both poliovirus and western equine encephalitis virus.

In a cell system identical to that used by Levintow et al. (1962) for poliovirus, Loh and Payne (1965) reported the inhibition of the incorporation of thymidine-H^3 into viral DNA in cells infected with vaccinia virus and treated with FPA. This would imply the need for protein synthesis in the synthesis of viral DNA (Appleyard and Zwartouw, 1965).

The effects of FPA were reversed when phenylalanine was added to the medium at an appropriate time or when FPA was removed from the medium. It has been suggested that one of the mechanisms of action of FPA is to cause the synthesis of fraudulent, nonfunctional proteins (Wilcox and Ginsberg, 1963; Baltimore and Franklin, 1963; Scharff et al., 1965); one such protein is viral RNA polymerase (Scharff et al., 1965).

Methoxinine inhibited the replication of influenza A (PR8) in the chorioallantoic membrane (Ackermann and Maassab, 1954b) and also inhibited the multiplication of vaccinia virus in chick embryo tissue in vitro (Thompson, 1947). The effect of methoxinine was nullified by L-methionine but not by DL-methionine (Ackermann, 1951a; Ackermann and Maassab, 1954b).

Thompson and Lavender (1953) showed that the survival time of mice injected intraperitoneally with Semliki Forest virus was increased by incorporating either D-, L- or DL-ethionine in the diet, or by injecting the amino acid intraperitoneally. It was found most effective when given 24 hours before the virus. Ethionine

did not completely suppress virus multiplication since the treated mice which survived were immune to a subsequent challenge by the virus. The inhibitory effect of ethionine was not counteracted by methionine.

The effects of L-canavanine, a naturally occurring amino acid in jack beans, on the multiplication of the Lee strain of influenza B in the chick embryo were studied in detail by Pilcher et al.(1955). The multiplication of the virus was inhibited both in ovo and in tissue cultures. The dose required to produce a complete suppression of virus multiplication in tissue culture was 1/20 of that required to inhibit multiplication of the virus in ovo. It neither stopped adsorption of the virus nor caused any virucidal effect. Although it was very effective in the chick embryo it was without effect on mice infected with the Lee strain. Among the myxoviruses, however, it showed some amount of selectivity since it was not active against both the PR8 strain of influenza A virus and mumps virus. The effect of L-canavanine was completely reversed by L-arginine and structurally it may be regarded as an analogue of L-arginine.

Puromycin inhibits protein synthesis by inhibiting the transfer of amino acids from sRNA into ribosomal protein and thereby interfering with the formation of the polypeptide chains at the ribosomal site (Darken, 1964). When puromycin was added early in the latent phase to cultures infected with poliovirus it prevented both the maturation of the virus and the synthesis of viral RNA (Levintow, 1962). However, when the antibiotic was added towards the end of the latent phase a limited synthesis of RNA was possible. The action of the antibiotic was reversed by removing it from the medium. This data has been interpreted as a further indication that the

synthesis of some "early" protein was necessary for the replication of RNA (Levintow, 1962; Sreevalsan and Lockart, 1964). In line with the above conclusion Scharff et al. (1965) have also suggested that puromycin interrupted the maturation of poliovirus by a direct inhibition of virus-directed protein synthesis.

iv. Inhibitors of energy-yielding mechanisms

In addition to the biochemical mechanisms for the synthesis of nucleic acid and protein other biochemical energy-yielding mechanisms of the host cell play an important role in the course of infection by various viruses. Although viruses differ in the extent to which they utilise the energy-yielding metabolic processes of the host cell for their replication (Koppelman and Evans, 1959), in general, since the replication of viruses is so intimately associated with the metabolic activities of the infected cell, any significant alterations of such host activities have a direct influence on the final outcome of virus infection.

Ackermann (1951b) showed that the Kreb's cycle played an important part in the replication of influenza virus. When sublethal doses of sodium fluoroacetate were administered to mice within the first 12 hours following infection the citric acid cycle was blocked, citrate concentration thereby increased in the lungs of the mice and the replication of the virus was inhibited. The salt had no virucidal effect on the virus in vitro.

The respiration of chorioallantoic membrane tissue fragments was stimulated by 2, 4-dinitrophenol (DNP) (Ackermann and Johnson, 1953). DNP uncouples oxidative phosphorylation and thereby prevents the formation of high energy phosphate bonds essential for cell metabolism. Under these conditions the multiplication of influenza virus was inhibited.

Butyl 3, 5-diiodo-4-hydroxy-benzoate, an antagonist of thyroxin which also uncouples oxidative phosphorylation, had effects similar to those of DNP in the course of influenza virus replication (Eaton et al., 1953).

c) Phase of Release of Newly Formed Virus

There is a significant paucity of information in the literature concerning antiviral substances that exert their effects by interrupting the final release of newly formed viral particles. It seems that when once the cycle of viral synthesis is allowed to go on to completion the release of these particles follows as a necessary consequence.

The only suggestion in the literature of a substance capable of inhibiting virus release was the one made by Ackermann and Maassab (1954a; 1954b). They suggested that ∝-amino-p-methoxy-phenylmethane sulphonic acid interfered with the release of newly formed influenza A virus from chorioallantoic membrane tissue culture. This effect was nullified by treatment with RDE (Ackermann and Maassab, 1954b); and on this basis it was suggested further that a function of the viral enzyme of myxoviruses is to facilitate the release of virus from the host cell.

No other studies with the compound have been reported. It is likely that the mechanism of release may differ from one virus group to the other. In the case of myxoviruses it is believed that the virus matures at the cell surface and is released in a "trickle", rather than a "burst", over a long period of time, without any major physical damage to the host cell. On the other hand, poliovirus is released in a burst, similar to the situation which occurs with bacteriophage; the cells are destroyed by this process. The adenoviruses occupy an intermediate position. These viruses have a tendency to assemble within the cell in characteristic crystalline form

and are released rather slowly.

3. Miscellaneous Substances Including the Antiviral Substance from Penicillium cyaneo-fulvum (McGill strain)

Several extracts of biological origin have been reported to possess some antiviral activity. None of these substances has so far been produced in a chemically pure form and, although an unequivocal antiviral activity has been demonstrated, the exact modes of action of most of these antiviral extracts remain essentially a matter of surmise.

Helenine

From a mould found growing on the back of his wife Helen's photograph in Guam, Shope (1953a) extracted a substance which he finally named "Helenine". The mould was later identified as Penicillium funiculosum. Two active principles appeared to be elaborated by the mould; one of these, the original antiviral principle, was active against the virus of swine influenza. In the course of maintenance of the mould in the laboratory over several years this principle was lost. However, another antiviral principle was demonstrated which was effective against Columbia SK encephalomyelitis virus. It was this substance which was designated helenine. Helenine was elaborated into the culture medium of the mould, but more of the active principle was extracted from the pellicle. Helenine was precipitated with 50% chilled acetone. The preparations showed periodic fluctuations in activity. This has been attributed to the presence of an inhibitor to helenine (Cheng and Shope, 1966) which was found present in varying amounts in the crude preparations. The inhibitor could be separated from helenine by depositing the active principle at 40,000 rpm for 2 hours in a Spinco Model L centrifuge.

Helenine exerted its maximum therapeutic effect in mice when given within the first 10 hours after infection with Columbia SK encephalomyelitis virus (Shope, 1953a). The best results were obtained when the mice were inoculated intraperitoneally or subcutaneously with helenine. It was also effective against Semliki Forest virus (Shope, 1953b). In both cases a "plateau effect" was observed in dose-response experiments where it was shown that beyond a certain dose level no corresponding increase in activity was observed with increase in the dose of helenine.

In mice infected with type 2 poliovirus helenine treatment delayed the onset of paralysis (Cochran and Francis, 1956). On the other hand helenine protected monkeys both from the onset of the disease and the paralysis following infection with type 1 poliovirus. These differences have been explained on the basis of differences in the susceptibility of the two types of poliovirus to the antibiotic or differences in the test systems.

Chemically helenine was shown to contain carbohydrate, protein, nucleotides and nucleosides. It was also found to be heat-labile (Shope, 1953c). Lewis et al. (1959) continued the purification of helenine, and identified the preparation as a ribonucleo protein, unstable to lyophilisation and repeated freezing and thawing.

Statolon, M-8450, and Agent 1758

These antibiotics are produced by strains of Penicillium stoloniferum. They were originally considered to be different types of antibiotics but are now known to be identical.

Statolon exhibited marked chemoprophylactic, and some chemotherapeutic activity, against MM encephalomyocarditis virus and Semliki Forest virus in mice (Powell et al. 1952). The filtrate inhibited the multiplication of the virus when the antibiotic was administered intraperitoneally, subcutaneously, or intravenously; but not orally. Its activity was demonstrated against virus inoculated subcutaneously, intramuscularly or intranasally, but not intracerebrally. Statolon was also effective against MEF 1 poliovirus in mice (Powell and Culbertson, 1953); in tissue culture it inhibited the cytopathogenic effect of all three immunologic types of poliovirus (Hull and Lavelle, 1953), and Salisbury virus H.G.P. (Powell et al. 1961). Antiviral activity was also reported against Mahoney type 1 poliovirus infection in monkeys (Cochran et al., 1954). The antiviral spectrum of statolon appears to be very broad since it inhibits the multiplication of representatives of picornavirus, arbovirus, papovavirus and poxvirus groups (Johnson and Baker, 1958; Powell et al., 1962; Cochran and Payne, 1964; Kleinschmidt et al., 1964a). In all instances statolon exhibited the greatest activity when the tissues were pretreated with the antibiotic prior to virus inoculation.

Chemically, statolon has been identified as a polyanionic polysaccharide (Probst and Kleinschmidt, 1961; Kleinschmidt and Probst, 1962), and it may exert its antiviral activity by stimulating interferon production (Kleinschmidt et al., 1964a; 1964b; Merigan and Kleinschmidt, 1965; Kleinschmidt, 1966).

In a recent study on the mode of action of helenine, Shope (1966), and Rytel et al. (1966), have come to the conclusion that the antiviral activity of helenine is mediated through the production of interferon. The general opinion now

seems to be that both helenine and statolon may be one and the same antibiotic, and that the recorded discrepancies in their chemical composition may be more apparent than real (Thompson, 1964; Tamm and Eggers, 1965).

Ehrlichin

Groupé et al. (1951a) reported the extraction of an antibiotic from a culture filtrate of Streptomyces lavendulae which was inhibitory to influenza A and influenza B viruses by the "Contact Test", in which virus and antibiotic were mixed in vitro and allowed to stand at room temperature for 2 to 3 hours prior to inoculation. An in vivo inhibitory effect was reported for only influenza B virus in the chick embryo; the antibiotic was effective in suppressing virus multiplication when it was given after virus inoculation but ineffective when given prior to virus inoculation. It was suggested that the substance was rapidly inactivated by the host's tissues. Ehrlichin also exerted a slight but detectable suppressive effect on pulmonary consolidation in mice. It was later found that two antiviral principles were involved in the culture filtrates; one of these was precipitable at pH 2, while the second principle was not . The latter was active against Newcastle disease virus, while the former (Ehrlichin) was active against influenza virus. There was no activity against pox viruses or bacteriophage.

Viscosin

A product of culture filtrates of Pseudomonas viscosa was found to exert a marked protective effect in embryonated eggs infected with infectious bronchitis virus (Groupé et al., 1951b). It also exerted a slight inhibitory effect on the multiplication of influenza A virus in the mouse lung and on influenza A, B and Newcastle disease viruses in embryonated eggs. It was ineffective against vaccinia

virus multiplication in the yolk sac. Viscosin exhibited its greatest activity when it was given an hour before virus.

Netropsin

Streptomyces netropsis elaborates an antiviral substance, netropsin (Schabel et al., 1953) which has been obtained in a pure form. The structure has been established as N- [5- [(2-carbamoylethyl) carbamoyl] - 1 - methylpyrrol - 3 - yl] - 4 - [(2 - guani-dinoacetimidoyl) - amino] - 1 - methylpyrrole - 2 - carboxamide (Thompson, 1964). Netropsin given intraperitoneally protected mice against vaccinia virus inoculated intracerebrally. The activity of netropsin is similar to that of the thiosemicarbazones, in that both inhibit the replication of intracerebrally inoculated vaccinia virus in mice but fail to prevent the development of vaccinial skin lesions in rabbits. Mice protected by netropsin develop antibodies against vaccinia.

Propionin

Cutting et al. (1960) reported an antiviral principle in cultures of Propionibacteria which reduced the mortality rate of mice inoculated intraperitoneally with Columbia SK virus. The antibiotic substance was given either subcutaneously or orally. It was effective when given a day after virus inoculation but seemed to show better results when given a day prior to virus. The material did not interfere with the development of specific immunity in the surviving and treated mice.

Cyclopin

Cyclopin, a constituent of Penicillium cyclopium, was shown to possess antiviral activity by Nacify and Carver (1963) and Nacify (1965). It was obtained by sonic disintegration of the mould mycelia. It showed some selective in-

hibitory activity against representatives of groups A and B arboviruses and acted at an intracellular stage of virus replication. Preliminary chemical characterisation suggested that cyclopin may be a protein or protein derivative (Nacify, 1965).

Cyclohexamide

Haff (1964) showed that cyclohexamide isolated from Streptomyces griseus cultures, inhibited plaque production by a wide spectrum of virus types- picorna,- myxo-, arbo-, vaccinia, Rous sarcoma and mouse hepatitis viruses. Cyclohexamide was not virucidal and did not prevent the release of virus from cells. It appeared to act during the initial four-fifths of the latent period. However, it has been suggested that the effects of cyclohexamide may not be specific since there was a possible effect on cell multiplication by the doses used. It may therefore act by interfering with general cell protein and DNA synthesis.

Xerosin

Originally termed "acid precipitable material" (APM) xerosin produced by Achromobacter xerosis, suppressed the development of pneumonia in mice infected with influenza A and influenza B viruses (Groupé et al., 1952; 1954). The most interesting biological activity of xerosin is its suppression of nontransmissible pneumonia in mice due to intranasal instillation of Newcastle disease virus (Groupé et al., 1952; Groupé et al., 1953). In pneumonia induced by Newcastle disease virus in mice there is no corresponding virus multiplication. Thus the effect of xerosin was considered not due to an antiviral effect per se but due to a modification of tissue reaction due to virus infection. Xerosin did not inhibit the multiplication of influenza virus in either the lungs of mice or the allantoic cavity of the embryonated egg (Groupé et al., 1952).

Subcutaneous injections of xerosin modified the neurotoxic effect of intracerebrally inoculated influenza A and B viruses in mice. However, the toxic effect consequent upon intravenous inoculation of virus was not prevented (Groupé and Herrmann, 1955). The Newcastle disease virus-induced neuropathic effect in mice was also modified by xerosin (Groupé and Dougherty, 1956). The anti-inflammatory reactions of xerosin were increased by autoclaving the preparation (Groupé and Herrmann, 1955).

Histological evidence for the action of xerosin in influenza infected mouse lungs was presented by Ginsberg (1955) who showed that, while xerosin neither had any action on the replicative cycle of the virus nor prevented damage to the infected epithelium, it suppressed the development of pulmonary lesions by inhibiting oedema, haemorrhage and cellular infiltration. On the basis of these studies it was concluded that at least two distinctive phases are likely in the pathogenesis of influenza in the mouse lung: injury to the bronchial and bronchiolar epithelium and pulmonary consolidation. Xerosin suppressed the latter and not the former.

MER-27 (9- (p-guanylbenzal) - fluorene hydrochloride

MER-27 is a synthetic organic compound developed by Ludwig et al. (1959) which suppressed pneumonia in mice induced by influenza virus (PR8). The mechanism of the suppression paralleled that of xerosin. However, MER-27 possessed certain advantages over xerosin since it did not cause any lung consolidation and could be given both parenterally and orally. Xerosin was ineffective by the oral route (Groupé et al., 1952).

Capsular Polysaccharide of Friedländer Bacilli (Klebsiella pneumoniae)

This polysaccharide has been shown to inhibit the multiplication of pneumonia virus of mice in mouse lung when virus and polysaccharide were given by the intranasal route (Horsfall and McCarty, 1947; Ginsberg and Horsfall, 1951); only limited activity was demonstrable after administering the polysaccharide by the intraperitoneal route. It also inhibited the multiplication of mumps virus in the allantoic cavity of the embryonated egg (Ginsberg et al., 1948b). There was no activity against influenza A and influenza B viruses nor against Newcastle disease virus. The polysaccharide was effective even when the inoculation of virus antedated it by as much as four days.

It was shown by chemical treatment of the polysaccharide with periodic acid that the structural configuration of the polysaccharide which is responsible for the antiviral activity is distinct from that which confers serological activity to the polysaccharide (Horsfall and McCarty, 1947; Ginsberg et al., 1948b) since the former could be destroyed without affecting the latter property.

Serial passage of mumps virus in the presence of the polysaccharide selected a resistant variant (Ginsberg and Horsfall, 1949). The variant reverted back to the sensitive strain upon passage in the absence of the inhibitor.

Mouse Intestinal Polysaccharide

A mucopolysaccharide extracted chiefly from the large intestine of the adult mouse inhibited both infectivity and haemagglutination of Theiler's encephalomyelitis virus (GDVII strain), but was ineffective against other strains of Theiler's virus as well as the Lansing strain of poliovirus (Mandel and Racker, 1953a). The

polysaccharide formed an electrostatic complex with the virus (Mandel, 1957), but there was no permanent alteration of either virus or the inhibitor by such interaction. The purified inhibitor proved to be a complex of carbohydrates and protein (Mandel and Racker, 1953b).

Interferon

Although interferon does not fit into the working definition of an antiviral substance proposed at the early part of this review a very broad account of interferon is included here in view of the current interest it has stimulated in the study of the suppression of virus replication. Moreover, the antiviral activity of one of the mould filtrates thus far studied has been definitely attributed to the induction of interferon production (Kleinschmidt et al., 1964a; 1964b; Rytel et al., 1966). It also seems a valid expectation that interferon induction may play a wider role than is at the moment known in the antiviral activities of the naturally occurring biological products, especially in those antiviral substances where pretreatment of the host tissue with the substance is a prerequisite for the demonstration of maximum antiviral activity.

The phenomenon of viral interference in mixed infections has been known for some time (Isaacs and Edney, 1950a; 1950b; 1950c; Henle, 1950; Schlesinger, 1951; 1959) but it was Isaacs and Lindenmann (1957) who provided for the first time a likely explanation for the phenomenon. Briefly, they showed that the incubation of pieces of chorioallantoic membrane along with heat-inactivated influenza virus in vitro for 24 hours, or even as short a time as 15 minutes, resulted in the elaboration of a protein-containing substance (Isaacs et al., 1957) into the culture fluid which possessed the ability to inhibit the multiplication of active

influenza virus in the same test system. Since this early significant discovery it has been shown that interferon production is a potential of all cells and that the production can be induced by the interaction of cells with the appropriate "foreign" stimulant, which may vary from active as well as inactivated viruses to a number of vastly different biological substances (Wagner, 1963; Larke, 1966). Because of its ubiquity it is widely held that interferon may play a significant part in the control of viral diseases (Wagner, 1963b; Larke, 1966; Paucker, 1964; Hilleman, 1965).

Among its most interesting physicochemical properties the stability at pH 2 has been a most distinguishing feature of classical interferon (Wagner, 1963). The fact that interferon is essentially nonantigenic is of immunological interest in view of its possible use in viral chemotherapy, although a number of obstacles still stand in the way of its usefulness in this regard (Hilleman, 1965). It should be mentioned however, that the induction of specific antibody against interferon has been reported (Pauker and Cantell, 1962). The interferon used in these studies was produced in L cells exposed to ultraviolet-irradiated Newcastle disease virus and the antiserum was prepared in the guinea pig. The anti-interferon antibody titre was not reduced after repeated adsorption of the antisera with normal L cells, a process that selectively diminished the anti-host cell antibody titre.

Although it is clear that interferon acts at an intracellular site, before the synthesis of viral nucleic acid in the infected cell (Paucker, 1964; Hilleman, 1965), its precise mechanism of action remains obscure. Several attractive hypotheses have been proposed but none seem to provide the exact answer. One of the most

popular hypotheses was the one put forward by Isaacs et al. (1961a; 1961b). They suggested that interferon exerted its inhibitory activity by uncoupling mitochondrial or nuclear oxidative phosphorylation. Such a mechanism would deprive the cell of the necessary energy-yielding mechanisms needed for virus synthesis.

Views contrary to the above have been expressed by Lampson et al. (1963) and Žemla and Schramek (1962). The latter workers showed that even under conditions of anaerobiosis, where oxidative phosphorylation cannot function, interferon caused the same degree of inhibition of the multiplication of Western equine encephalitis virus as it did in the presence of air. Using a highly purified interferon preparation the former workers failed to demonstrate any uncoupling of oxidative phosphorylation and no detectable effect of interferon on the respiration of isolated liver mitochondria from chicken. The purified interferon also failed to have any effect on aerobic or anaerobic glycolysis of Ehrlich ascites cells in vitro. However, a crude interferon preparation did stimulate glycolysis of chick embryo cells in vitro. It was, therefore, suggested that the uncoupling of oxidative phosphorylation reported by other workers may be due to some extraneous contaminants of the interferon preparations used.

Because interferon inhibits the multiplication of both RNA-and DNA-containing viruses, Joklik (1965) has proposed an "economical hypothesis" for the mechanism of action of interferon. It was proposed that interferon acts by blocking the translation of RNA-nucleotide sequence into polypeptide sequence. However, although this hypothesis would embrace a reaction common to both types of viruses that are susceptible to the action of interferon it also involves the interference of energy-yielding mechanisms of the biosynthetic processes proposed.

Antiviral Substance from Penicillium cyaneo-fulvum (McGill strain)*

Diena (1954, 1956) precipitated at full saturation with ammonium sulphate, a toxin neutralizing substance, "Noxiversin", from glucose beef heart infusion broth culture filtrates of a strain of Penicillium cyaneo-fulvum (Biourge) first isolated at McGill University as a contaminant of a Lowenstein's slope (Murray et al., 1948). In preliminary experiments it was demonstrated that noxiversin exhibited some marginal activity against influenza A virus (PR8) in ovo. Noxiversin also possessed some haemagglutinating (HA) ability, but failed to exhibit haemagglutination inhibiting (HI)activity. No activity was demonstrated with Czapek-Dox broth culture filtrates of the mould.

Cooke (1958) studied the antiviral activity of noxiversin as well as some methods of production and partial purification of the active substance. Roux bottles of glucose beef heart infusion broth, pH 7.2, were seeded with 5 drops of spore suspension and incubated at room temperature for 10 days and were harvested when the pH had risen to between 8.0 to 8.5. In order to follow the pH changes phenol red indicator was added to a reference bottle. Fractional precipitation with ammonium sulphate was done on the crude culture filtrates and noxiversin was found to precipitate out at 65 per cent of saturation with the salt. Antiviral activity against influenza A virus (PR8) was demonstrated in ovo with both the noxiversin fraction and the remaining filtrate. In these studies equal volumes of the fraction under test and the virus dilution were mixed and incubated at 37°C for 1 hour prior to inoculation. After a series of experiments it was concluded that the antiviral factor was separate and distinct from noxiversin, since the former was not precipitated

*This culture is deposited as P. cyaneo-fulvum(Biourge) McGill strain, in The Commonwealth Mycological Institute, Ferry Lane, Kew, England, as No. I.M.I. 58138; in the American Type Culture Collection, 2029 M Street NW, Washington, D.C., as No. 11923; and in Le Musée National d'Histoire Naturelle, Laboratoire de Cryptogamie, 12 rue de Buffon, Paris V. Can. J. Microbiol. 4 (1958)

at 65 per cent saturation with ammonium sulphate. However, the antiviral factor was reported to be precipitated at full saturation with the salt. In future studies, however, it was found that the antiviral substance did not precipitate at any concentration of ammonium sulphate. A small fraction of antiviral activity was seen in various fractions on occasion but the bulk of the activity was in the filtrate remaining after saturation, with the salt (Cooke, 1960). In an attempt to precipitate the material ethanol precipitation was tried. Once again the bulk of the activity remained in the filtrate. Although these procedures failed to precipitate the active substance they did remove a number of impurities. As a first approximation of the nature of the antiviral substance various procedures, including electrophoresis, one-dimensional paper chromatography and ultraviolet absorption spectrum were done. Electrophoresis in borate buffer at pH 8.6 showed two bands on the paper both of which were stained by mucicarmine and by bromphenol blue. The chromatography experiment demonstrated the presence of ten amino acids. There was no definite peak in ultraviolet absorption spectrum although a slight hump was seen at 230 mμ. The results of these preliminary tests as well as the fact that the material did not precipitate with either ammonium sulphate or ethyl alcohol indicated that the active material was neither a protein nor a polysaccharide but that it could be an amino acid- carbohydrate complex. However, since no corresponding runs of uninoculated broth were done it is not possible to state whether the bands found on electrophoresis or the amino acids detected were from the active substance.

A number of experiments to determine the range of antiviral activity of the substance were done. Influenza A virus (PR8) was studied extensively in a

variety of test systems and its growth was inhibited in ovo, in monkey kidney tissue cell cultures, in Maitland-type tissue cultures and in mice. In the mouse experiments the virus and antiviral substance were mixed and incubated in vitro for 30 minutes at room temperature prior to intranasal instillation of the mixture and also the virus was given intranasally followed by intraperitoneal injection of the active material. There was a sparing effect and a delay of about 24 to 48 hours in the onset of death in the treated groups with the most marked effect seen in those groups which received the mixture of virus and antiviral substance intranasally. Some antiviral activity was also reported against mumps virus multiplication in embryonated eggs although the results were not conclusive because of the low potency of the virus used.

The cytopathogenic effect caused by poliovirus type 1, Mahoney strain, in monkey kidney cell cultures was suppressed by the antiviral agent. The effect was observed when the virus and the substance were mixed and added immediately to the cell cultures.

Syeklocha (1962) studied the biological activities of the antiviral substance, using the methods of production and purification worked out earlier. However, the activity was not uniformly produced or extracted and tended to be erratic. A seasonal effect, notably during the summer months, on the production was suspected (Syeklocha; Cooke; Personal Comm., 1965). In embryonated eggs the AVS was either not active at all or showed only slight activity. A variable activity was also reported for the precipitates after both the extraction with full saturation ammonium sulphate and subsequent precipitation with about eight volumes of ethanol.

It was suggested that some activity was trapped in the precipitates. In mice infected with influenza A virus (PR8) although the results were not as promising as those of Cooke (1960), a delaying effect (24 to 48 hours) and a sparing effect were demonstrated in some experiments, while in others little or no effect was seen. In these studies, the mice were also usually, treated with several intraperitoneal injections of the antiviral substance after the initial dose. In summary, the best results were obtained when the virus and the AVS were given after in vitro mixing and incubation. In Maitland cultures it was shown that the antiviral substance was active either when added 60 minutes prior to virus or 60 minutes after virus and in one experiment it was active even when added 2 hours after virus inoculation. Nevertheless, it was suggested that the substance acted by stopping virus adsorption to the host cell either in the first cycle or later cycles of virus multiplication, with a possible action also on release of newly formed virus particles. Syeklocha (1964) conducted further studies with the substance. In growth curve experiments with about 200,000 EID_{50} of influenza A virus (PR8) per flask and with an antiviral substance dose of about 0.164 mg per flask, of Maitland culture, it was found that the latent period of virus multiplication was extended by about 9 hours. No antiviral activity was demonstrated against vaccinia virus grown in tissue cultures of chick fibroblast cells or in HeLa cells. There was also no activity against T2 bacteriophages.

In order to establish the chemical composition of the antiviral substance, several different types of enzymes were tried in attempts to inactivate the activity. The effect of potassium periodate was also tested. All of the enzymes except alpha amylase were unsuccessful in destroying the antiviral activity. There was no

effect with periodate. After hydrolysing the most purified preparation with 12N HCl at 121°C for 24 hours, 13 amino acids were detected in two-dimensional paper chromatography; but since there was no report of a corresponding chromatogram of the uninoculated broth, it is not certain if these acids are part of the active substance. No aromatic amino acids were located. No definite ultraviolet absorption peak was found, other than a small hump between 260 and 280 mμ.

4. General Comments on the Problems of Antiviral Chemotherapy

In most virus infections, because of the intimate dependence of the processes of viral replication upon the host's physiological mechanisms, by the time clinical signs become obvious the virus has already established itself in its milieu. Related to this problem is the length of time required for accurate laboratory diagnosis. Thus it is not uncommon that by the time the laboratory investigations are completed the disease has already spent itself or else the patient has died.

Another problem in the quest for effective antiviral chemotherapy is the laborious and extensive screening procedures required in the study of possible antiviral agents. Moreover, a demonstration of antiviral activity in in vitro screening experiments does not necessarily imply a corresponding activity in vivo.

The development of drug-resistance poses a serious problem in the search for an efficacious antiviral agent, a problem not less important than that posed at the moment in antibiotic therapy of bacterial infections. Thus, in addition to the intrinsic technical limitations in the study of antiviral agents, it has been shown that viruses, like bacteria, can exist in three genetically stable forms: drug-sensitive, drug-resistant and drug-dependent.

The possibility of radiosensitization following the incorporation of IUDR and BUDR into DNA may also very greatly hamper the use of the halogenated nucleosides which have so far been one of the few antiviral agents of any clinical promise.

III. MATERIALS AND METHODS

For the purpose of clarity, only those procedures which are more involved and which were used routinely are included in this section on materials and methods. Other methods used less frequently are included along with the particular experiments where they occur. This ensures a more coherent presentation of the experimental results.

The composition and the preparation of all the media and solutions referred to in this section are described in the Appendix.

A. Production of the Antiviral Substance from P. cyaneo-fulvum

i. Maintenance of the mould

Penicillium cyaneo-fulvum was maintained on malt extract agar slopes and stored at between 4°C and 5°C. The stock cultures were prepared by inoculating the agar slopes with 1 to 2 drops of spore suspension prepared in either sterile distilled water or sterile phosphate-buffered saline (PBS) pH 7.2 (Dulbecco and Vogt, 1954). In the preparation of the stock cultures only those slopes showing a uniformly green sporulation of the mould were selected. After seeding the tubes were plugged with cotton wool plugs and incubated at room temperature, at about 22°C to 25°C, until a uniform carpet of mycelia with extensive sporulation covered the entire surface of the slopes. This took usually three to four days.

ii. Preparation of batches of the culture filtrates of the mould

Two methods were used for the production of the culture filtrates of the mould. The first was a method originally devised by Diena (1954; 1956) for the production of noxiversin and later modified by Cooke (1958; 1960) and Syeklocha

(1962; 1964) for the production of the antiviral substance. This method involved the use of McGill glucose beef heart infusion broth. The second method, used for the first time in the present studies, involved the use of Czapek-Dox broth. The initial operations for the setting up of the batches of the culture filtrates were similar in both cases; details of differences in the use of the two broths will be discussed in later sections of the thesis.

A spore suspension of the mould maintained on malt extract agar slopes was made using 3 ml of either sterile distilled water or sterile PBS per slope. The mycelia were dislodged from the surface of the slope with the aid of an inoculation wire. The harvests from the slopes were pooled in a sterile glass vial with a screw cap. Sterile glass beads were added to the vial and after thoroughly shaking the contents, to effect as uniform a spore suspension as possible, the suspension was filtered through two layers of sterile muslin gauze. The filtrate was then used as the seeding inoculum of the broths.

Roux bottles containing 225 ml of glucose beef heart infusion broth, pH 7.2, were prepared. The glucose was added to a final concentration of one per cent, from a 50 per cent sterile stock solution of the sugar, just before seeding of the bottles with the spore suspension. Two dozen Raschig rings (Cooke, 1960) were inserted into each bottle and sterilised along with the broth before the addition of the sugar solution.

In order to follow the pH changes during the growth of the mould phenol red at a final concentration of 0.002 per cent was added to one of the bottles from a sterile 0.2 per cent stock solution (Cooke, 1960). The indicator was not added to batches prepared with the Czapek-Dox broth.

Each Roux bottle was inoculated with 1 ml of the spore suspension. The bottle was gently rocked after the inoculation to effect both a mixing of the inoculum and the distribution of the Raschig rings over the surface of the bottle. The bottles were incubated stationary at room temperature. The cotton wool plugs were slightly loosened to allow sufficient aeration but maintaining sterile conditions. The bottles were incubated in a well lit room and growth of the mould was allowed to proceed to the 10th day when the pH, which first dropped to the acid range, had risen to about 8.0. The culture fluids were harvested at this stage and filtered through wetted Whatman No. 2 filter paper. The mycelial felt was harvested separately into weighed beakers.

B. Extraction and Partial Purification of the Antiviral Substance.

i. Ammonium sulphate - Ethyl alcohol method of fractionation

a. Culture filtrates of the mould

Ammonium sulphate - ethyl alcohol fractionation, hereafter referred to as the ammonium sulphate method, as used by Cooke (1960) and Syeklocha (1962; 1964) was originally used. It was later replaced by the acetone method of fractionation.

After adjusting the pH to 7.2 the crude culture filtrate was divided into 250 ml portions. The amount of the ammonium sulphate required for full saturation (190.36 gm per 250 ml of the culture filtrate) was added a bit at a time, with vigorous shaking to dissolve each portion before the next was added. After the required amount of salt had been added the contents were shaken very vigorously and allowed to sit at room temperature for about 2 hours to complete

the precipitation.

A yellowish amorphous precipitate rose to the surface like a thick scum. The supernatant was separated from the precipitate and the undissolved salt and filtered through Whatman No. 2 filter paper. The filtrate was dialysed against running tap water for 48 hours in dialysis tubing measuring 5/8'' diameter, 1'' flat width, 0.0008'' wall thickness and average pore diameter of 48Å. Further dialysis was carried out at about 4°C against distilled water for an additional 48 hours, changing the distilled water after the first 24 hours. The completion of the dialysis was determined using the barium chloride-hydrochloric acid test for sulphate ions (Appendix).

The contents of the dialysis bags were harvested and, since there was a 3 - to 4 - fold increase in volume, the harvested fluid was concentrated to about 1/20 th of the original volume of the crude filtrate by flash evaporating under reduced pressure in a 50°C water bath using an aspirator. The resulting concentrated supernatant was used for the subsequent purification steps.

Anhydrous ethyl alcohol was added to the concentrated material until there was no further increase in cloudiness. The alcohol was delivered slowly from a burette and the flask swirled vigorously at each addition of the alcohol. A total of about four volumes of alcohol were required. The flask was left at 4°C over-night (12 hours) to complete the precipitation. A sticky brownish precipitate settled out and the clear yellow supernatant was separated by filtration through Whatman No. 2 filter paper.

The supernatant was flashed to reduce the volume to the original volume before reprecipitating with alcohol; about six volumes of alcohol were required

for this second alcohol precipitation. A whitish floccular precipitate settled out after leaving the contents at 4°C over-night. The precipitate was removed and the supernatant concentrated to the starting volume and dialysed as previously described.

b. Mycelial felt of the mould

The harvested mycelia were ground in a chilled Waring blendor with PBS for about 10 minutes. In order to reduce aerosols the blendor was well encased in several layers of wetted filter paper, leaving only a portion at the bottom half uncovered to follow the grinding process.

The green gruel-like mixture was poured into an Erlenmeyer flask and the blendor thoroughly rinsed with PBS and the washings added to the flask. The amount of PBS used was about equal to the volume of the crude culture filtrate harvested. The contents of the flask were shaken vigorously and allowed to sit at 4°C overnight. The mycelial extract was filtered through wetted Whatman No. 2 filter paper and the greenish cloudy filtrate centrifuged at 1100 x G for 30 minutes. The supernatant was removed and the pH adjusted to 7.0 to 7.2.

The processes of ammonium sulphate precipitation and ethyl alcohol precipitation were essentially similar to those with the crude culture filtrates. But after the ammonium sulphate step the ensuing precipitate was greenish and floccular and the supernatant was clear and light yellow in colour. After the first as well as the second ethyl alcohol precipitation a whitish floccular precipitate settled out. About ten volumes of alcohol were required in this case

for the second alcohol precipitation.

The final product was dialysed and treated as described for the culture filtrates

ii. Acetone method of fractionation

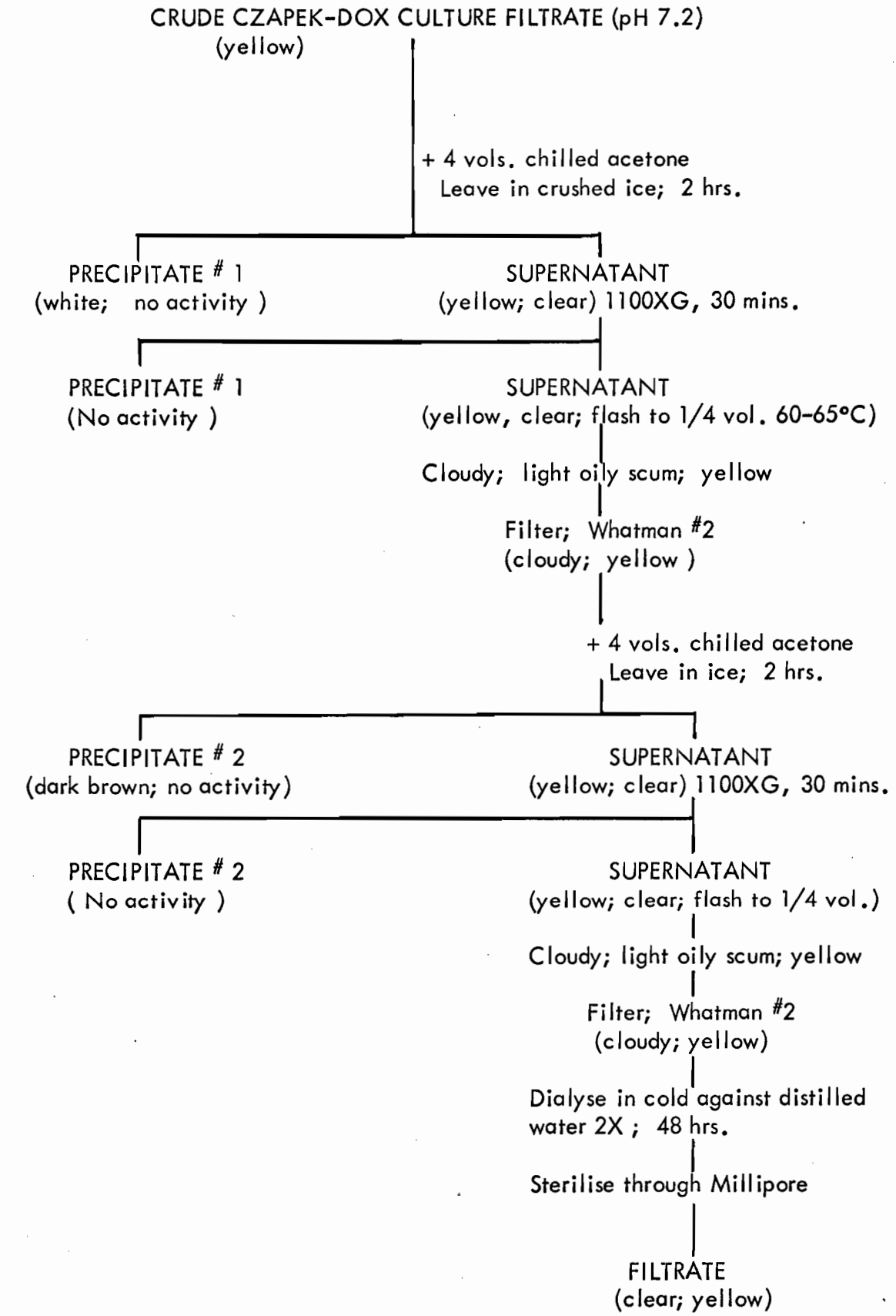
a. Culture filtrates of the mould grown in Czapek-Dox broth

The acetone method of fractionation and partial purification of the culture filtrates of P. cyaneo-fulvum was developed in an attempt to obtain an alternative and simpler procedure to replace the ammonium sulphate method described earlier. It was a modification of the method used by Shope (1953a) who precipitated helenine from culture filtrates of P. funiculosum with 50 per cent acetone. A flow sheet showing the fractionation steps is illustrated in Fig. 4.

The crude culture filtrate, after adjusting to pH 7.2, was precipitated with 4 volumes of chilled acetone, added a little at a time. After all of the acetone was added the contents were swirled vigorously and left to stand in crushed ice for 2 hours. The yellow supernatant was filtered through Whatman No. 2 filter paper, leaving behind a whitish precipitate. The supernatant was centrifuged at 1100 x G for 30 minutes, with more precipitate settling out. The clear, yellow supernatant was flashed under reduced pressure in a 60°C to 65°C water bath, to reduce the volume to 1/4 of the starting volume of culture filtrates.

After filtration an additional 4 volumes of acetone were added. A second type of precipitate, brownish in colour, settled out after standing in crushed ice

Figure 4



Extraction and partial purification of the antiviral substance from Penicillium cyaneo-fulvum .

Antiviral activity

for 2 hours. The processes of centrifugation and concentration were repeated. The final product was then dialysed against about 50 volumes of distilled water at 4°C for 48 hours, changing the distilled water after the first 24 hours.

Dialysis was complete when acetone could no longer be detected in the processed culture filtrate. Acetone was tested using "ACETEST" reagent tablets (Appendix). A negative result with these tablets was equivalent to less than 0.1 per cent acetone.

b. Mycelial felt (Czapek-Dox broth)

The mycelia were processed as was previously described. The methods of acetone precipitation were identical to those used for the crude culture filtrates. However, after the addition of the first 4 volumes of acetone, two types of precipitates settled out; a coarser greenish precipitate which formed the greater bulk and a whitish sticky precipitate. The addition of the second 4 volumes of acetone produced virtually no precipitation. The subsequent processes of concentration and dialysis were the same as those previously described.

c. Culture filtrates and mycelial felt from McGill glucose beef heart infusion broth

Although the newer methods of production and purification of the antiviral substance using Czapek-Dox broth and acetone fractionation were in subsequent studies used in preference to the older methods,a batch of culture filtrates prepared in McGill glucose beef heart infusion broth was processed with acetone to compare the results directly with a similar batch prepared in Czapek-Dox broth.

The processes of fractionation were similar to those described for the Czapek-Dox broth, with the following differences: the precipitate formed after the addition of the first 4 volumes of acetone to the culture filtrate was sticky and dark brown. The precipitates formed from the mycelial extracts were, however, similar to those obtained with the Czapek-Dox broth.

iii. Processing of uninoculated Czapek-Dox broth for antiviral activity control

A sample of uninoculated Czapek-Dox broth was processed by the acetone method. Except for a small amount of whitish floccular precipitate which settled out after the addition of the first 4 volumes of acetone, no precipitates were obtained. The subsequent processes were similar to those described earlier.

iv. Sterilization of the antiviral extracts

Before testing for activity the processed filtrates were sterilized by filtration through Millipore filters of pore size 0.22 microns. Sterility tests were carried out by inoculating tubes of trypticase soy broth with the filtrates and incubating at 37°C for 24 hours.

v. Dry weight determinations

Small beakers of about 10-ml capacity were weighed and aliquots of the sterilized preparations put into each beaker. The beakers were then placed in a desiccator over anhydrous $CaCl_2$ and evacuated, taking care to avoid any spattering of the samples. This may be accomplished by not evacuating for more than 12 mm Hg.

The samples were dried for exactly seven days. The weight of the sample was calculated by difference and expressed as mg per ml of sterilised antiviral substance preparation. Before testing for activity this result was expressed in

terms of ml. per mg of sample since the experiments were done using appropriate volumes of the preparations.

C. Maintenance, Passage and Titration of Influenza A Virus (PR8 strain)

i. Maintenance and passage of influenza A virus

The PR8 strain of influenza A virus used in this investigation was originally obtained from Dr. Robert S. Gohd of the Virus Research Laboratory, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts in 1956 and has since been maintained by allantoic passage in embryonated hens' eggs.

Embryonated eggs from white leghorn hens were supplied by a commercial hatchery (Appendix) and incubated for 10 days at 38°C in a humidified egg incubator. Before inoculation all eggs were candled and two positions were marked with pencil on the shell; one over the air sac and the other on the side below the margin of the air sac, in an area free from large blood vessels. The eggs were then swabbed generously with 70 per cent ethyl alcohol and the positions marked were carefully drilled with an electric egg drill just to the shell membrane. After drilling the eggs were again swabbed with the alcohol.

During inoculation the area over the air sac was bored gently with a sterile needle to equalize the pressure. The eggs were inoculated through the drilled area on the side with 0.1 ml of a 10^{-5} dilution of the virus prepared in PBS. The holes were sealed with cellophane tape and the eggs incubated at 35°C for 48 hours.

At the termination of the incubation period the eggs were chilled for about 24 hours at 4°C before harvesting the allantoic fluid. The allantoic fluid from each egg was aspirated off separately into sterile tubes. The content of each

tube was spot tested separately for HA activity and the tubes showing positive HA activity were later titrated. The contents of all tubes with similar HA titres were pooled and stored at -20°C. Fresh passages of the virus were done every two months.

ii. Determination of the haemagglutinin titre

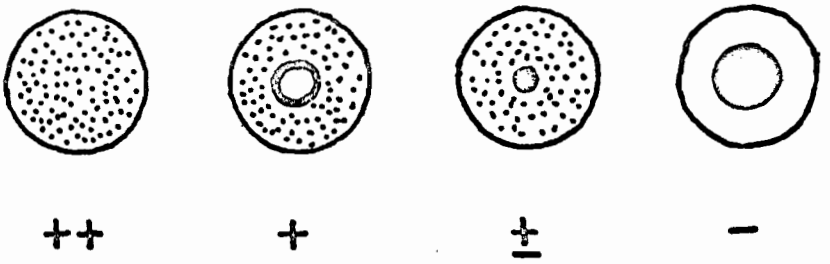
a. Preparation of chicken red blood cells

Blood was collected from an adult fowl by cardiac puncture and stored in Alsever's solution. The red blood cells were stored in the Alsever's solution at 4°C for 24 hours before use, and were not kept longer than two weeks after preparation. Prior to use they were washed three or four times in PBS and after the final wash a 10 per cent, v/v, suspension was prepared. From this stock suspension a 0.25 per cent suspension was prepared and used for all titrations.

b. Titration of the virus

The haemagglutinin titration was set up as illustrated in Table 1. This was a method originally recommended by Dr. Robert S. Gohdand used by Cooke (1958, 1960) and Syeklocha (1962, 1964). Round bottom test tubes measuring 7.5 mm by 75 mm were used. The test was read after 2 hours at 4°C and the "patterns" of the settled red blood cells were recorded (Salk, 1944). Since it is essential that the pattern selected as the end point be consistent in this investigation the end point was taken as the tube showing the so-called "Bull's eye" or "doughnut" pattern of settled cells (David-West, 1962) (Fig. 5).

Figure 5



Legend for reading patterns of haemagglutination test (David-West, 1962):

+ = Positive for haemagglutination

- = Negative for haemagglutination

Second pattern taken as the endpoint.

TABLE 1

Haemagglutinin Titration

Tubes	1	2	3	4	5	6	7	8
Allantoic fluid 1:1000 (ml.)	0.50	0.33	0.25	0.20	0.16	0.12	0.06	0.00
PBS	0.00	0.17	0.25	0.30	0.34	0.38	0.44	0.50
Final dilution 1:	1000	1500	2000	2500	3000	4000	8000	0
0.25% red blood cells (ml.)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

D. Screening of Culture Filtrate Extracts for Antiviral Activity

i. Preparation of Modified Maitland cultures

a. Preparation of the chorioallantoic membrane

Maitland and Maitland (1928) infected pieces of chicken kidneys, maintained in a mixture of Tyrode's solution and hen's serum, with vaccinia virus. In this system, although the tissues showed no evidence of growth, they supported multiplication of the virus. Fulton and Armitage (1951), Tamm et al. (1952) and Fazekas de St. Groth and White (1958a) adapted the chorio-allantoic membrane (CAM) of embryonated chicken eggs to Maitland-type cultures for the multiplication of influenza virus. The pieces of CAM were maintained in simple glucosol medium (Appendix).

In the present studies several methods were tried for the preparation of the pieces of CAM, including that used by Cooke (1960) and Syeklocha (1962; 1964), in which the eggs were opened with forceps over the air sac and after carefully pouring off the contents of the eggs the CAM was left adhering to the shell. The CAM was then washed in situ with medium and the shell along with the CAM was cut into the required bits. With this method in the hands of the candidate, difficulties arose, due to dislodging of the CAM along with the other contents of the eggs during the pouring out process, in a number of the eggs. There was also an interference with the albumin even when a successful pouring of the contents was achieved. The method described here, which was a modification of those used by Tamm et al. (1953a) and Fazekas de St. Groth and White (1958a) proved more satisfactory.

Ten-day-old embryonated hen's eggs were used in the preparation of the Maitland cultures because of their greater sensitivity in supporting the multiplication of influenza virus in this system (Fazekas de St. Groth, 1959). The eggs were swabbed with 70 per cent ethyl alcohol and held in an horizontal position. A light crack was made about $\frac{1}{2}$ of the distance from the pointed albumin end of the egg. With a sterile pair of scissors inserted through the crack, the shell and CAM were cut through. The cut was continued around the shell, always maintaining the same distance. The egg was then turned to a perpendicular position with the albumin end pointing downwards and with a gentle swirling motion the contents of the upper half were poured out and discarded

along with the albumin half of the egg. Almost invariably the embryo was held back in the air sac half of the egg and was removed with a pair of forceps after cutting the connecting tissue. The air sac half was then cut into three or four equal pieces, depending on the size of the egg. The pieces of CAM were removed from the shell with a sterile pair of fine forceps. The pieces of tissue were rinsed three consecutive times in the glucosol medium used for the Maitland cultures and pooled.

Although individual pieces of membrane derived from the same egg did not vary significantly in either their susceptibility to infection by influenza virus or the final yield of virus particles (Fulton and Armitage, 1951; Fazekas de St. Groth and White, 1958b), variations were reported for membranes from different eggs of the same age (Fazekas de St. Groth and White, 1958b). It was therefore necessary to pick the pooled pieces of CAM into each flask at random.

b. Setting up the Maitland cultures

Sterile 25-ml Erlenmeyer flasks containing the appropriate volume of the glucosol medium were set up. The amount of the medium varied depending upon the ml per mg relationship of the extract to be tested; usually this was between 2.0 ml and 2.5 ml. Each flask received a piece of CAM picked out at random from the pool. After the addition of the desired amount of the mould culture filtrate preparation and the appropriate test virus dilution the flasks were stoppered and incubated on a temperature controlled water bath shaker set at 35°C. The platform of the shaker was adjusted to move horizontally at a frequency of about 80 to 90 strokes per minute. The flasks were in-

cubated for 40 to 44 hours. At the end of the incubation period the CAM pieces were picked out of the flasks and were either discarded or reserved for wet weight determinations and the assay of cell-associated virus, depending upon the experiment. The culture medium in each flask was titrated for virus activity . Continuous agitation of the cultures during incubation prevented a local accumulation of metabolites on the cell surfaces and also effected an adequate aeration (Tamm et al., 1953a; Fazekas de St. Groth and White, 1958a).

The sizes of the CAM pieces used for the Maitland cultures may influence the final virus yield (Fazekas de St. Groth and White, 1958a). However, in the present studies it was found that with weights of CAM pieces between 10 mg and 20 mg the final yield of virus was not significantly different; nevertheless, pieces of CAM weighing about 20 mg were used routinely.

ii. Determination of the profile of activity of the extract

A new method for screening the mould culture filtrate extracts for antiviral activity was developed. Essentially, the method consisted of testing different doses of the extract for activity thereby establishing a profile of activity for each batch screened. For each dose tested 5 flasks of Maitland cultures were set up and each dose was tested against 100 EID_{50} (Egg Infective Dose) of the test influenza A virus (PR8). The virus dose was calculated by the method of Reed and Muench (1938).

This method has a number of advantages. By establishing the profile of activity, which is similar to dose-response assays, toxic doses as well as non-

active doses can be eliminated in subsequent tests with the particular batch of extract. The chances of scoring any particular batch as nonactive if it fails to show activity in an arbitrarily decided dose is also eliminated. The method also makes possible a direct comparison of the potency of the different batches prepared.

At the end of the incubation period of the Maitland cultures the virus content of each flask was titrated separately and expressed in terms of per cent inhibition relative to the titre of the virus control flasks. The HA test was used to assay the virus and the scheme illustrated in Table 1 was followed, with the exception that with the Maitland cultures the range of dilution was from undiluted to 1:800.

Although assay for infectivity is more sensitive than titrations for haemagglutinin, the latter method recommended itself because of the greater stability of the influenza virus haemagglutinin under the experimental conditions (Tamm et al., 1953a; Horsfall, 1954; Horsfall, 1955). Moreover, the HA test has the advantages of simplicity as well as precision (Tamm et al., 1953a).

Fazekas de St. Groth and White (1958b) demonstrated that the Maitland-type cultures were consistently more precise than the titration of influenza virus in the allantoic cavity of embryonated eggs.

iii. Toxicity tests

At the end of the incubation period the contents of all the flasks in which the pieces of CAM had turned whitish and phlegmatic were discarded. This was taken as a presumptive evidence for toxic doses of the extract tested. After

the selection of an appropriate test dose proven non toxic in CAM the lack of toxicity was further checked by inoculating the allantoic cavity of 10-day old embryonated chicken eggs with a suspension of the ground pieces of CAM. The eggs were incubated at 35°C for 48 hours and the allantoic fluid aspirated and tested for haemagglutinin titre.

Another presumptive test used was the colour of the CAM in those flasks with nontoxic doses of the extract. The CAM in these flasks was pink in colour, with healthy blood vessels.

iv. Testing the extracts for haemagglutinating and haemagglutination-inhibition activities

Since the HA test was routinely used to assay for the activity of the extract of the mould culture filtrates, it was essential to know if the extract per se possessed HA activity as well as haemagglutination-inhibition (HI) activity.

The HA test was set up by preparing serial doubling dilutions of the extract in 0.5 ml amounts in PBS; an equal volume of 0.25 per cent chicken red blood cells was added to each tube. The tubes were incubated at 4°C for 2 hours and the patterns of settled cells recorded.

The HI test was set up according to David-West (1963). Serial two-fold dilutions of the extract were prepared in PBS in 0.25 ml amounts. To each tube was added 0.25 ml of an influenza A virus (PR8) suspension calculated to contain 4 agglutinating doses (AD). The AD calculation was made according to Hsiung (1964). The racks were shaken and left at 4°C for 30 minutes. Each tube then received 0.5 ml of 0.25 per cent chicken red blood cells and after shaking the racks a further incubation at 4°C was carried out for 2 hours. The end point

was taken as the highest dilution of the extract that prevented haemagglutination by the 4 AD of the virus used.

E. Preparation of Tissue Cultures

i. Chick fibroblast cultures

a. Preparation of glassware and stoppers

Glassware was soaked overnight in a nontoxic detergent solution, 7X (Linbro Chemical Co.). After brushing it was rinsed thoroughly in tap water, then in three changes of distilled water and finally given an additional rinse in glass distilled water. It was then dried, stoppered and sterilised by autoclaving at a pressure of 15 lb. per sq. in. (p.s.i.) for 15 minutes.

Glass coverslips were washed essentially as described above but after the final rinse they were immersed in absolute alcohol and after flaming they were thoroughly wiped with a soft tissue. They were sterilised along with the appropriate glassware.

Nontoxic white rubber stoppers were used. They were treated like the glassware, but before sterilisation they were individually packed in domestic aluminium foil.

b. Preparation of the tissue

Embryonated 10-day chicken eggs were used. The eggs were wiped with 70 per cent alcohol and opened aseptically over the air sac with a pair of sterile forceps. The embryos were collected into a sterile container and were decapitated and the legs removed. The torsos were washed in cold Hanks' balanced salt solution (BSS). After mincing, the tissue paste was

transferred into a trypsinization flask with teflon-coated stirring bar and washed with Hanks' BSS, to remove as much of the blood as possible. The tissue was drained of the BSS and about 10 ml of a 0.25 per cent trypsin solution per embryo processed was added to the flask. The trypsin was prepared in Medium 199, Hanks' base, (Microbiological Associates) and warmed at 37°C before use.

Trypsinization was carried out for 45 minutes at room temperature. The cell suspension was filtered through 6 layers of sterile gauze into centrifuge bottles and centrifuged at 80 x G for 15 minutes.

The supernatant was discarded and after sucking off the layer of fatty material on the surface of the packed cells, the cells were washed in pre-warmed BSS, filtered and centrifuged. The packed cells were given a final wash in pre-warmed Medium 199 and after re-packing at 80 x G for 15 minutes, a 10 per cent suspension, v/v, was made in pre-warmed Medium 199 supplemented with 10 per cent calf serum.

A sample of the cell suspension was mixed with nigrosin stain and placed in an haemocytometer. The viable cells, i.e., the unstained cells, were counted and the stock cell suspension adjusted to contain 3×10^5 cells per ml. Three-ounce prescription bottles were seeded with 10 ml of the cell suspension and stoppered. Leighton tubes were seeded with 2 ml of the suspension. The bottles were incubated at 37°C for the establishment of cell monolayers. Usually this took about 24 hours. The medium was changed when the pH became acid.

ii. Monkey kidney cells

Kidney cells prepared from Rhesus monkeys were obtained from Dr. Anne M. Masson of the Virus Laboratory, McGill University. The cells, when received, were already appropriately suspended in nutrient medium and contained 6×10^5 cells/ml. They were seeded without further treatment. After the cells were established, the medium was changed when necessary and replaced with Medium 199 without serum.

F. Techniques for Bacteriophage Studies

Two different types of bacteriophage (phage) were used; the single-stranded DNA phage, ØX174 and the RNA phage, f2. The former was obtained through the courtesy of Dr. Irwin Tessman of Purdue University and the latter from Dr. Norton D. Zinder of the Rockefeller Institute. The indicator bacteria strains for the two phages, E. coli C for phage ØX174 and E. coli K13 for phage f2, were also sent along with the phages.

i. Titration of phage

Before titrating the phage the indicator bacteria were grown in order to obtain a fresh stock of the strains. E. coli C was grown in McGill beef heart infusion broth and three consecutive passages of 24-hour cultures were made. From the last passage a 16-hour culture was prepared and from this a 2-hour culture was grown. The 2-hour culture was set up by preparing a 1/5 dilution of the 16-hour culture in medium 3XD. All incubations were at 37°C on the platform of an electrically-operated culture shaker. The bacteria content of the 2-hour culture was determined with a photonephelometer. For the phage titration 1×10^8

bacteria were used per plate to give a confluent bacterial growth.

The scheme for the passage of E. coli K13 was similar to that of E. coli C with the following exceptions. After maintaining two consecutive 24-hour cultures in McGill glucose beef heart infusion broth, the final 24-hour culture was grown in the medium proposed by Loeb and Zinder (1961). Both the 16-hour culture and the 2-hour culture were also set up in this medium.

The agar-layer method of Adams (1950) was used to titrate the phage. Nutrient agar plates were prepared with 1.5 per cent Difco agar solution in McGill beef heart infusion broth. A 0.7 per cent agar solution in phosphate buffer, pH 7.2, was also prepared and dispensed in 2 ml amounts into test tubes. The agar was melted and held at 45°C before it was used. To the molten agar was added the indicator bacteria and the dilution of the phage to be titrated prepared in phosphate buffer, pH 7.2. The contents of the tube were mixed thoroughly but quickly between the palms and poured over the surface of the agar plate. After uniformly spreading the agar the plates were incubated at 37°C. The plates were inspected at intervals for the appearance of plaques. The plaques were counted after four hours.

Agar plates for the titration of phage f2 were prepared in the medium of Loeb and Zinder (1961).

ii. Preparation of standard phage pool

To each of the plates showing confluent phage plaques were added 2 ml of phosphate buffer. The buffer was spread evenly over the surface and the plates were left at 4°C for 10 minutes. The surface of the agar was then gently scrubbed

with a glass rod and the harvests from the plates were pooled in a sterile test tube. The plates were rinsed with a further 1 ml of buffer and the rinse was added to the original harvest. This served as a standard phage stock and was titrated before use.

G. Chromatographic Techniques

Thin-layer chromatography was used for the detection of amino acids and sugars present in the mould culture filtrate extracts. The method recommended itself more than paper chromatography , especially for the separation of amino acids, because of its greater rapidity, greater sensitivity, greater resolution and requirement for only small amounts of the sample to be chromatographed (Randerath, 1964). Thin-layer chromatography has also proved to be particularly useful in the separation of sugars (Stahl, 1965; Jacin and Mishkin, 1965).

i. Preparation of standard silica gel plates

a. Coating of the plates

Standard silica gel G plates were prepared according to Stahl (1965) and Randerath (1964). Glass plates measuring 20 cm x 20 cm were cleaned in 7X detergent, rinsed several times in distilled water and dried in a 110°C oven.

The gel was weighed,placed in a flask and dissolved in distilled water. Two ml of distilled water were required to dissolve each gram of the gel. The contents of the flask were mixed by vigorous swirling, avoiding bubbles, to produce a uniform slurry. The process of mixing was carried on for exactly 90 seconds. The slurry was then poured into a Desaga spreader equipped with

a tipping mechanism and adjusted to produce a layer of gel 250 microns in thickness. After spreading the plates with the slurry they were left to set at room temperature before they were transferred into a desiccator containing $CaCl_2$.

For the separation of sugars the slurry was prepared in 0.02 M borate buffer, pH 8.0 (Jacin and Mishkin, 1965) and the plates were activated at 100°C for 30 minutes before use. Activation of the plates was not necessary for the separation of amino acids.

b. Setting up the plates

A margin of about 5 mm was rubbed off from either edge of the plate along the direction of spread of the gel. Another margin of about 20 mm., perpendicular to the first one, was also rubbed off from the top edge of the plates; this space was used for writing the codes of the samples and for holding the plates. A distance of 20 mm was marked from the lower edge. This served as the point of origin of the samples. Another spot 100 mm from the origin was also marked. The samples were applied on spots 20 mm equidistant from each other along the origin. Five microlitres of each sample were delivered with 20 microlitre - capillary tubes. Each sample was applied as several superimposed spots and dried with a hair drier.

The plates were placed in a perpendicular position in a chromatographic tank filled to a depth of 5 mm with the appropriate freshly prepared solvent system. The tank was lined with Whatman filter paper. The run was stopped as soon as the solvent front reached the 100 mm mark and the time taken for the run was recorded. The plates were dried again with the hair drier and

finally dried in the oven at 100-105°C for 5 minutes. As soon as they were removed from the oven, they were sprayed with the appropriate reagents and returned to the oven for another 5 minutes. The Rf's of the spots were then recorded. Before and after the run the spots were viewed under ultraviolet lights of wavelengths 366 mμ and 260 mμ.

Each sample was run simultaneously with reference compounds. Reference amino acid solutions were purchased from the Consolidated Laboratories Ltd. The reference sugar solutions were locally prepared as 1 per cent solutions in 10 per cent isopropyl alcohol. These solutions were further diluted 1:5 with the alcohol before use.

c. Solvent systems used

A solvent system of n-butanol - acetic acid - water in the proportions of 4: 1: 5 v/v (Partridge, 1948) was used for the separations of both amino acids and sugars. The same solvent system but in the proportions of 5: 4: 1 v/v (Jacin and Mishkin, 1965) was used for sugars alone. Two layers separated out after mixing and after standing at room temperature the top layer was used.

For two dimensional chromatography of amino acids, n-propanol-water (7:3, v/v) was used as the first solvent system and 96 per cent ethanol - water (7:3, v/v) was used as the second solvent system. Two dimensional chromatography was not used for the separation of the sugars (Smith, 1960).

d. Developing reagents

The polychromatic spray of Moffat and Lytle (1959) was particularly useful in the detection of amino acids. With this spray the characteristic colours

produced by the individual amino acids provided additional criteria for the identification of the spots. Sugars were developed with aniline hydrogen phthalate spray (Partridge, 1949) and benzidine spray (Horrocks, 1949).

ii. Hydrolysis of the samples

a. Amino acids

The hydrolysis of the sample, for amino acid detection was carried out by the method used by Matheson and Reed (1959). Hard glass tubes containing the samples and 6N HCl were partially evacuated of oxygen by flushing with nitrogen gas and after sealing with flame the samples were hydrolysed at 120-121°C for 24 hours. After the hydrolysis the contents of the tubes were poured into test tubes and centrifuged to remove humin substance formed during the hydrolysis (Neurath, 1963). The supernatant was aspirated into small beakers and evacuated in a desiccator over NaOH pellets. After the hydrolysates were evaporated to dryness the amino acid chlorides were decomposed by five consecutive treatments with distilled water (Neurath, 1963; Stahl, 1965). The hydrolyzates were finally taken up to half the starting volume with 10 per cent isopropyl alcohol. Hydrochloric acid was used in the hydrolysis in preference to sulphuric acid because of the increased rate of the cleavage of peptide bonds in the former (Neurath, 1963).

b. Sugars

For the detection of sugars, the method of hydrolysis of Adams (1965) was used. The samples were hydrolyzed in sealed glass ampoules with 0.5N HCl. The ampoules were heated in a boiling water bath for four hours. After neutralizing with 0.5N NaOH the contents were evacuated to dryness over

NaOH pellets and finally made up to half of the original volume with 10 per cent isopropyl alcohol.

IV. EXPERIMENTAL PROCEDURES AND RESULTS

Since the use of the McGill glucose beef heart infusion broth and the Ammonium sulphate Method (Cooke, 1960; Syeklocha, 1962; 1964) for the production and the purification of the antiviral substance (AVS) have been replaced respectively by Czapek-Dox broth and the Acetone Method, which were developed during the current investigation, the data to be presented are those obtained with these newer methods and references will be made to the older methods where necessary. Also, although it was the practice during the initial stages of the investigation to test all the fractions of the purification steps, only the results obtained with the final supernatant fraction will be reported since all the other fractions were inactive (Fig. 4).

A. Biological Studies with the Culture Filtrates of Penicillium cyaneo-fulvum

i. Antiviral activities

a. The effect of the antiviral substance on the infectivity of influenza A virus in Modified Maitland Cultures

The modified Maitland cultures prepared from the chorioallantoic membranes of 10-day old embryonated hens' eggs were used routinely as the principal tissue system in the present investigations; influenza A virus PR8 strain was also used as the routine test virus. The activity of the antiviral substance was assayed in terms of the suppression of the multiplication of 100 EID_{50} of the virus, as determined by the titration of viral haemagglutinins (HA) (Table I) and expressed as per cent inhibition in relation to the virus control.

Table II illustrates typical results obtained with one of the batches of

TABLE II

The Effect of the Antiviral Substance on Influenza A Virus (PR8) in Modified Maitland Cultures

Flask No.	Virus Control	AVS Dose per Flask*		
		0.25 mg	0.1 mg	0.05 mg
	Haemagglutinin Titre			
1	250	10	10	200
2	100	10	10	100
3	200	10	10	100
4	100	100	150	150
5	100	0	10	10
Average HA titre per flask	150	26	38	112
% Inhibition	0	83	75	25

* 1. 0.5 mg per flask was toxic.

2. the dose of 0.25 mg/flask (Batch 251165) is equivalent to 0.07 mg/ml of culture medium.

the AVS. As stated previously, each batch of AVS was tested in different doses in order to establish a profile of activity for the AVS. There was some fluctuation in potency among the different batches processed; such variations only affected the degree of inhibition produced by some doses of the AVS. In all the batches tested better than 80 per cent inhibition of virus multiplication was produced by doses of 0.2 mg or 0.25 mg per flask.

In one experiment, 0.125 mg of AVS was effective in causing 96 per cent suppression of the multiplication of 10^5 or 10^6 EID_{50} of the virus. In all of these experiments the virus inoculum was diluted below the level of detection of any HA activity. Therefore, any HA detected at the end of the experiments must have been due to virus multiplication.

b. The effect of the antiviral substance on the infectivity of Newcastle Disease Virus and Influenza B Virus in Modified Maitland Cultures

The successful suppression of the multiplication of influenza A virus in Maitland cultures by the antiviral substance occasioned a trial of the AVS against other myxoviruses. Two other viruses were chosen; influenza B virus (Lee strain) which is known to multiply readily in Maitland-type cultures prepared from the chorioallantoic membranes CAM (Tamm et al., 1952; 1953a; Womack and Kass, 1953) and the B_1 strain of Newcastle disease virus (NDV) which multiplies to high titres in the allantoic sac but which has not been used before in the Maitland cultures. The former was obtained through the courtesy of Dr. Igor Tamm of the Rockefeller Institute, New York and the latter from the American Type Culture Collection, Washington, D.C. Both viruses were main-

TABLE III

The Effect of the Antiviral Substance on Influenza B Virus (Lee Strain) and Newcastle Disease Virus (B1 Strain) in Modified Maitland Cultures

Flask No.	Influenza B Virus Control	Influenza B Virus plus AVS	Flask No.	Newcastle Disease Virus Control	Newcastle Disease Virus plus AVS*
	Haemagglutinin Titre				
1	250	10	1	40	<10
2	200	10	2	80	<10
3	250	10	3	80	<10
4	150	10	4	80	<10
5	250	10	5	80	<10
Average HA titre per flask	220	10		72	1**
% Inhibition	0	96		0	99

* AVS Batch 30365, 0.25 mg per flask (0.125 mg/ml)

** Titre of less than 10 with nontoxic doses taken as unity.

tained, on arrival, by serial allantoic passages in 10-day old embryonated hens' eggs and 100 EID_{50} of each were used in the Maitland cultures. The tests were set up as for influenza A virus.

Table III illustrates that 0.25 mg of the AVS caused 96 per cent inhibition of the multiplication of influenza B virus and 99 per cent inhibition of NDV. In separate experiments it was shown that influenza A virus was also similarly suppressed by this batch of AVS.

c. In ovo studies with Influenza, Newcastle Disease, Mumps, Vaccinia, and Herpes Simplex Viruses

In a further attempt to extend the spectrum of activity of the AVS as well as trying other test systems, the AVS was used in embryonated hens' eggs against influenza A and B, Newcastle disease, mumps, vaccinia and herpes simplex viruses.

Influenza A and B, Newcastle Disease and Mumps Viruses

Previous reports on the activity of the AVS against the multiplication of influenza A virus (PR8) in ovo have not been in complete agreement. Cooke (1960) reported good suppression of the multiplication of the virus in eggs. However, Syeklocha (1962), using the same virus, failed to obtain significant antiviral activity; some of the batches of the AVS were not active at all in ovo. With mumps virus, Enders strain, both workers reported difficulties in obtaining a high virus titre and consequently only a limited number of experiments was conducted with this virus; the results were not conclusive in either case.

The Enders strain of mumps virus was again used in the present investigations and was obtained from Dr. Masson of the Virus Laboratory, McGill University, as an allantoic fluid preparation. A high titred virus pool was obtained by passing a 10^{-3} dilution of the virus, prepared in PBS, several times in the amniotic sac of 7-day old embryonated eggs and incubating the eggs at 35°C for 7 days. Subsequent serial allantoic passages were carried out with a 10^{-5} virus dilution. After such passages most of the eggs had HA titres between 1/640 and 1/1280. The maintenance of the other myxoviruses was described earlier.

Several in ovo experiments were conducted with 100 EID_{50} of each these myxoviruses. The virus was inoculated into the allantoic sac and followed immediately by inoculation of the AVS. No antiviral activity was demonstrated; even when the virus dose was decreased 10-fold and the AVS concentration increased 10-fold of the dose used in Maitland cultures. The possibility that the failure to observe antiviral activity in eggs might be due to dilution by the allantoic fluid was considered. It has been estimated that the volume of allantoic fluid of 10-day old embryos is 5 ml (McLimans et al., 1957). But similar volumes of culture medium were used in Maitland cultures with good suppression of virus replication with the same AVS dose. Furthermore, in ovo tests in the amniotic sac also failed to show any antiviral activity against these viruses. The volume of amniotic fluid is much less at this age of embryonic development; it was found to be about 2 ml (David-West, 1962). The results of the experiments in eggs are shown in Table IV.

TABLE IV

The Effect of the Antiviral Substance on the Infectivity of Selected Myxoviruses in Embryonated Eggs

Egg No.	Influenza A Virus Control	Influenza A Virus + AVS 4565 2 mg/egg	Egg No.	Influenza B Virus Control	Influenza B Virus + AVS 30365 3 mg/egg	Egg No.	Newcastle Disease Virus Control	Newcastle Disease Virus + AVS 4565 2 mg/egg	Egg No.	Mumps Virus Control	Mumps Virus + AVS 30365 2 mg/egg
	Haemagglutinin Titre										
1	10,240	10,240	1	640	1,280	1	5,120	2,560	1	320	640
2	10,240	20,480	2	80	2,560	2	2,560	5,120	2	320	160
3	10,240	20,480	3	1,280	80	3	10,240	10,240	3	320	640
4	20,480	10,240	4	1,280	640	4	5,120	2,560	4	640	320
5	5,120	10,240	5	N.T.	N.T.	5	320	2,560	5	640	160
Average HA titre	11,264	14,336		820	1,140		4,672	4,608		448	384

1. N.T. stands for Not Tested.
2. Influenza A Virus: 10 EID_{50}.
   Influenza B Virus: 100 EID_{50}.
   Newcastle Disease Virus: 10 EID_{50}.
   Mumps Virus: 100 EID_{50}.

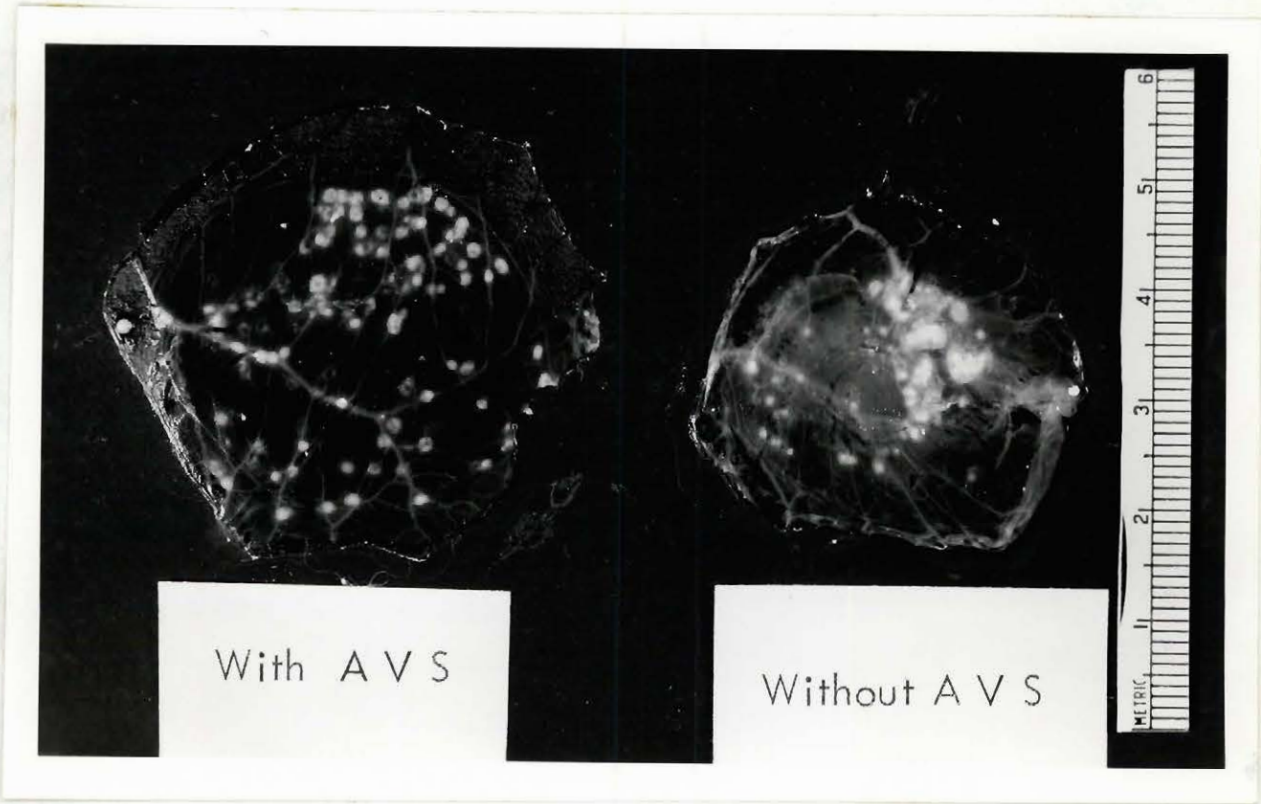
Vaccinia Virus and Herpes Simplex Virus

These two DNA-containing viruses are treated together in this section because the same in ovo technique was used. The effects of the AVS were studied by the pocks suppression technique.

The viruses were supplied by Dr. Masson, as monkey kidney tissue cell culture preparations.

Twelve-day old embryonated eggs were used as recommended by Westwood et al. (1957). The eggs were wiped with 70 per cent alcohol after candling. Two spots were marked on the shell; one over a relatively avascular area of the CAM, above the position of the embryo, and the second on the centre of the airspace. Holes were drilled through the shell at both spots, taking great care not to break through the shell membrane. After drilling, the eggs were wiped again with alcohol. Each egg was placed horizontally on an egg cup; the lateral hole was rimmed with melted paraffin wax and the shell membrane over the airspace was punctured. The virus inoculum was placed on the lateral spot and the shell membrane was gently torn with a needle. A light suction was applied to the airspace, while simultaneously candling the egg and, as an artificial airspace was created below the lateral spot, the inoculum was sucked in onto the CAM. The AVS was then delivered through a 1 ml syringe. The holes were sealed with cellophane tape. The eggs were given a gentle rocking from side to side, to mix and distribute the virus inoculum and the AVS,and incubated at 35°C for 48 hours. The eggs were candled every 24 hours during the incubation period and any embryo dying within the

Figure 6



The effect of the antiviral substance on vaccinia virus multiplication on chorioallantoic membrane in ovo:

Primary and secondary pock formation.

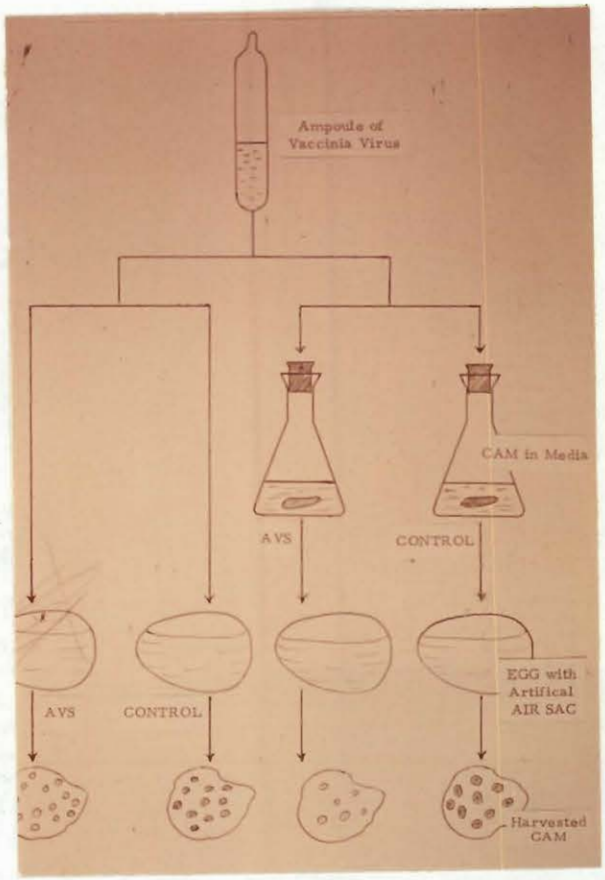
first 24 hours was discarded. If the artificial airspace became closed additional suction was applied. At the end of the incubation period, the eggs were chilled, wiped with alcohol and the CAM was harvested into saline wash solution; the pocks were counted against a black background (Kempe, 1956). In the enumeration of the pocks for vaccinia virus, both primary pocks and the smaller secondary or satellite pocks (Overman and Tamm, 1956) were counted. With herpes simplex, the pocks were much smaller and showed less tendency to become necrotic.

The AVS was essentially ineffective against the two DNA viruses used. In a preliminary experiment with herpes simplex virus, there was some evidence of activity but this was not reproduced in subsequent experiments. With vaccinia virus, the AVS seemed to induce the production of several small, secondary-type pocks or select for such variants (Fig. 6). These small pocks appeared stable since they were reproduced in two consecutive subcultures of the corresponding CAM in the absence of AVS. When the treatment of vaccinia virus with the AVS was first carried out in modified Maitland cultures (Fig. 7) before titrating the ground CAM pieces in ovo, a more definite suppression of the virus was observed (Table V). No pocks were produced in the CAM of the Maitland cultures.

d. In vivo studies with Influenza and Vaccinia Viruses

The ultimate goal for any antiviral agent is the demonstration of activity in humans. Animal test systems for the study of antiviral agents offer conditions that very closely approximate what may be expected in man, hence

Figure 7



Diagrammatic model of experiments on the effects of the antiviral substance on vaccinia virus.

TABLE V

Effect of the Antiviral Substance on Pock Production by Vaccinia Virus after *in vitro* Interaction in Maitland Cultures

Egg No.	Numbers and Types of Pocks			
	0.25 mg AVS*	Control	0.125 mg AVS	Control
1	0	30 small	3 small 1 confluent	6 large 1 confluent
2	0	18 small	10 small 1 confluent	38 large
3	0	10 small	3 small 1 confluent	35 large 1 confluent
4	0	21 small	10 small	13 large

* AVS Batch 4565

effective antiviral activity in infected animals has been described as the "crucial test" for any antiviral agent (Staehelin, 1959). It was desirable, therefore, to test the AVS in animals, since it had given very promising results in in vitro tests.

Two in vivo systems were selected for investigation; mice, for the study of antiviral activity against the RNA-containing influenza virus and rabbits, for the DNA-containing vaccinia virus.

Mice

In previous studies with the AVS in mice infected with influenza virus there was usually a delay in the onset of death of the animals and in a few instances there was a definite sparing effect (Cooke, 1960; Syeklocha, 1962). In some instances no significant activity was demonstrated (Syeklocha, 1964). In these studies it appeared in some instances that in vitro incubation of the virus and the AVS at room temperature for 30 minutes prior to inculation was essential, while in other cases this was not necessary.

In the present studies, random-bred white female Swiss mice with an average weight of 20 gm supplied by a commercial firm were used. The mice were divided into eight groups and the experiment was designed as follows:

Group A: Virus inoculated intranasally; AVS inoculated intranasally immediately afterwards. 10 mice

Group B: Virus inoculated intranasally; AVS inoculated intraperitoneally immediately afterwards. 10 mice

Group C: Virus inoculated intranasally; AVS inoculated intraperitoneally 1 hour afterwards. 10 mice

Group D:	AVS inoculated intraperitoneally; virus inoculated intranasally 1 hour afterwards.	10 mice
Group E:	Virus inoculated intranasally; PBS inoculated intranasally immediately afterwards.	10 mice
Group F:	Virus inoculated intranasally; PBS inoculated intraperitoneally immediately afterwards.	10 mice
Group G:	AVS inoculated intranasally; PBS inoculated intranasally immediately afterwards.	5 mice
Group H:	AVS inoculated intraperitoneally; PBS inoculated intraperitoneally immediately afterwards.	5 mice

The mice were prepared for inoculation by light ether anaesthesia and a virus inoculum of 0.05 ml., containing 6400 Haemagglutination Units, was employed. The virus inoculum was prepared by sedimenting a fresh allantoic fluid harvest at 2600 x G for 1 hour in Spinco Model L ultracentrifuge and finally suspending the pellet in PBS. Each treated mouse received a single dose of 2.8 mg AVS. After the inoculations, the mice were inspected twice daily for 14 days; only those found dead during the first inspection of the day were recorded as deaths for the particular day and those found dead at the second inspection were recorded as deaths for the following day. All mice were autopsied on the day of death.

Mouse lungs were examined at autopsy and the lesions scored in terms of the following arbitrary standards:

4 Plus: Complete consolidation of all lobes. The appearance was that of red hepatization.

3 Plus: 3/4 of the total lung tissue consolidated.

2 Plus: 1/2 of the total lung tissue consolidated.

1 Plus: 1/4 of the total lung tissue consolidated.

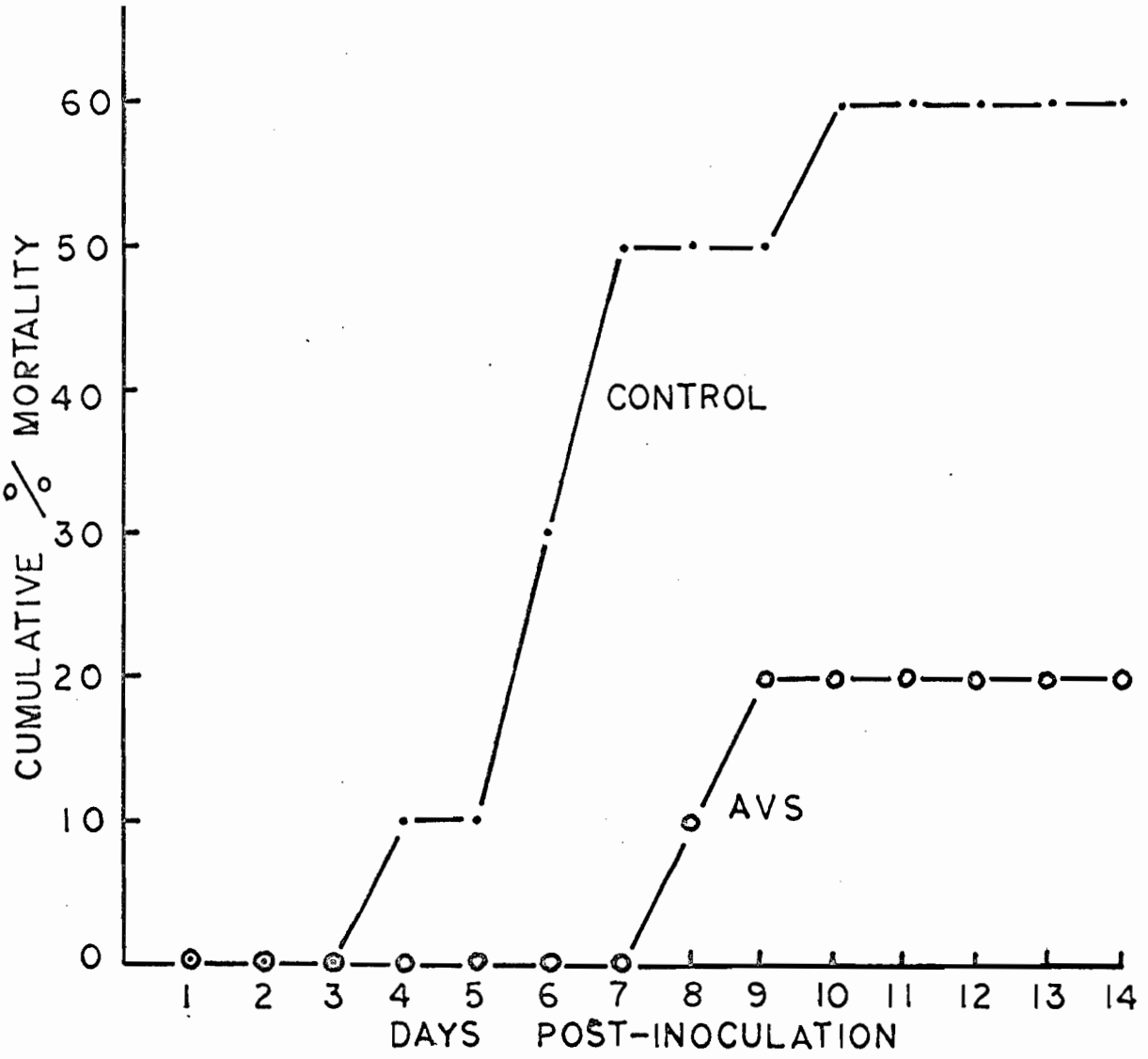
0 : None.

The lungs were weighed, after absorbing as much of the blood as possible on filter paper. A 20 per cent lung suspension in PBS was prepared from representative samples from each group. All the mice surviving at the end of the 14-day inspection period were sacrificed and autopsied.

The results of the different experiments are shown in Figures 8, 9 and 10. Figures 8 and 9 show that the AVS was very effective against intranasally inoculated influenza virus in mice when it was given immediately after the virus, by either the intranasal or the intraperitoneal routes. Under these circumstances there was both a delaying effect of 3 to 4 days in the onset of death in the treated groups and a sparing effect of 30 to 40 per cent. However, it would seem that when the AVS was inoculated intraperitoneally 1 hour before inoculating the virus intranasally it was not effective; however, some slight delaying and sparing effects were demonstrated when the AVS was given intraperitoneally 1 hour after the virus (Fig. 10).

The lungs lesion scores were high for both the controls and the test series although they were generally higher in the treated groups. The average lung weights were also generally higher in the treated groups (Table VI). It

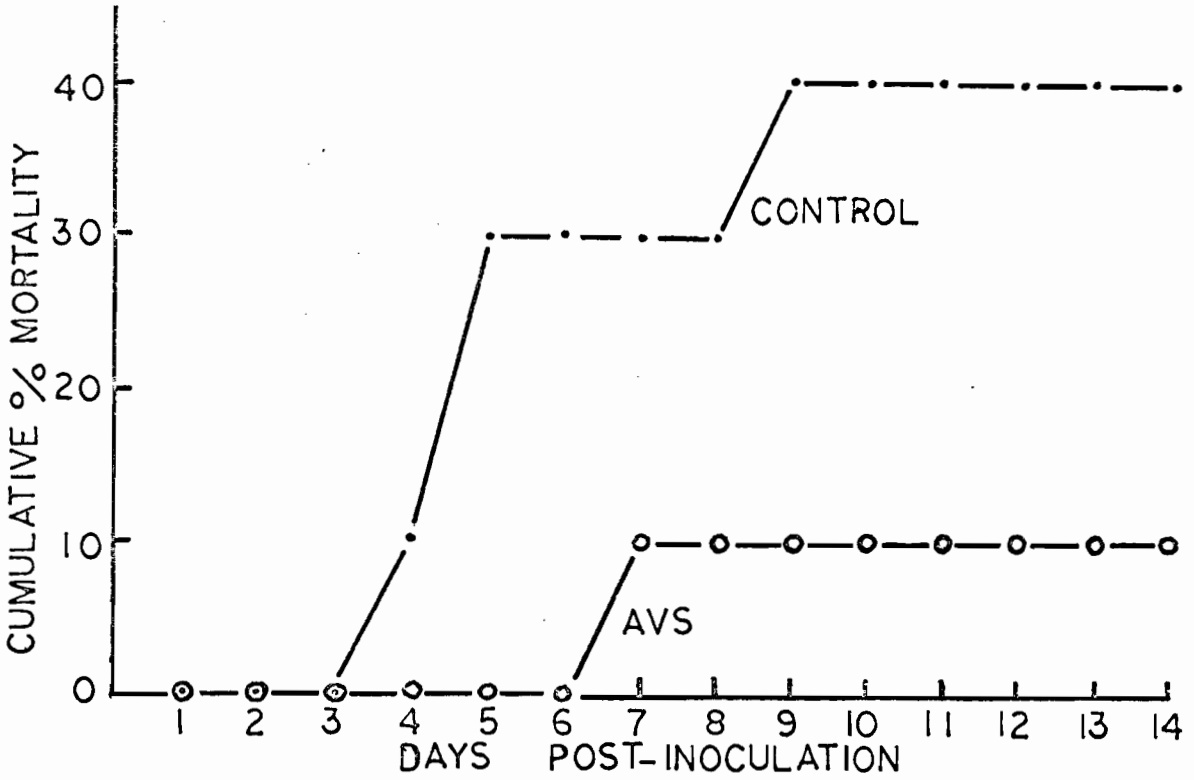
Figure 8



Effect of the antiviral substance on mice infected with influenza A virus (PR8).

i. Virus inoculated intranasally, followed immediately with intranasal instillation of the antiviral substance.

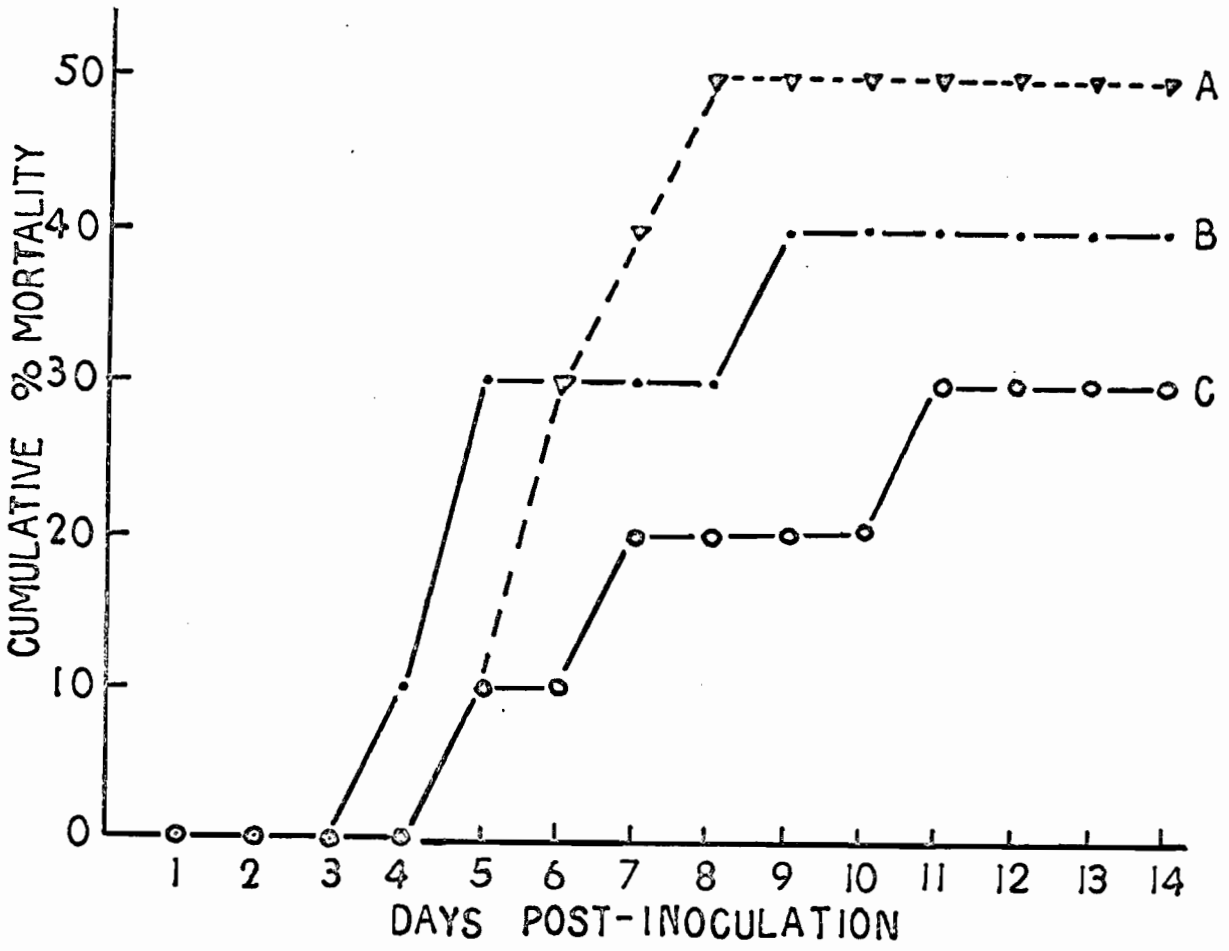
Figure 9



Effect of the antiviral substance on mice infected with influenza A virus (PR8).

ii. Virus inoculated intranasally, followed immediately with intraperitoneal injection of the antiviral substance.

Figure 10



Effect of the antiviral substance on mice infected with influenza A virus (PR8)

iii. Virus inoculated intranasally and the antiviral substance given intraperitoneally at different times.

A = Antiviral substance given one hour before virus
B = Control
C = Antiviral substance given one hour after virus

TABLE VI

Weights and Relative Consolidation of Lungs of Mice Infected with Influenza Virus (PR8) and Treated with Antiviral Substance

Mice Group	Average wt. of Lungs (mg.)*		Average Lung Score	
	Total	Sacrificed	Total	Sacrificed
A	442.4	423.5	3.3	3.25
B	384.5	375.9	3.2	3.2
C	366.4	327.5	3.4	3.4
D	367.9	303.3	3.4	2.8
E	416.8	376.8	3.6	2.3
F	310.2	258.9	2.4	2.3
G	-	231.7	-	1.4
H	-	245.3	-	0.8

Group A: Virus and antiviral substance intranasally (simultaneously).
Group B: Virus intranasally, antiviral substance intraperitoneally (simultaneously).
Group C: Virus intranasally, antiviral substance intraperitoneally (1 hour afterwards).
Group D: Virus intranasally, antiviral substance intraperitoneally (1 hour before).
Group E: Virus intranasally, PBS intranasally (simultaneously).
Group F: Virus intranasally, PBS intraperitoneally (simultaneously).
Group G: Antiviral substance intranasally.
Group H: Antiviral substance intraperitoneally.

* Average weight of lungs of normal mice - 166.0 mg.

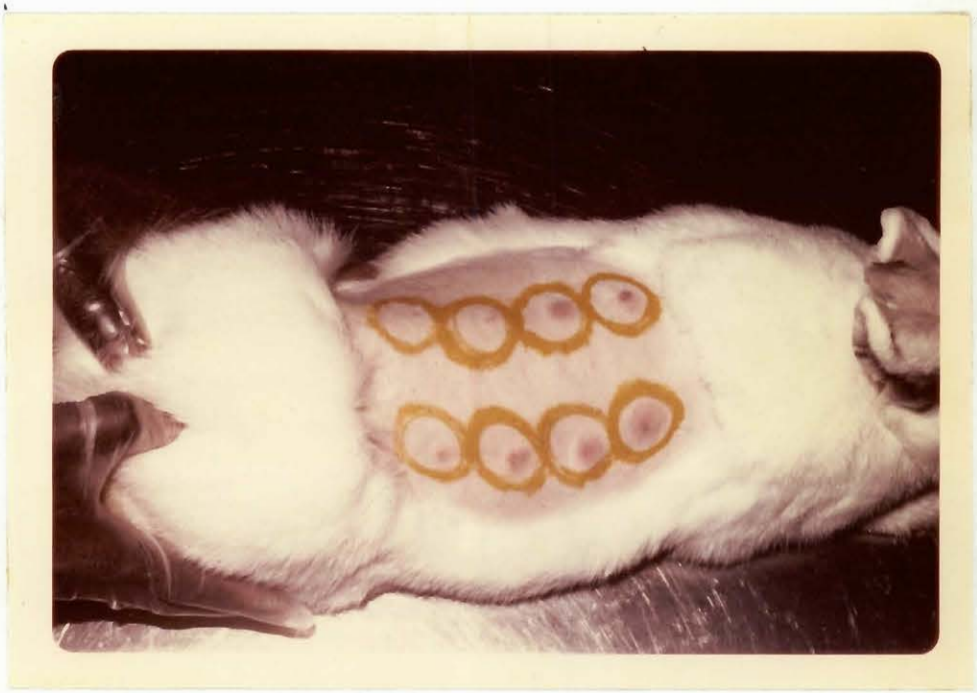
was observed that some mice which looked grossly healthy did, on autopsy, show high pulmonary scores. Finally, because there did not seem to be any obvious correlation between the gross state of health of the mice, the degree of lung consolidation and survival, it was decided to try other methods of evaluation which may be more sensitive. The determination of mean lung HA titre and the titration of infectivity have been reported to be very sensitive (Smorodintseff and Ostrovskaya, 1937; DeLong et al., 1965). The 20 per cent lung suspensions were titrated for both HA and HI activities; infectivity titrations were also carried out in 10-day old eggs, by allantoic inoculations. As a control, a similar suspension of normal mouse lungs was prepared. No HA activity was demonstrated in any of these suspensions. No infectious virus was demonstrated in two egg passages. However, in the HI tests, both the infected lung suspensions and the control suspension, showed an HI titre of 1/80.

Rabbits

The activity of the AVS was tested against vaccinia virus in white New Zealand rabbits weighing about 2 kg. The hair over the dorsal surface of the animals was clipped and the inoculation sites marked with a solution of picric acid (Appendix). The following experimental design was used:

Rabbit A: Varying doses of virus and a constant dose of AVS: virus dilutions of 10^{-1}, 10^{-2}, 10^{-3}, or 10^{-4}, in 0.1 ml volumes, were inoculated intradermally into each site followed immediately by the inoculation of 1.0 mg of AVS into each site on the left dorsal surface of the animal and an equal volume of PBS into each site on the right dorsal surface.

Figure 11



Effect of the antiviral substance on the development of vaccinial lesions on rabbit skin. Left side with AVS and virus. Right side with virus only.

Rabbit B: A constant dose of virus and varying doses of AVS: A 10^{-3} dilution of virus was inoculated into each site followed immediately by the inoculation of 2.0 mg, 1.0 mg, 0.5 mg and 0.25 mg AVS into each test site on the left dorsal surface of the animal. Control sites on the right side received PBS.

Rabbit C: A 10^{-3} dilution of virus was given intradermally. After 24 hours the animal received 2 mg AVS intravenously into each ear vein.

The virus inoculum used was a CAM preparation of the virus. The animals were kept under observation for one week; lesions began to develop after the second day post-inoculation and pictures were taken when the lesions were well developed.

No activity was demonstrated in rabbit C; the results with rabbit B were not uniform enough for a meaningful interpretation. However, with rabbit A, there seemed to be some suppression of the vaccinial lesions with the higher dilutions of the virus, i.e. with log dilutions 3 and 4 (Fig. 11).

These experiments were repeated with higher doses of AVS of another batch (Batch 11266). For the intradermal inoculations of the AVS, 2.6 mg per site was employed, while for the intravenous inoculations, three doses of 2.6 mg were given hourly.

There was no suppression of lesions when AVS was administered intravenously; the interpretation of the lesions on the animal which received the intradermal inoculations of the AVS was complicated by the erythema caused by the high dose of the AVS used such that no definite conclusion could be

drawn. A third series of experiments was discontinued because the test sites on the rabbits were overgrown with hair before the termination of the observation.

e. Tissue culture studies with vaccinia virus and poliovirus

Two tissue culture techniques were used to study the effect of the AVS on poliovirus and vaccinia virus; the plaque technique and the use of fluid cultures for the production of CPE. Rhesus monkey kidney cultures were used for both viruses; chick embryo fibroblast cultures were also used for vaccinia virus.

Sabin type 1 poliovirus was obtained through the courtesy of Dr. Masson, as monkey kidney cell preparations.

Plaque Technique

Plaque formation by vaccinia virus was carried out on chick embryo fibroblast cell cultures. The method of Lindenmann and Gifford (1963) was used. The virus inoculum contained about 20 pock forming units in 0.1 ml. Following the inoculation of the virus, 0.2 mg of AVS was added to the cultures immediately. The control cultures received an equal volume of Medium 199 instead of AVS. The cultures were set up in duplicate. After 2 hours at 37°C Medium 199 was added so that the cell sheets were just covered. The cultures were then incubated at 37°C for 72 hours and after decanting the medium, the cell sheets were stained with crystal violet (Appendix) and the plaques counted. There was no suppression of plaques. Two more experiments on plaque suppression were carried out using the same dose of AVS, with similar results.

Leighton Tube Cultures

Fluid cultures of monkey kidney cells and chick embryo fibroblast cells were prepared in Leighton tubes. The antiviral activity of 0.2 mg of AVS was tested against about 5 pock forming units of vaccinia virus. The control tubes received an equal volume of Medium 199 instead of AVS. Five tubes were used for each test. The cultures were incubated at 37°C for 72 hours, with daily inspection. At the end of the incubation period, the coverslips were removed and the cells stained with May-Grünwald Giemsa stain and mounted in permount (Negroni, 1964)(Appendix). There was no inhibition of cytopathogenic effect (CPE) in any of the tubes; the AVS control tubes did not show any obvious CPE. Similar results were obtained in three repeated experiments.

Similar tissue culture experiments were carried out with poliovirus (Sabin type 1) in Rhesus monkey kidney cell cultures. For the plaque technique the method of Dulbecco and Vogt (1954), as modified by Hsiung and Melnick (1955), was used; with agar overlay medium as suggested by Hsiung (1964) (Appendix). No plaque suppression by the AVS was observed in two preliminary experiments. However, these results may not be significant due to the technical limitations of not obtaining a confluent monolayer of cells. Although there was also no inhibition of CPE in three Leighton tube experiments, these too were more of a preliminary nature. These studies were not pursued further due to the limitations of time. It is suggested that a detailed study of the effect of the antiviral substance on poliovirus is necessary in view of the apparent contradiction between the present preliminary

observations and the observations reported earlier by Cooke (1960), who was able to inhibit the formation of CPE by type 1 poliovirus, Mahoney strain, using either 4 mg or 6.3 mg of AVS per tube. Moreover, the plaque technique would provide a more quantitative assay system than the recording of CPE.

f. The effect of the antiviral substance on bacteriophage

The activity of the AVS was tested against selected bacteriophages. It was felt that if anti-phage activity could be demonstrated, the phage system would provide a very convenient system to study the substance, since the different phage techniques are more thoroughly worked out than the corresponding techniques in animal virology.

Syeklocha (1964) failed to demonstrate activity against both T2 coliphage and the lytic phage, PKV5, of Bacillus subtilis (strain H, tryptophan minus mutant).

In the present investigation, two different phages were selected; the RNA-containing phage, f2 (supplied by Dr. Zinder) and the single-stranded DNA-containing phage, ØX174 (ØX) (obtained from Dr. Tessman). These were chosen because they share a number of properties with the animal viruses (Tessman, 1959; Zinder,1963). The f2 RNA phage was considered a suitable prototype for the study of the multiplication of RNA viruses (Cooper and Zinder, 1962);since the AVS inhibited the multiplication of a number of RNA-containing animal viruses it was hoped that the AVS would inhibit the f2 phage.

The Agar-Layer Method of Adams (1950) (Materials and Methods) was used in all the studies. The experimental procedure carried out in triplicate,

was as follows:

Group A Test: AVS plus phage incubated at 37°C for 30 minutes, then added to 0.7% melted agar containing indicator bacteria; the contents were mixed, poured over the surface of an agar plate and incubated at 37°C.

Group B Control: Buffer plus phage incubated at 37°C for 30 minutes, then added to 0.7% melted agar containing indicator bacteria; the contents were mixed, poured over the surface of an agar plate and incubated at 37°C.

Group C Test: AVS plus indicator bacteria incubated at 37°C for 30 minutes, then added to 0.7% melted agar containing phage; the contents were mixed, poured over the surface of an agar plate and incubated at 37°C.

Group D Control: Buffer plus indicator bacteria incubated at 37°C for 30 minutes, then added to 0.7% melted agar containing buffer; the contents were mixed, poured over the surface of an agar plate and incubated at 37°C.

Group E Control: Buffer plus indicator bacteria added to 0.7% melted agar, mixed, poured over the surface of an agar plate and incubated at 37°C.

Group F Test: AVS plus phage plus indicator bacteria added to 0.7% melted agar, mixed, poured over the surface of an agar plate and incubated at 37°C.

TABLE VII

The Effect of the Antiviral Substance * on the Infectivity on Bacteriophage f2

Groups **	Plaque Count After 4 Hours		
	Plate 1	Plate 2	Plate 3
A	94	105	100
B	104	101	107
C	100	108	97
D	Confluent	Confluent	Confluent
E	Confluent	Confluent	Confluent
F	107	112	115
G	98	104	118

* The antiviral substance used was 1.0 mg of Batch 4565; 0.1 mg of this preparation completely suppressed the multiplication of 100 EID_{50} of influenza virus (PR8) in Maitland Cultures.

** Group A: AVS plus phage incubated 37°C for 30 minutes, before adding indicator bacteria.
Group B: Buffer plus phage incubated 37°C for 30 minutes, before adding indicator bacteria.
Group C: AVS plus indicator bacteria incubated 37°C for 30 minutes, before adding phage.
Group D: Buffer plus indicator bacteria incubated 37°C for 30 minutes.
Group E: Buffer plus indicator bacteria without reaction at 37°C.
Group F: AVS plus phage plus indicator bacteria without reaction at 37°C.
Group G: Buffer plus phage plus indicator bacteria without reaction at 37°C.

All the plates were finally incubated at 37°C for 4 hours.

TABLE VIII

The Effect of the Antiviral Substance on the Infectivity of Bacteriophage ØX174

Groups	Plaque Count After 4 Hours		
	Plate 1	Plate 2	Plate 3
A	46	45	50
B	52	54	49
C	104	111	110
D	Confluent	Confluent	Confluent
E	Confluent	Confluent	Confluent
F	97	100	104
G	101	107	100

The antiviral substance and the Groups are as for Table VII.

Group G Control: Buffer plus phage plus indicator bacteria added to 0.7% melted agar, mixed, poured over the surface of an agar plate and incubated at 37°C.

The phages were used in concentrations of either 50 plaque forming units or 100 plaque forming units; the bacteria inoculum contained 1×10^8 cells. For phage f2, the indicator bacteria, E. coli K13 was used and E. coli C was used for phage ØX. The activity of the antiviral substance used was checked in Modified Maitland Cultures against influenza A virus (PR8); 10X the active Maitland Culture doses were used in the phage experiments.

No anti-phage activity was demonstrated in six repeated experiments; typical results are shown in Tables VII and VIII. In one preliminary experiment with ØX there seemed to be a 30-minute delay in the appearance of plaques in the series of plates with the AVS. But this was not reproduced in any of the subsequent experiments.

ii. Haemagglutination and haemagglutination-inhibition studies with the antiviral substance

Experiments were conducted to investigate possible haemagglutination (HA) and haemagglutination-inhibition (HI) activities of the antiviral substance (AVS). These experiments were essential for two main reasons. First, since the HA test has been used routinely to assay for virus multiplication in the myxovirus test systems, it was essential to know if the AVS per se possessed HA activity. Secondly, if the AVS possessed HA and/or HI activity, this would in itself indirectly suggest certain modes of action for the substance, based on known steric

TABLE IX

Haemagglutination and Haemagglutination Inhibition Tests with the Antiviral Substance from Penicillium Cyaneo-Fulvum

AVS Batch	AVS Dilution						
	1/2	1/4	1/5	1/10	1/20	1/40	1/80
14 11 65 (McGill: $(NH_4)_2$ SO_4-ETOH)	++	+	-	-	-	-	-
HI with 4 HAU PR8	++	++	++	++	++	++	++
4 5 65 (Czapek-Dox:Acetone)	-	-	-	-	-	-	-
HI with 4 HAU PR8	++	++	++	++	++	++	++

+ = Positive for Haemagglutination.

considerations and receptor sites requirements similar to other known myxo-virus inhibitors (David-West, 1962).

The HI test was set up according to Jensen (1956) and modified by David-West (1963) for the titration of non-specific inhibitor. In the HA test, serial 2-fold dilutions of the AVS were prepared in PBS and tested with 0.25 per cent chicken red blood cells. The results are shown in Table IX, which illustrates that the Czapek-Dox broth preparation of the AVS was negative for HA activity at all the dilutions used; the McGill glucose beef heart infusion broth culture filtrates of the mould processed by the ammonium sulphate method, were positive for haemagglutinating activity at lower dilutions. However, these concentrations were much higher than those used in the Maitland culture studies. The results with the McGill broth agreed with those of Syeklocha (1962). No demonstrable HI activity was detected in the two preparations of AVS used. The HA test was also repeated twice with identical results. It is suggested that impurities in the earlier preparations accounted for the haemagglutination elicited when unusually high concentrations were used.

iii. Mode of action of the antiviral substance

Experiments were designed to continue and extend the work of Syeklocha (1964) on the mode of action of the antiviral substance. The following hypotheses were proposed to serve as the bases for the experimental design:

1. If the AVS acts primarily by blocking virus adsorption and entry into the cell, its effect should be greatest when it is added prior to the virus.
2. If its activity is after the above stages, it should be effective even after virus adsorption and penetration. In order to test this, it would be

essential to establish the minimal time for effective adsorption of virus to the cell. Furthermore, if the AVS is not stopping the adsorption of virus to the cells, its action could be reversed by washing the cells after an interaction between cells, AVS and virus through the adsorption phase.

3. If it impedes virus release, it should be possible to demonstrate that at the peak of its activity, there is more virus within the cells than outside in the culture medium. Also, although there is no unanimity among the experts on the exact role of the influenza virus enzyme, neuraminidase, there seems to be little disagreement that it plays some part in the infective processes. Moreover, the release of the virus from red blood cells has been constantly used as a model of virus penetration and/or release from host cells (Hirst, 1943; Fazekas de St. Groth, 1950; Ackermann and Maassab, 1954b; Schlesinger and Karr, 1956; Rubin, 1957; Rubin and Franklin, 1957; Hoffmann et al., 1965; Kathan, 1965). It should be possible, therefore, to demonstrate a decreased rate of virus elution from red blood cells in the presence of the AVS if it is involved in the processes of virus release.
4. If it is viricidal, it should be possible to establish that it is a contact viricide, by incubating AVS and virus in vitro for an appropriate length of time.
5. If it exerts some effect on the host cells, this may be demonstrated by reacting the cells and the AVS for various time intervals, washing off the AVS and then inoculating with virus.

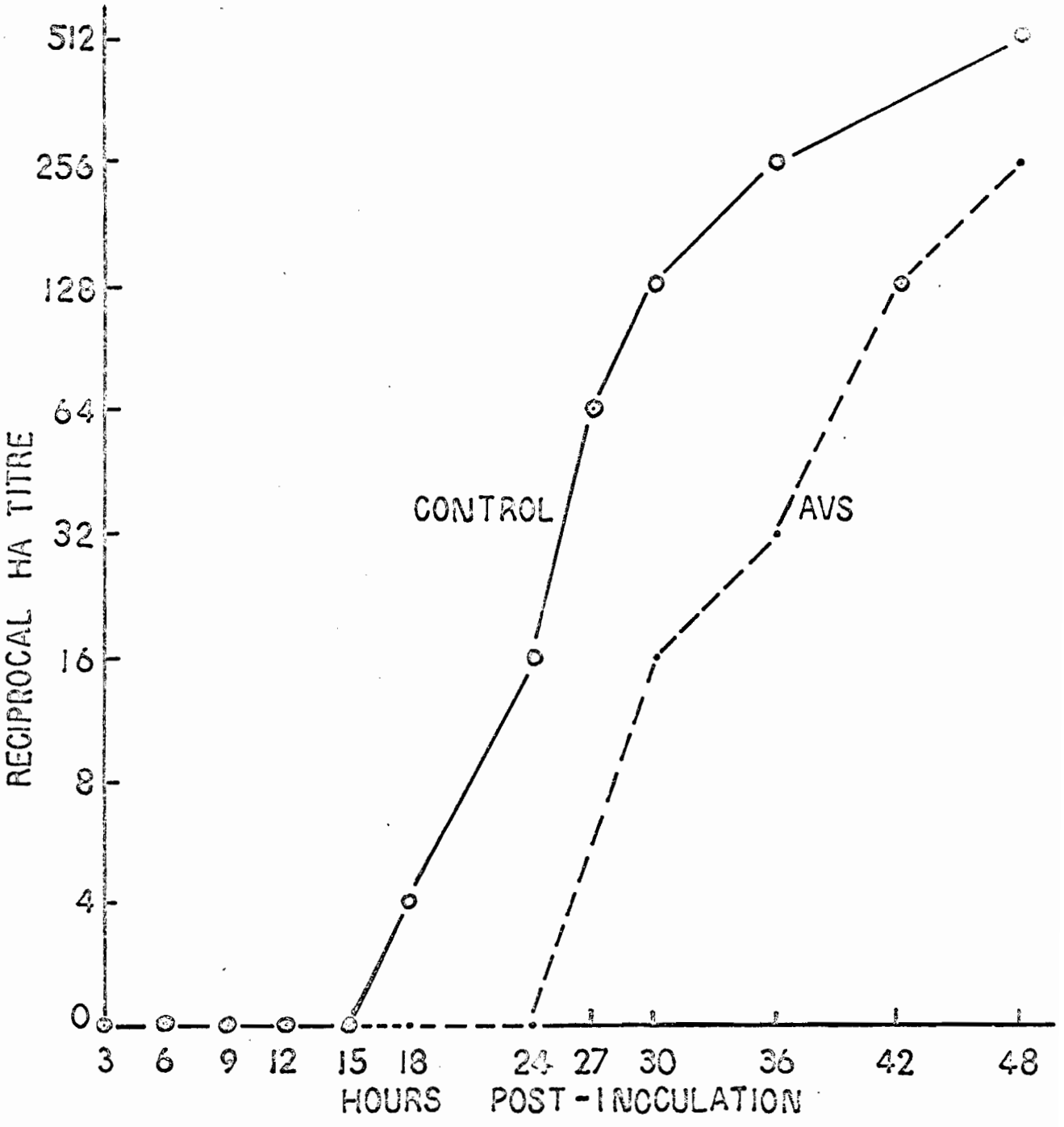
a. The effect of the antiviral substance on the growth curve of influenza virus (PR8)

A growth curve experiment was first conducted with the test virus, the PR8 strain of influenza A virus. This would provide a general picture of the patterns of the multiplication of the virus in both the presence and absence of the AVS.

Maitland cultures were set up in two series, with and without AVS. Each flask was inoculated with 100 EID_{50} of influenza A virus (PR8); at intervals two flasks were removed from each series. The culture medium and the CAM were titrated for HA activity. The ground CAM was suspended in a volume of PBS equal to the corresponding culture medium. The results of two of the growth curve experiments are shown in Figures 12 and 13. The AVS caused an extension of the lag period by 6 to 9 hours. This is in general agreement with the results of Syeklocha (1964). The figures also show that a lower dose of the AVS prepared by the new methods was required to produce the effects reported.

The patterns of the results obtained with the corresponding CAM were similar to the above with the exception that virus multiplication was demonstrated with the CAM earlier than with the corresponding culture medium, although the virus titre of the culture medium was consistently higher than the CAM titre at the termination of the experiments. This growth pattern is characteristic of influenza viruses (Cairns, 1952; Tamm et al., 1953a; Tamm and Tyrrell, 1954).

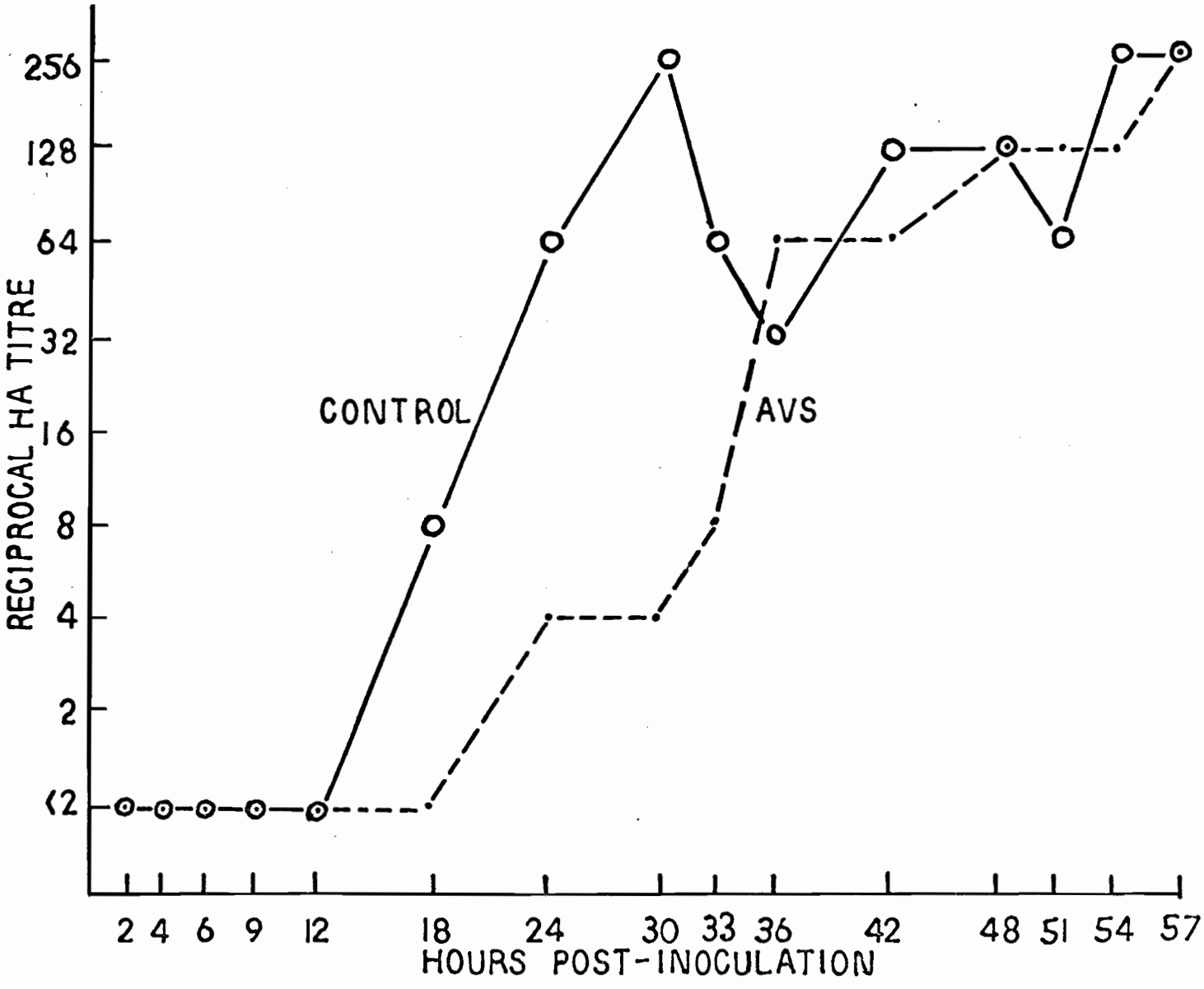
Figure 12



Effect of the antiviral substance on the growth cycle of influenza A virus (PR8) in Modified Maitland Cultures (culture medium).

i. Antiviral substance (Batch 251065) prepared in Czapek-Dox broth: 0.2 mg per flask.

Figure 13



Effect of the antiviral substance on the growth cycle of influenza A virus (PR8) in Modified Maitland Cultures.
(culture medium).
ii. Antiviral substance (Batch 211064) prepared in McGill glucose beef heart infusion broth: 0.7 mg per flask.

b. Minimal time for effective adsorption of influenza virus to chorioallantoic membrane

The design of this experiment is illustrated in Figure 14. It was established that after 2 minutes, sufficient virus had effectively adsorbed to the CAM to initiate infection. This is in accord with the results of Hirst (1942; 1943) who showed that more than 90 per cent of influenza virus inoculum was adsorbed irreversibly within the first five to ten minutes.

c. The effect of the antiviral substance on the adsorption and the penetration of influenza virus

Six groups of Maitland cultures, with five flasks per group, were set up according to the following scheme:

Group A: AVS plus virus plus CAM, incubated 44 hours at 35°C.

Group B: AVS plus virus plus CAM, reacted for 1 hour at 35°C; CAM washed 3X with medium, transferred to fresh flask containing medium and incubated 44 hours at 35°C.

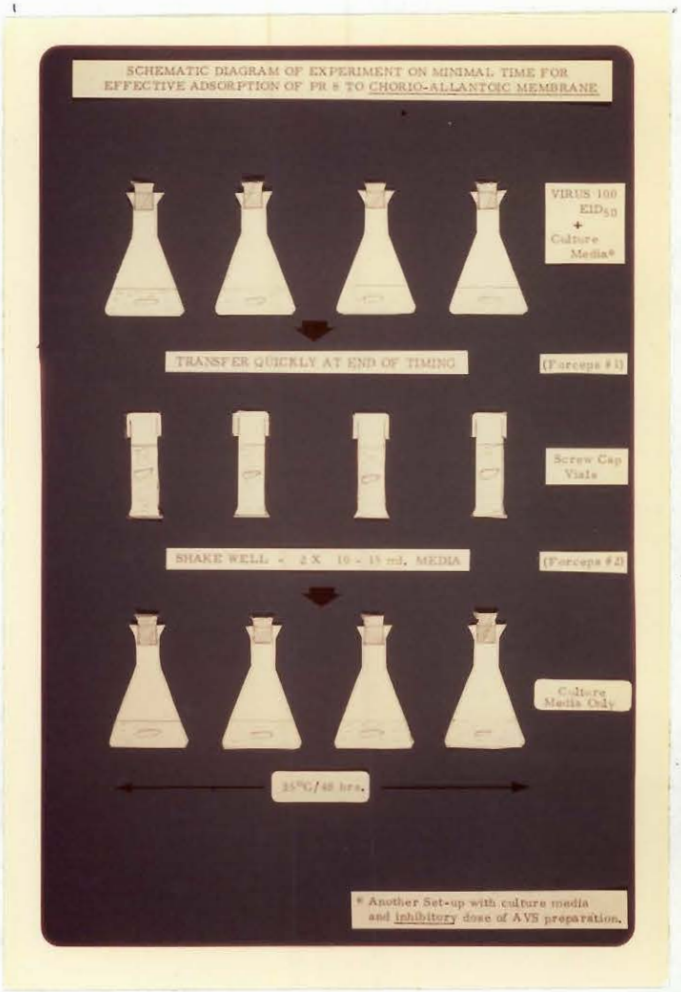
Group C: AVS plus CAM reacted for 1 hour at 35°C; CAM washed then transferred to fresh flask, inoculated with virus and incubated 44 hours at 35°C.

Group D: Virus plus CAM reacted for 1 hour at 35°C; CAM washed, transferred to fresh flask, AVS added and incubated 44 hours at 35°C.

Group E: Virus plus CAM reacted for 1 hour at 35°C; CAM washed, transferred to fresh flask and incubated 44 hours at 35°C.

Group F: Virus plus CAM incubated for 44 hours at 35°C.

Figure 14



Diagrammatic model of experiments on minimal time for effective adsorption of influenza virus to chorioallantoic membrane.

Groups A, E and F served as controls; 100 EID_{50} of influenza virus and 0.25 mg of AVS were used for each flask. The experiment was done with both PR8 strain of influenza A virus and Lee strain of influenza B virus. At the termination of the incubation period, the culture media were titrated separately for HA activity. Figures 15 and 16 show the results obtained with two viruses. The picture that emerged in these experiments was that the AVS was effective in suppressing virus multiplication even when it was added to the cultures 1 hour after virus inoculation. Furthermore, its inhibitory activity was reversed by washing the CAM after 1 hour of interaction with AVS.

d. The effect of the antiviral substance on virus release

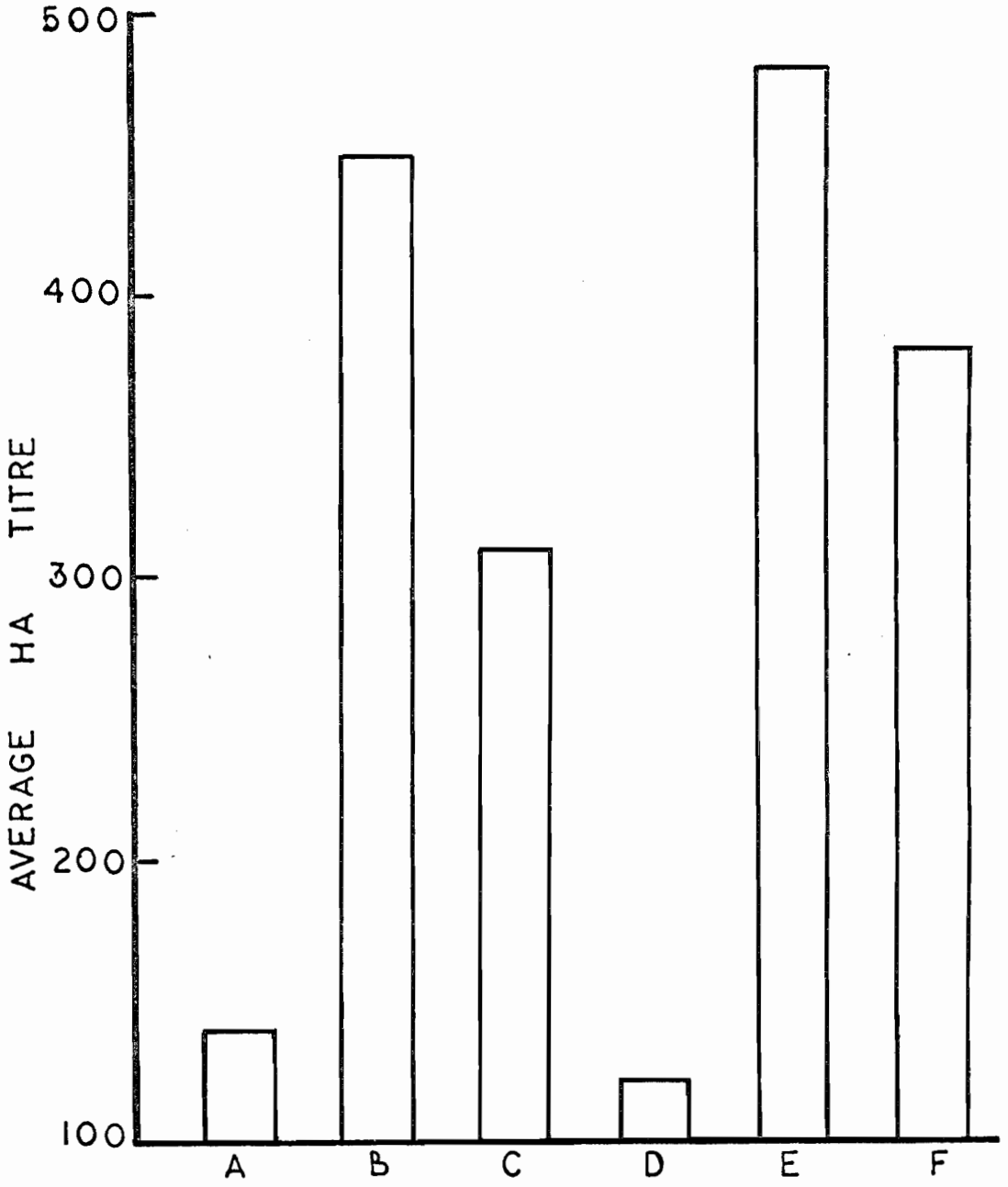
Comparison of intracellular and extracellular virus titres

At the termination of some of the routine Maitland culture experiments, it was the practice to titrate both the culture medium and the ground CAM for virus activity. The ground CAM was suspended in a volume of PBS equal to the corresponding culture medium. The results of three of such experiments are shown in Table X. In all of these experiments the virus titre of the CAM was consistently lower than that of the culture medium. An interpretation that would be consistent with the data shown on Table X would be, that the AVS is not stopping the release of newly formed virus from the cells, since at no time was the virus titre within the cells higher than that in the culture fluid, at the peak of the activity of the AVS.

Effect of the antiviral substance on virus elution

The rates of elution from chicken red blood cells of influenza A virus, PR8, and Newcastle disease virus (NDV) were studied in both the presence

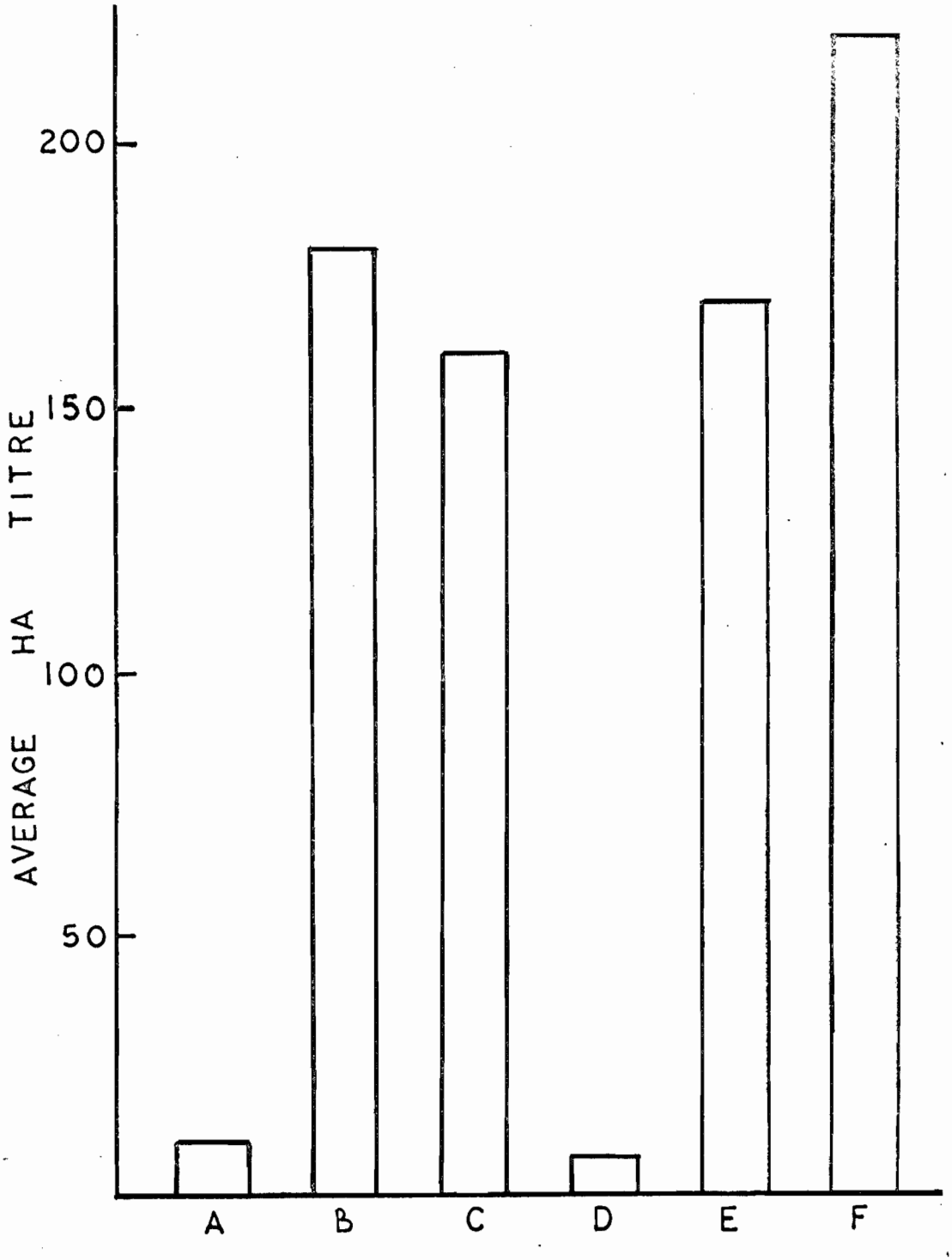
Figure 15



Effect of the antiviral substance on adsorption and penetration of influenza A virus (PR8)

A: AVS plus virus plus CAM
B: AVS plus virus plus CAM interacted for 1 hour at 35°C, washed CAM and transferred to fresh medium
C: AVS plus CAM interacted for 1 hour at 35°C, washed CAM, transferred to fresh medium and inoculated virus
D: Virus plus CAM interacted for 1 hour at 35°C, washed CAM, transferred to fresh medium and added AVS
E: Virus plus CAM interacted for 1 hour at 35°C, washed CAM and transferred to fresh medium
F: Virus plus CAM
All flasks were finally incubated at 35°C for 44 hours.

Figure 16



Effect of the antiviral substance on adsorption and penetration of influenza B virus (Lee).

Note: Groups as for Figure 15.

and the absence of an inhibitory AVS dose. The procedures used were similar to experiments on neuraminidase activity (David-West, 1963). Each virus pool was adjusted to contain 800 haemagglutinating units per 0.5 ml. This was mixed with a freshly prepared red blood cell suspension to give a final cell concentration of 1 per cent. Adsorption of virus was carried out in crushed ice for 2 hours; at the end of this time, the cells were sedimented and the supernatant was aspirated without disturbing the red cell pellet and saved. The AVS was added to the test tubes and an equal volume of PBS was added to the control tubes; the red cells were resuspended and the tubes left in a 37°C water bath for elution of virus. Aliquots of 0.5 ml were removed from each tube at intervals and were replaced with equal volumes of AVS or PBS. The different aliquots as well as the aspirated supernatants were titrated for HA activity. Typical results representing two repeated experiments are illustrated in Table XI, which shows that the presence of the AVS did not influence the respective rates of elution of both viruses.

e. The effect of the antiviral substance on the susceptibility of chorioallantoic membrane to influenza virus infection

The effect of the antiviral substance on the chorioallantoic membrane (CAM) was studied by exposing the membranes to an inhibitory dose of AVS for varying lengths of time, washing the membranes and finally infecting with 100 EID_{50} of PR8 virus in fresh flasks. The membranes were then incubated for 40 hours at 35°C for virus multiplication. Five flasks of Maitland cultures were set up for each group of experiments; 0.3 mg of AVS (Batch 251065) was used

TABLE X

Mode of Action of Antiviral Substance: Effect of Antiviral Substance on Virus Release

Comparison of Influenza A Virus Titres of Culture Fluid and Ground CAM at Peak of AVS Activity.

Batch of AVS	Average HA Titre	
	Culture Fluid	CAM
4 5 65 (Czapek-Dox: Acetone Prep.)	42	10
6 7 65 (Czapek-Dox: Acetone Prep.)	74	10
14 11 64 (McGill: $(NH_4)_2$ SO_4-ETOH)	128	20
Representative Virus Control	270	100

TABLE XI

Mode of Action of Antiviral Substance: Effect of Antiviral Substance on Virus Release

	PR8 (HAU per 0.5 ml)		NDV (HAU per 0.5 ml)	
Control*	64		64	
Unadsorbed	0		0	
Elution Time (mins.)	AVS	PBS	AVS	PBS
5	0	0	Not done	Not done
10	4	4	4	4
20	8	4	8	8
30	8	8	8	8
120	64	64	64	64

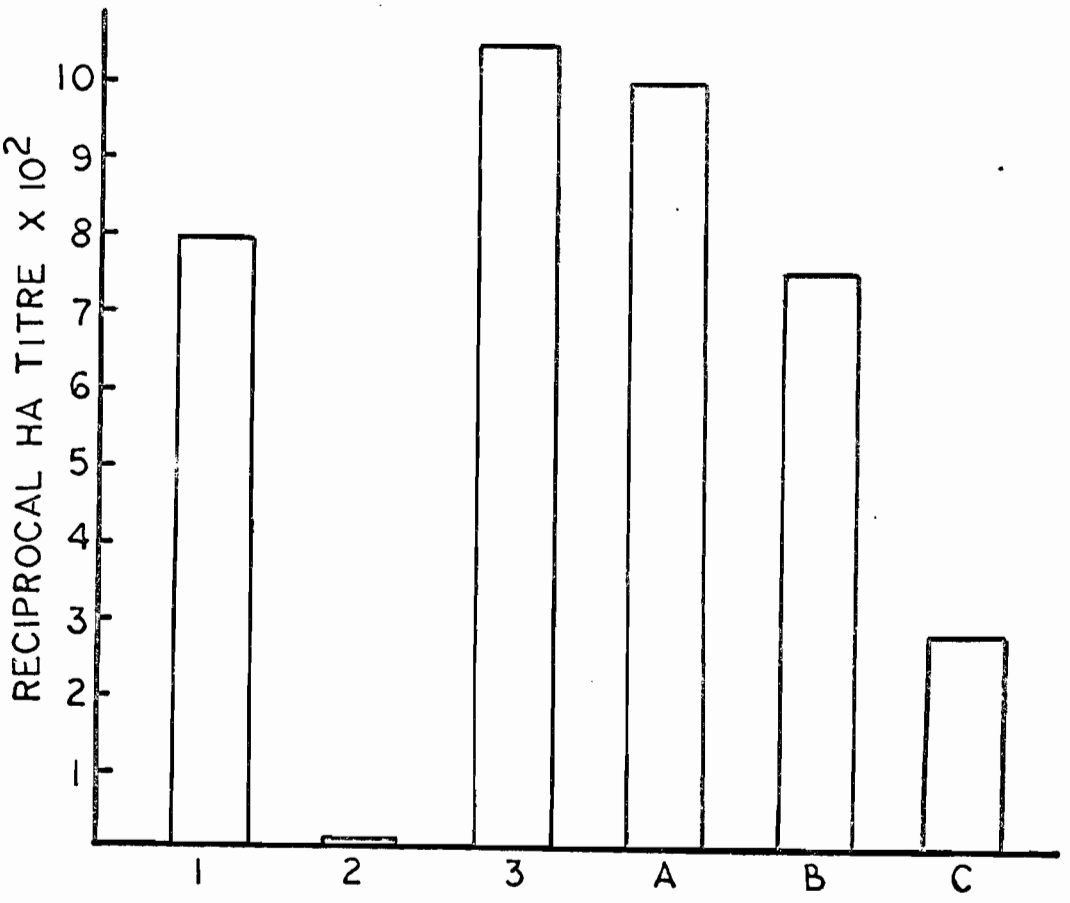
* Control Titre was calculated from 2 ml of virus dilution (800 HAU/0.5 ml), plus 2 ml of PBS. This mixture was then titrated for HA. These volumes corresponded to the volume of virus dilution and the volume of 2% RBC suspension used for the adsorption step.

and at the end of the 40-hour incubation the culture fluid in each flask was titrated separately for HA activity. In two of such experiments it was observed that pretreatment of the CAM for a maximum of 6 hours at 35°C before virus inoculation, reduced the susceptibility of the membranes to infection; while incubating the CAM for an equal length of time in medium without AVS did not result in a significant reduction of virus multiplication. It would appear from these observations that for maximum susceptibility to virus infection the physiological integrity of the membranes had to be maintained during the initial stages of virus replication. Figure 17 illustrates these points.

f. The study of viricidal effect of the antiviral substance

The possibility that the AVS might be acting by a viricidal effect was considered. Ten milligrams of AVS (Batch 30365) were mixed and incubated for 24 hours at room temperature with an equal volume of influenza A virus. The control experiment received an equal volume of PBS in the place of the AVS. After the incubation period serial ten-fold dilutions were prepared in PBS; 0.1 ml of each dilution was inoculated by the allantoic route into 10-day old embryonated eggs; 4 eggs were used per dilution. The eggs were incubated at 35°C for 48 hours and the allantoic fluid from each egg was harvested separately and tested for HA activity. The EID_{50} for each group was calculated by the method of Reed and Muench (1938). The results showed that the EID_{50} per 0.1 ml of the control was $10^{5.75}$ while that of the test was $10^{5.0}$. The difference was not considered significant and may indicate that the AVS is not acting by directly inactivating the virus. The activity of the AVS used

Figure 17



Effect of the antiviral substance on susceptibility of chorio-allantoic membrane to influenza A virus infection.

TEST A: CAM plus AVS interacted for 1 hour at 35°C before virus inoculation.
TEST B: CAM plus AVS interacted for 3 hours at 35°C before virus inoculation
TEST C: CAM plus AVS interacted for 6 hours at 35°C before virus inoculation
CONTROL 1: CAM incubated for 6 hours at 35°C before virus inoculation
CONTROL 2: CAM plus AVS plus virus
CONTROL 3: CAM plus virus
All flasks were finally incubated at 35°C for 40 hours.

in these experiments was rechecked in Maitland cultures and 0.2 mg caused 95 per cent suppression of the multiplication of 100 EID_{50} of the test virus.

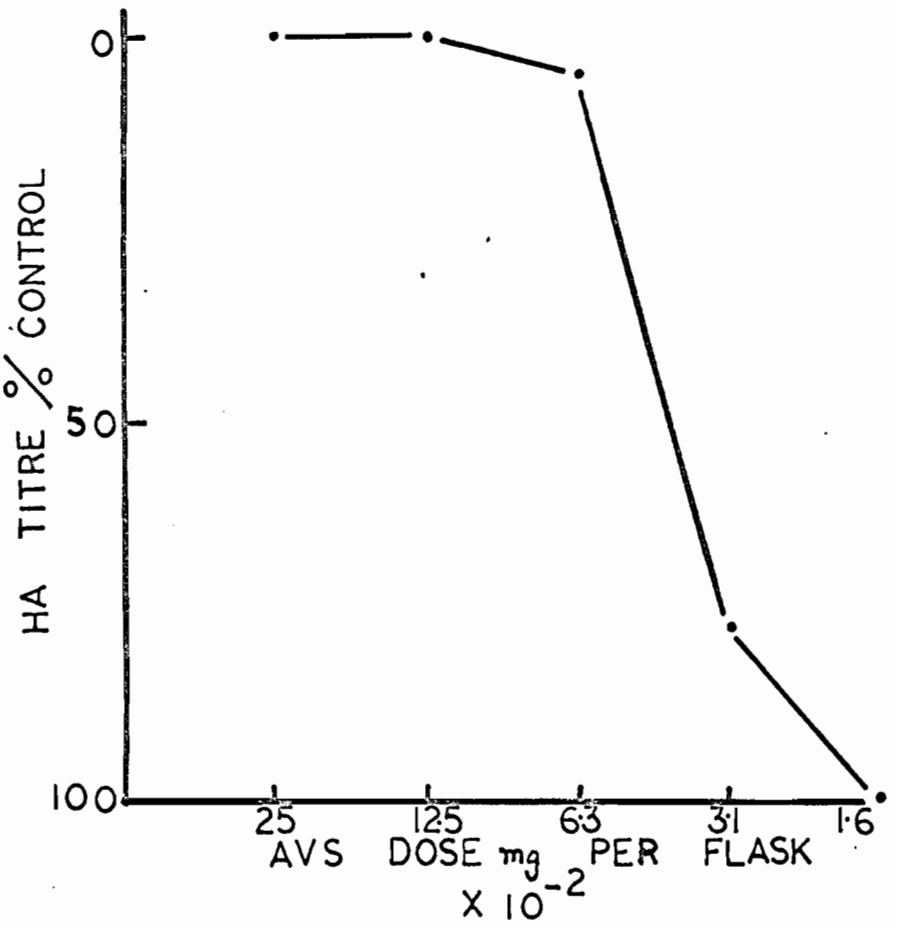
From these experiments it would appear that the antiviral substance is neither stopping virus adsorption nor virus release; it is also not viricidal. It is suggested that the AVS is acting at some intracellular stage of virus multiplication. This contrasts with the conclusions of Syeklocha (1964) who suggested that the AVS may be acting by stopping virus adsorption and virus release.

iv. The dose-response curve with the antiviral substance

As was stated earlier (Materials and Methods) every new batch of the AVS preparation was screened for activity with varying doses before selecting the particular dose or doses that would be used for subsequent studies. This procedure established a profile of activity for each batch which was essentially a dose-response curve. However, since the doses selected for the screening were usually not very closely spaced it was decided to conduct a regular dose-response experiment using more closely spaced doses.

An autoclaved sample of batch 4565 was used in amounts ranging from 25×10^{-2} mg per flask to 1.6×10^{-2} mg per flask; for each dose 10 Maitland culture flasks were set up and each was tested against 100 EID_{50} of influenza A virus. At the termination of the experiment, after 44 hours, the culture medium of each flask was titrated separately for HA activity. The titres of each group were averaged and expressed in terms of per cent inhibition of the controls; Figure 18 illustrates the dose-response curve obtained. Several other similar experiments were conducted with different batches of AVS with similar dose-response patterns.

Figure 18



Dose-Response Curve with the antiviral substance (Czapek-Dox Batch 4565)

The curve was essentially similar to that expected of a substance which acts by an all-or-none reaction. This emphasizes the value of the preliminary screening for a profile of the activity of all batches of the antiviral substance such as has been performed routinely throughout the present series of investigations. Shope (1953b) described a "plateau effect" for helenine where, after a critical dose, further increases in dose failed to elicit more inhibition. A dose-response curve similar to the one obtained was also reported for the antiviral agent, M-8450, from Penicillium stoloniferum (Johnson and Baker, 1958).

B. Chemical Studies on the Antiviral Substance *

Although the antiviral extract is in a semi-purified form, it was necessary to obtain some information about its chemical nature. In previous studies with McGill glucose beef heart infusion broth preparations (Syeklocha, 1964) 13 amino acids were located on two-dimensional paper chromatograms with acid hydrolysates of the partially purified material; the presence of carbohydrate was also demonstrated by a positive mucicarmine staining of paper electrophoresis strips, while a quantitative estimation by the tetrazolium method (Fairbridge et al., 1951) gave 21 to 25 per cent reducing sugar per mg of the antiviral substance. It was concluded that the antiviral substance may be a carbohydrate-amino acid complex.

i. Chemical composition of the antiviral extract

In order that the results of the different chemical determinations may be directly comparable, the same preparation of the antiviral substance was used in each of the following investigations.

a. Determination of carbohydrate

Total hexose was determined by the anthrone reaction, as modified by Fairbairn (1953). Two millilitres of AVS preparation were carefully layered over 10 ml of anthrone reagent (Appendix) in a hard glass tube measuring 20 mm x 175 mm. The contents of the tubes were mixed, heated in a boiling water bath of 98°C for 12 minutes, cooled in tap water for 10 minutes and the colour read at 625 mμ in a Beckman DU spectrophotometer using glass cuvettes of 1-cm light path. Blank and glucose standards were included; the determination was carried out in triplicate. Quantitative values for hexoses were calculated from the optical density readings using a standard curve prepared with

* The candidate acknowledges with grateful thanks the correspondences of Dr. Elvin A. Kabat, of Columbia University, New York.

(D+) glucose (Analar). The amount of total hexoses was estimated to be 7 per cent.

In another series of determinations an attempt was made to see if half of the volume of sample used in the above determinations would suffice. This was done because one of the drawbacks in the use of the anthrone test is the need to use relatively large amounts of the test sample, which may vary from 2 ml (Fairbairn, 1953) to 5 ml (Dische, 1955). The present modification using 1 ml of the sample and 5 ml of the anthrone reagent gave values very close to those obtained with the method of Fairbairn (1953).

Hexose was also determined by a modified diphenylamine reagent (DPA) (Appendix) (Segovia et al., 1965); with this test nucleic acid sugars are not measured. One millilitre of AVS preparation was mixed with 3 ml of DPA reagent, heated in a boiling water bath for 40 minutes and the colour read at 635 mμ. About 4 per cent hexose was estimated by this test, using a standard curve prepared with glucose.

b. Determination of protein

Colorimetric determination of protein was carried out with the Folin phenol reagent as modified by Lowry et al. (1951). A 2 per cent solution of Na_2CO_3 (anhydrous) in 0.1 N NaOH and a 0.5 per cent solution of $CuSO_4.5H_2O$ in 1 per cent sodium potassium tartrate were prepared; 50 ml of the former and 1 ml of the latter were mixed (Reagent A). Commercial Folin reagent was diluted 1 : 2 with distilled water (Reagent B).

The determinations, in triplicate, were done by mixing 1 ml of AVS preparation and 5 ml of Reagent A, reacting them at room temperature for 10

minutes, adding the Folin reagent, reacting for a further 30 minutes at room temperature and reading the colour at 750 mμ in a Beckman DU spectrophotometer. Blanks of distilled water and reagents and bovine albumin standards were included. A standard curve prepared with bovine serum albumin (Nutritional Biochemicals) was used in the estimation of the protein. The test indicated the presence of protein in an overall concentration of some 75 per cent. However, one of the limitations of this test is that the Folin reagent reacts with proteins as well as peptides and even free amino acids.

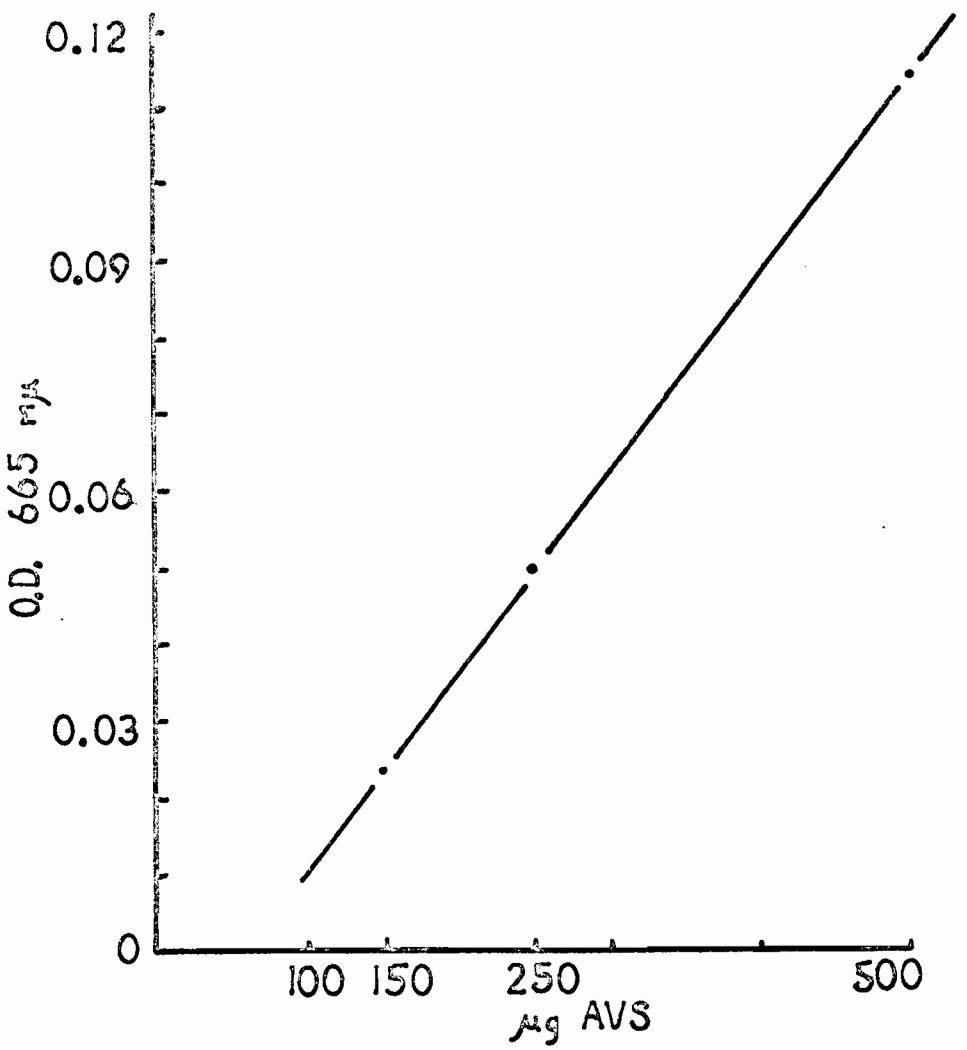
c. Determination of ribonucleic acid

Ribonucleic acid (RNA) was determined by the orcinol reaction as modified by Dische (1960). For each determination 1.5 ml of AVS preparation were mixed with 3.0 ml of orcinol reagent made up with 3.5 ml of 6 per cent twice recrystallized orcinol in ethyl alcohol, 100 mg $FeCl_3.6\ H_2O$ and 100 ml concentrated HCl. The reaction mixture was heated in a boiling water bath for 3 minutes, cooled in tap water and the colour read at 665 mμ in a Beckman DU spectrophotometer. Water blanks and nucleic acid standards were included. Using a standard curve plotted with commercially prepared ribonucleic acid (Nutritional Biochemicals) RNA was estimated to be present in a concentration of about 4 per cent.

d. Determination of deoxyribonucleic acid

The diphenylamine method for the determination of deoxyribonucleic acid (DNA) (Burton, 1956) was employed. The test was set up by mixing 1 ml of AVS extract with 1 ml of 0.5 N perchloric acid and after adding 4 ml of DPA reagent (Appendix) the tubes were left at 30°C in the dark for 18 hours. The

Figure 19



Orcinol reaction for the determination of ribonucleic acid: Linear relationship between concentrations of the antiviral extract (Batch 131265) and optical density readings.

colour was read at 600 mu in a Beckman spectrophotometer. Blanks of water and DNA standards were included. From a standard curve plotted with sodium deoxyribonucleate (Nutritional Biochemicals) DNA was estimated at a concentration of about 2 per cent.

In each of the above determinations there existed a linear relationship between AVS concentration and optical density. A typical result, obtained with the orcinol reaction for RNA determination is shown in Figure 19. The linear relationship would imply that the different substances assayed for were quantitative.

ii. Enzyme and chemical inactivation studies

a. Trypsin

A stock solution of 2X crystallized trypsin (Worthington Biochemicals Co.) was prepared in 0.001 M HCl. The enzyme was diluted in tris buffer containing 0.046 M tris and 0.0115 M $CaCl_2$, pH 8.1, to give a final enzyme concentration of 5 µg in 0.5 ml. Two ml of AVS, containing 1 mg were added. The inactivation of the AVS was carried out at 37°C for 18 hours. A control tube containing AVS and 0.5 ml of buffer was treated in the same manner. The enzyme was destroyed by heating in a boiling water bath for 15 minutes and residual AVS activity was determined in Maitland cultures. The results are shown in Table XII and indicate that the enzyme treatment caused 22 per cent inactivation of the AVS.

b. Lipase

Lipase was procured from Nutritional Biochemicals, Cleveland, Ohio. The enzyme stock solution was prepared in 0.075 M $NaHCO_3$ buffer, pH 7.3,

TABLE XII

Enzyme Inactivation of the Antiviral Substance*

TEST	Haemagglutinin Titre Flask 1	Flask 2	Flask 3	Flask 4	Average	% Inhibition
Virus Control	400	250	300	400	337	0%
AVS + Trypsin	200	150	300	150	200	40%
AVS Control	250	100	150	10	127	62%
AVS + Lipase	400	100	100	150	187	45%
AVS Control	150	100	100	100	113	66%
AVS + RNAase	150	100	400	100	187	45%
AVS Control	10	100	10	10	33	90%
AVS + DNAase	250	150	200	150	187	45%
AVS Control	200	250	200	150	200	40%
AVS Activity Control	0	0	0	10	25	97%

* AVS Batch 5466, 0.25 mg per flask

and was used in a concentration of 10 μg per mg of AVS, since the enzyme was not supplied in crystalline form. The reaction was at 37°C for 4 hours. After the reaction the enzyme was destroyed in a boiling water bath; residual AVS activity was tested in Maitland cultures. An appropriate control was included. Table XII illustrates the results, and shows that lipase treatment caused 21 per cent inactivation of the AVS.

c. Ribonuclease

Crystalline ribonuclease (Worthington Biochemical Co.) was used in a concentration of 5 μg per mg of AVS; the enzyme was prepared in physiological saline solution. The inactivation was carried out at 37°C for 3 hours and after destroying the enzyme in a boiling water bath, residual AVS activity was tested in Maitland cultures. An appropriate control was included. The results are shown in Table XII. The enzyme reduced the activity of the AVS by 45 per cent.

d. Deoxyribonuclease

Crystalline deoxyribonuclease was obtained from the same commercial source as the ribonuclease. The treatments of the AVS were similar to those of the ribonuclease inactivation, with the exception that, the enzyme was prepared in a buffer system made up of 0.85 per cent NaCl in 0.005 M $MgCl_2$. $6H_2O$. The results are shown in Table XII. There was no effect of the enzyme on the activity of the AVS.

e. Periodate

Two concentrations of periodate were employed, 0.1 M and 0.01 M. One volume of AVS was reacted with three volumes of periodate solution for

three hours in the dark. Excess periodate was inactivated with three volumes of 0.25 M sucrose solution before testing for residual AVS activity. However, the attempt to inactivate the residual periodate did not appear to be successful, since the reaction mixture was toxic to the CAM in Maitland cultures.

With the exception of RNAase none of the enzymes appeared to have any significant effect on the antiviral substance; the about 20 per cent decrease in the activity caused by both trypsin and lipase may not be significant. No conclusions could be drawn from the periodate treatment since the test was discarded because of a toxic effect of the salt on the membranes. Although the antiviral substance is still in a partially purified form, it does seem from the above data that RNA, possibly in the form of nucleotides or nucleosides may be playing some part in the overall activity. The variations in percentage inhibition of the AVS controls may be due to differences in the test conditions.

Syeklocha (1964) tried the effects of proteolytic, carbohydrate, and nucleic acid enzymes, as well as lipase on the antiviral substance. With the exception of alpha-amylase, none of the enzymes used had any significant effect on the activity of the antiviral substance. One experiment was also conducted with periodate inactivation but the results were inconclusive.

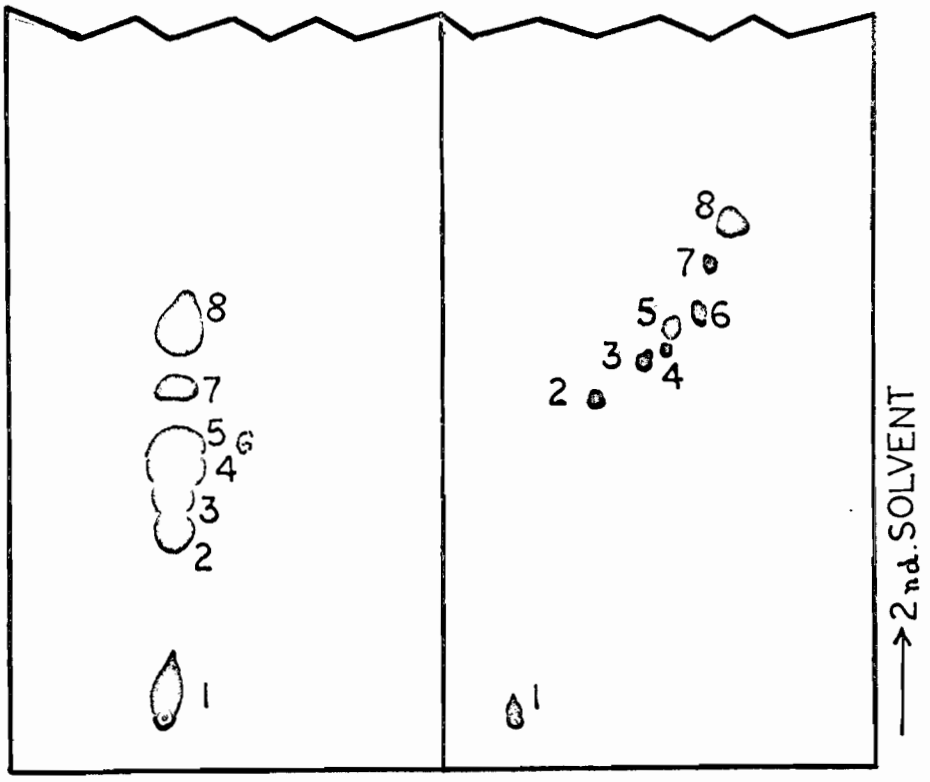
iii. Chromatographic studies

The preparation of thin-layer chromatography plates and hydrolysis of the samples were described in the section on Materials and Methods. Instant thin-layer chromatography sheets from two different commercial sources, Eastman chromatogram sheets and Gelman sheets Type SG, were not as satisfactory as the silica gel plates that were locally prepared.

a. Amino acids

Several chromatographic runs were done with different batches of the AVS extracts processed by the new acetone methods. Initially, amino acid standards, representing different amino acid groups, were chromatographed simultaneously with the sample. From such preliminary runs, some of the reference amino acids were eliminated; sometimes it was necessary to include other reference acids in the run after the elimination of those whose Rf values and colour reactions did not correspond to any of the spots produced by the sample. Finally, the following eight amino acids were detected and identified: arginine, proline, aspartic acid, glycine, alanine, serine, methionine and leucine. These acids were checked several times with the corresponding reference amino acids in both one- and two-dimensional chromatographic runs using different solvent systems and they were consistently detected. In the detection and identification the polychromatic stain of Moffat and Lytle (1959) proved particularly useful. With this stain the different amino acids produced characteristic colours, which together with the Rf values gave more reliable identification. In one-dimensional runs, overlapping colours could easily be distinguished. On two occasions a faint spot was located behind the proline spot, but since this was not consistently produced, it was not included in the list of acids detected. Proom and Woiwod (1953) emphasized the importance of not recording faint chromatographic spots that are located by the imagination; only unequivocal and reproducible spots are of any significance. Furthermore, Woiwod (1949) and Proom and Woiwod (1953) showed that the use of both Rf values and characteristic colour reactions gave more precise identification of

Figure 20



Diagrammes of amino acid patterns on thin-layer chromatograms.
One-dimensional (LEFT). Solvent system: n-propanol-water (Approx. proportions).
Two-dimensional (RIGHT). Solvent systems: n-propanol-water and 96% ethanol-water.

	R_f		Reference		*Text	
1: Arginine		0.03		0.03		0.04
2: Proline		0.45		0.45		0.35
3: Glycine		0.51		0.51		0.43
4: Serine		0.50		0.50		0.48
5: Alanine (α)		0.52		0.54		0.47
6: Aspartic		0.55		0.56		0.55
7: Methionine		0.61		0.65		0.59
8: Leucine		0.67		0.68		0.61

* Second solvent

chromatographic spots than the use of either of these alone.

The R_f values of the amino acids identified and diagrams of the chromatograms are illustrated in Figure 20. As a comparison the values reported by Randerath (1964) for the R_f of the different reference acids are included, but since experimental variations may affect the results obtained by different people for the same system, more significance was given to the reference values obtained during this investigation.

The uninoculated McGill glucose beef heart infusion broth, used by previous investigators (Cooke, 1960; Syeklocha, 1964) and used also for the earlier experiments in the present studies, was also chromatographed in order to obtain a control for the neat broth; at least the following amino acids were present in a one-dimensional thin-layer chromatogram: arginine, aspartic acid, glycine, serine, methionine and leucine. This list agrees very closely with that reported by Syeklocha (1964) for the semi-purified AVS chromatogram and all were present in the semi-purified AVS reported above.

Before the run the sample spots fluoresced strongly under ultraviolet lights of wavelengths 260 mμ and 366 mμ; most of the reference amino acid spots also showed weak fluorescence. However, after the run and before and after heating the plates in the oven, only the samples fluoresced. The fluorescence patterns of the samples were rather complex. Usually, there was a streak of fluorescence with knots of deep fluorescence along the streak; the spots of deep fluorescence did not correspond with the stained spots of the amino acids and fluorescence was detected even after the staining of the chromatograms.

These observations are interesting in the context of fluorescence patterns reported in the literature for amino acids. Philips (1948), Pereira and Serra (1951), Opienska-Blauth et al., (1960), and Stahl (1965) reported that amino acid spots on chromatograms fluoresced only after heating. Furthermore, since fluorescence, in the systems studied, was reported to be due to a reaction between the amino groups of the amino acid and the aldehydic groups of the carbohydrates in the paper (Woiwod, 1950; Stahl, 1965) it was suggested that the fluorescence test is not applicable to chromatograms run on silica gel (Stahl, 1965).

Because alkaloid spots on chromatograms give strong fluorescence under 365 mμ wavelength ultraviolet light before and after staining (Stahl, 1965), an attempt was made to stain the fluorescent spots with an iodoplatinate spray modified for alkaloids (Appendix). With this spray, although two white spots were located the majority of the fluorescent streak was not developed.

A control spot of uninoculated Czapek-Dox broth showed no fluorescence and did not stain with the polychromatic ninhydrin stain (Moffat and Lytle, 1959).

b. Sugars

For the detection of sugars aniline hydrogen phthalate reagent (Partridge, 1949) and benzidine reagent (Horrocks, 1949) were used; at least three spots were located. However, the identification was difficult since most of the reference sugar standards had very close Rf values and the colour reactions were not as diverse as those for amino acids reported above. The

spots located gave the colour reactions for hexoses and pentoses which have been reported for the sprays used (Partridge, 1949; Horrocks, 1949).

iv. Colorimetric detection of tryptophan

None of the aromatic amino acids was detected in the chromatograms of amino acids. This may be due to destruction during hydrolysis. Tyrosine and tryptophan are known to be labile during acid hydrolysis (Duggan and Udenfriend, 1956; Neurath, 1963; Stahl, 1965; Kabat, 1966; Personal Communication). However, the presence of aromatic amino acid was suggested by the Folin phenol method for protein determination, which chiefly measures the tyrosine and tryptophan content (Kabat and Mayer, 1961), especially tyrosine (Kabat, 1966; Personal Communication) and a further colorimetric test for tryptophan was done (Tauber, 1949). Three millilitres of perchloric acid (C.P.) and 0.1 ml of 10 per cent potassium dichromate solution were added to 0.5 ml of AVS. The reaction was carried out in 20 mm x 175 mm glass tubes; after standing at room temperature for 5 to 10 minutes, the fluorescence produced was observed with 366 mμ wavelength ultraviolet light. The AVS was positive for the test. The control tubes of all the reagents without tryptophan and those with the acid but without the reagents were negative. The AVS alone, at this concentration, was not fluorescent.

An attempt was made to find out the limit of sensitivity of the test. This was done by conducting the test with varying concentrations of tryptophan (Nutritional Biochemicals Corp.). It was found that the test could detect as little as 10 micrograms of the amino acid. The colour and fluorescence were stable at room temperature for at least 72 hours.

v. Trichloroacetic acid precipitation

Two ml. of trichloroacetic acid (TCA) were added to 2 ml of AVS (dry weight 11 mg) to give a final TCA concentration of 10 per cent. The tube was left in crushed ice for 30 minutes and then centrifuged. No detectable precipitate was observed. Cold TCA, at a final concentration of 5 or 10 per cent, precipitates proteins and nucleic acids. However, nucleotides and smaller peptides are soluble in the acid (Hutchison and Munro, 1961; de Verdier and Agren, 1948).

vi. Lipid analysis

Evidence for the presence of lipid in the antiviral extract was afforded by an oily scum that invariably floated on the surface during the concentration process in the flash evaporator. This was checked by the transluscence test on paper. Lipids could be extracted efficiently from biological materials with n-butanol (Morton, 1950; Mechman and Mohammad, 1955). This method of extraction was therefore used. The AVS was extracted several times with half the volume of n-butanol. All the extracts were pooled and evaporated to dryness under vacuum. The lipid content of this material was determined by silicic chromatography, using solvent systems of increasing polarity, ranging from diethyl ether and petroleum ether to methanol. With increasing polarity of the solvent, more lipid was fractionated. Through the courtesy of Dr. B.I. Weigensberg and Mrs. Anna Zushmanis of Pathology Department, McGill University, a lipid analysis showed that most of the lipid material was present in the form of phospholipid.

The production of lipids by Penicillium cyaneo-fulvum was also reported by Woodbine et al.(1951) and Woodbine (1959); less lipid was formed in simple

media like Czapek-Dox than in complex media.

C. Physical Treatments of the Antiviral Substance

A sample of the AVS containing about 20 mg was autoclaved at 15 p. s.i. for 15 minutes at 120°C and tested for activity in Maitland cultures. The treatment did not destroy the activity. Usually after such heat treatment the activity increased and in one batch, 30365, autoclaving increased the activity about 10 fold. The AVS did not become cloudy during the heat treatment.

Syeklocha (1964) also showed that the AVS, at pH 7.0, withstood autoclaving at 121°C for either 15 minutes or 30 minutes.

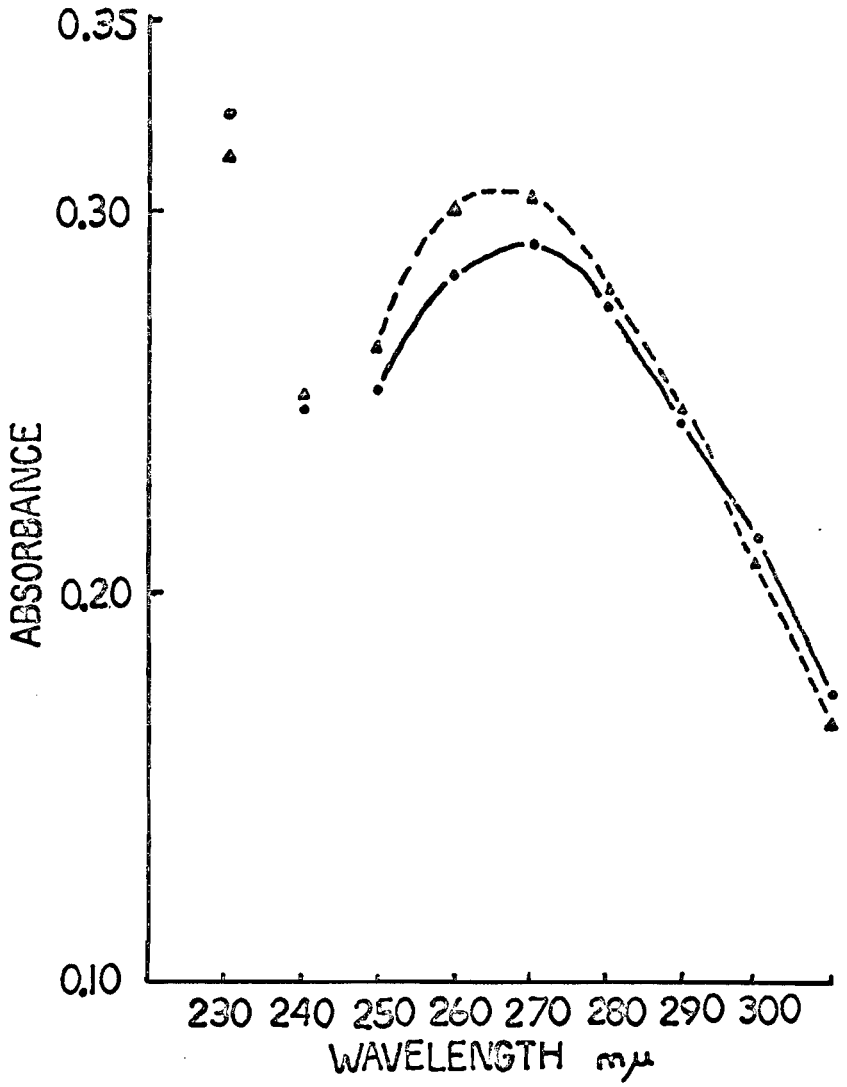
The AVS was frozen and thawed five times in a mixture of dry ice and 70 per cent ethyl alcohol and tested for activity. The treatment did not reduce the activity.

A sample of the AVS was lyophilized and after a month at 4°C it was reconstituted with distilled water and tested for activity. The activity was retained after such treatment. Another sample kept for 2 months, also did not show a decrease in AVS activity.

The AVS kept very well at 4°C. The stability at 4°C was especially checked with Batch 30365, which was found to be active even after 8 months at 4°C; 0.2 mg per flask caused 95 per cent inhibition of influenza virus multiplication in Maitland cultures.

Before storing at 4°C all the preparations were sterilized. This was considered necessary since microbial contamination might otherwise decompose the substance.

Figure 21



Ultraviolet absorption spectrum of the antiviral substance.

i. Czapek-Dox broth preparation (Seitz-filtered) Batch 11266 (0.17 mg/ml)
Dotted lines indicate readings after heating at 98°C for 3 minutes.

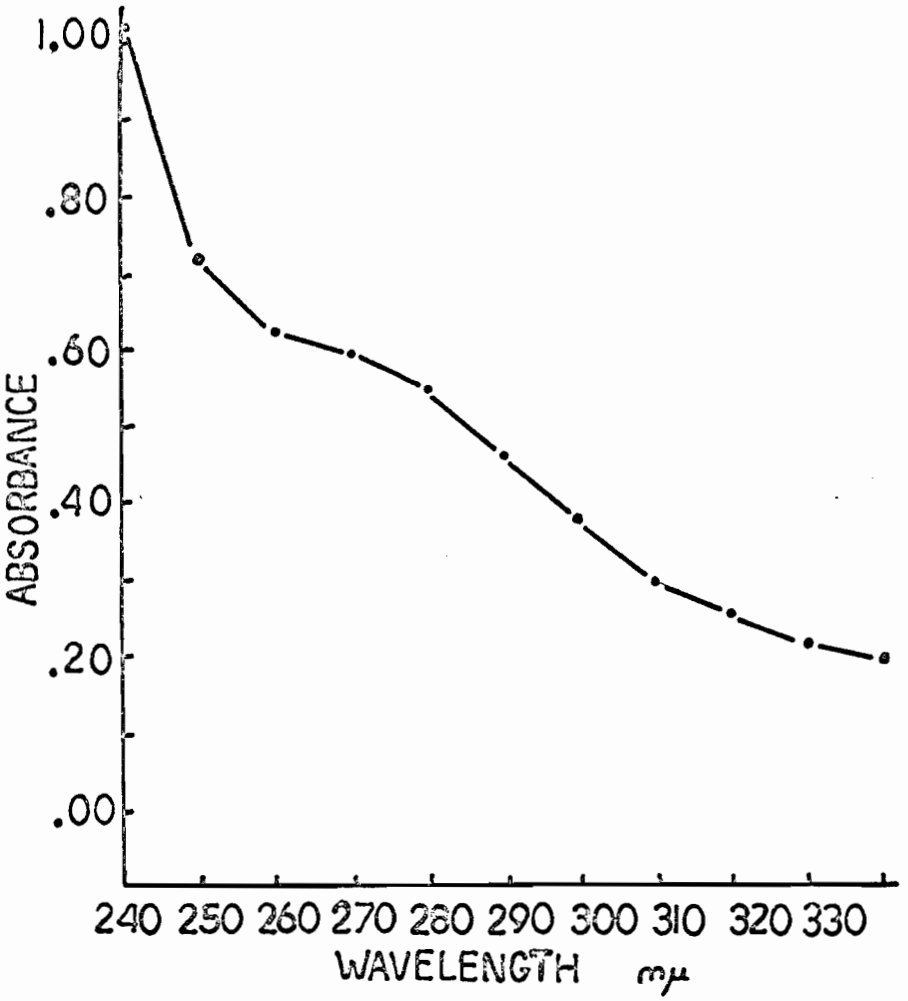
D. Ultraviolet Absorption Spectrum of the Antiviral Substance

The ultraviolet absorption spectrum of the antiviral substance was determined with a Beckman DU spectrophotometer using quartz cuvettes of 1-cm. light path. The absorbance values were recorded over a range of 230 mμ to 310 mμ. The antiviral substance was diluted with distilled water to contain 0.17 mg per ml; a distilled water blank was used. The spectra of several AVS preparations were determined and the absorption patterns were consistently reproduced. The results with one of the batches prepared by the new methods are illustrated in Figure 21. The peak of absorbance was at 270 mμ; on heating the sample at 98°C for 3 minutes, the absorbance shifted to higher values when the readings were taken within five minutes of the heat treatment. The water blank was also similarly heated for this determination. However, if the heated samples were allowed to stand at room temperature for 20 minutes before the determination, such shifts were not observed. The 280 mμ / 260 mμ ratio was 0.97 for the unheated samples and 0.93 for the corresponding heated samples.

During the present investigation it was observed that the absorbance peak was more clearly defined in Czapek-Dox broth preparations of the AVS which were filtered through a Seitz filter; in the process some of the pigment was removed. It did appear that a component of the pigment was interfering with the absorbance.

Although neither the characteristic 280 mμ absorption peak for proteins nor the characteristic 260 mμ peak for nucleic acid was observed, it is suggested that the 270 mμ peak observed may represent a mixture of both protein and nucleic acid components. This suggestion is consistent with the 280 mμ / 260 mμ ratio of 0.97 or 0.93. The hyperchromic effect observed on heating the samples also suggested the presence of nucleic acid components. This effect is characteristic of nucleic acids and

Figure 22



Ultraviolet absorption spectrum of the antiviral substance

ii. McGill glucose beef heart infusion broth preparation (ammonium sulphate-ethanol method) Batch 211064 (0.175 mg/ml).

even of nucleotides and nucleosides (Kunitz, 1950; Sinsheimer, 1954; Privat de Garilhe, 1956; Falk, 1965). The extent of hyperchromicity reflects the integrity of the fragments involved in the phenomenon (Privat de Garilhe, 1956; Ginsberg, 1961; Albertson, 1962); with double-helix DNA, for instance, the increase in absorbance may be as much as 30 per cent (Kunitz, 1950).

As a comparison the spectra of samples of AVS prepared in McGill glucose beef heart infusion broth were determined. Figure 22 shows the results obtained with Batch 211064 and diluted to contain 0.174 mg per ml. With the batches prepared by the older methods, no definite absorbance peak was observed, although a hump did appear between 260 mμ and 280 mμ. This result compares with that of Syeklocha (1964) but contrasts with that of Cooke (1960) who reported highest absorbance at 230 mμ.

E. Comparison of Czapek-Dox Broth and McGill Glucose Beef Heart Infusion Broth for the Production of the Antiviral Substance

Czapek-Dox broth (Appendix) is a simple chemically defined medium of easily reproducible composition, with 3 per cent saccharose as the only organic substance and 0.3 per cent $NaNO_3$ as the sole source of nitrogen. For a period of six months, a record was kept of the pH values of batches of the broth after preparation; the values were consistently 8.1 ± 0.1. This was adjusted to 7.2 before use. In comparison, the McGill glucose beef heart infusion broth (Appendix) is a complex medium.

The uninoculated Czapek-Dox broth did not exert any inhibitory effect on the multiplication of influenza A virus in Maitland cultures. However, some activity was found with uninoculated McGill glucose beef heart infusion broth. The latter

TABLE XIII

Comparison of Czapek-Dox Broth and McGill Glucose Beef Heart Infusion Broth in Terms of Yield*

	Czapek-Dox broth	McGill glucose beef heart infusion broth
Volume of crude culture filtrates	170 ml	170 ml
Total dry weight of crude filtrates	1411.0 gm	3400.0 gm
Dry weight per ml of acetone** processed culture filtrates	18.9 mg	48.7 mg
Total wet weight of mycelia	26.7 gm	25.4 gm

* Batch 30365

** 0.5 mg of the Czapek-Dox preparation caused 100% inhibition of 100 EID_{50} of influenza A virus. The same dose of McGill glucose beef heart infusion broth preparation caused 56.5% inhibition.

observation is in accord with Syeklocha (1964) who also reported insignificant activity with the uninoculated McGill glucose beef heart infusion broth.

Direct comparisons of identical batches of the antiviral substance prepared with both broths are shown in Table XIII and Table XIV. Table XIII shows that the McGill glucose beef heart infusion broth preparation contained about 2X as much solid matter as the Czapek-Dox preparation. This relationship was reflected in both the crude culture filtrates and the acetone processed culture filtrates. Table XIV shows that on dry weight to dry weight basis the Czapek-Dox preparation was more active than the corresponding McGill glucose beef heart infusion broth preparation. This along with the differences in the dry weights of the two preparations, indicates that the Czapek-Dox broth preparation is in a purer form. Table XIV also indicates that the production is better with the latter broth.

Although Czapek-Dox broth has a much simpler composition than McGill glucose beef heart infusion broth, approximately the same wet weights of mycelia were produced in similar culture batches in the two broths. This is shown in Table XIII.

The Acetone Method of extraction of the active principle is more economical in time; the entire process could be completed in less than 1 week as compared to the Ammonium Sulphate Method, which may take as long as two weeks. Furthermore, with the Acetone Method, there was more complete separation of the active principle from the inactive precipitates. In the ammonium sulphate extracts, activity was always detected in the precipitates even after elaborate washing with a saturated solution of the salt.

TABLE XIV

A Direct Comparison of the Antiviral Substance* Prepared in Czapek-Dox Broth and in McGill Glucose Beef Heart Infusion Broth

Test dose Per flask	Per cent inhibition	
	Czapek-Dox broth preparation	McGill glucose beef heart infusion broth preparation
1 mg crude	100	Inactive
1 mg purified	100	83.9
0.5 mg crude	100	Inactive
0.5 mg purified	100	56.5
0.1 mg crude	Inactive	Inactive
0.1 mg purified	95.6	Inactive
0.05 mg crude	Inactive	Inactive
0.05 mg purified	Inactive	Inactive

* Batch 30365 processed with acetone

F. Growth Characteristics of Penicillium cyaneo-fulvum in Czapek-Dox Broth and Temporal Elaboration of the Antiviral Substance

Ajemian (1957) extended the studies of Diena (1954) on some of the cultural changes of Penicillium cyaneo-fulvum in McGill glucose beef heart infusion broth in relation to the production of "Noxiversin". It was found that the best production of "Noxiversin" was about 10 days after seeding of the cultures, when the pH, which first dropped to the acid range, had risen to about 8.0. Cooke (1960) found these conditions ideal also for the production of the antiviral substance(AVS). A change of medium from that used in previous studies to Czapek-Dox broth made it necessary to study the growth characteristics of the mould in the new medium and to attempt a correlation of these characters with the elaboration of the AVS. In a wider context, it was felt also that a detailed study and careful observation of growth conditions might contribute some information to the solution of the controversy that seems to exist among mycologists about the relationship of P. cyaneo-fulvum and P. notatum (Raper and Thom, 1949).

Fresh malt extract agar slopes of the mould were prepared and Roux bottle cultures were set up as described under the section on Materials and Methods; two uninoculated bottles of medium served as the controls. One of the control bottles was processed on the day the cultures were set up, while the second bottle was kept under identical conditions with the test bottles and was processed on the day the last test bottle was processed. The test bottles were harvested at two day intervals. The temperature of incubation was recorded at the same time each day and the bottles were also harvested at an identical hour of the day throughout the investigation. The temperature varied between 22°C and 24°C.

Processing of the culture filtrate consisted of the following operations:

i) Determination of pH

ii) Determination of optical density

iii) Determination of volume of filtrate

iv) Calculation of dry weight

v) Observation of fluorescence

vi) Determinat ion of ammonium nitrogen

vii) Assay of antiviral activity profile

The pH was determined with Beckman electric pH meter; from a reading of 7.2 on the day of seeding, the pH dropped to 4.5 on the second day, when growth of the mould was first observed. A further drop in pH to 3.7 was recorded on the fourth day and on the sixth day the pH started to rise, attaining a reading of 8.6 on the fourteenth day, when the experiment was terminated. There was a buffering effect in cultures of the eighth to the fourteenth day; the greatest effect was from the twelfth to the fourteenth day.

During the cultivation of the mould a yellow pigment diffused into the culture medium with a tinge of green in later cultures. Pigment production as reflected by changes in optical density of the medium was followed at each harvest. A change in the optical density was first noticed on the second day of the culture and gradually increased to maximum on the tenth day. There was no further increase in the optical density in the later days of the growth. The readings were made with a Beckman Spectronic 20 photometer using uninoculated Czapek-Dox broth as a blank; 380 mμ wavelength was chosen because the samples gave the highest readings at this point. The

optical density was determined for all the samples on the same day after the termination of the investigations.

The pigment was easily extracted with n-butanol after the culture filtrate was adjusted to pH 0.7 with HCl (Koffler et al., 1945). When it was examined in a Beckman DK-2 spectrophotometer, absorbance peaks were observed at 280-290 mμ and at 370 mμ.

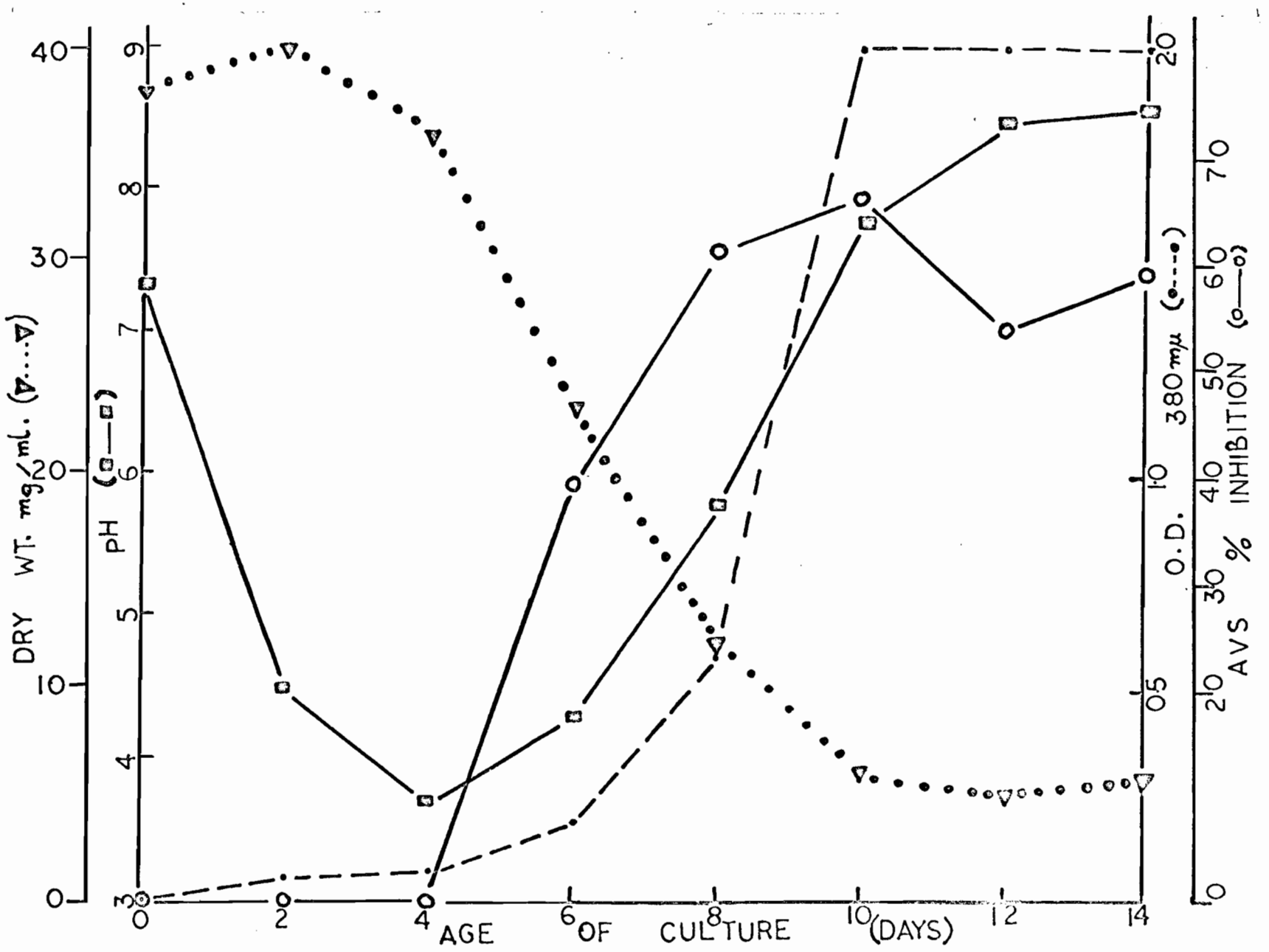
The volume of culture filtrates harvested on each of the test days did not vary significantly from the initial volume of 200 ml.

Dry weight of each of the harvests was calculated as described in the section of Materials and Methods. Depletion of the nutrients with age of the culture was shown by the progressive drop in the dry weight values.

In one of the early culture filtrates a weak fluorescence was observed when the filtrates were examined under 360 mμ ultraviolet light. It was therefore decided to include this study in the present series of investigations; fluorescence was not noticed earlier than the sixth day of growth of the mould. The fluorescence spectrum of a sample containing 2 mg per ml was determined in a Beckman DU spectrophotometer using a mercury lamp with maximum peak at 360 mμ. A dome-shaped pattern was plotted with maximum per cent transmittance at about 490 mμ. This peak was confirmed with a fluoremeter.

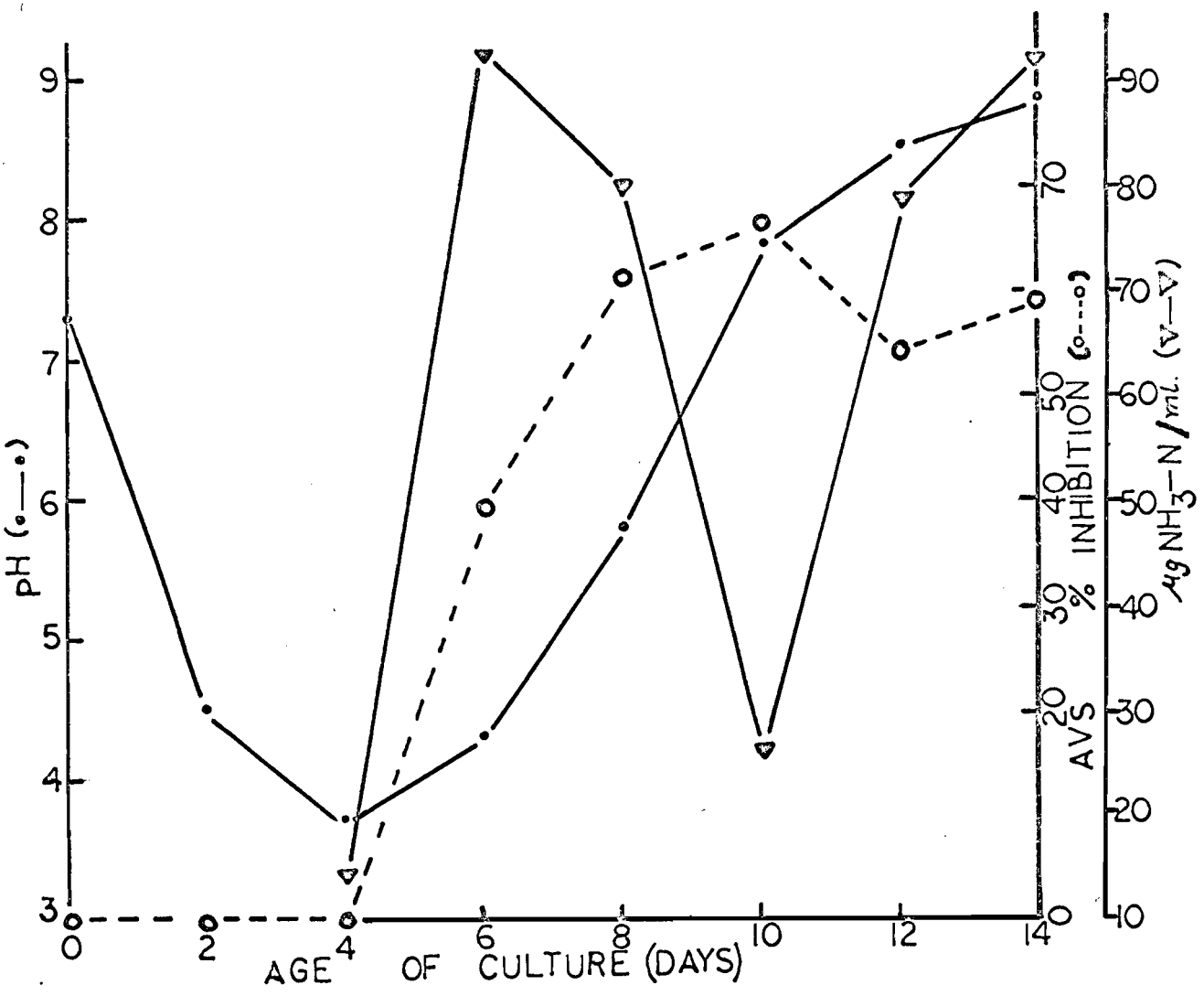
A titration of ammonia nitrogen was prompted by the expectation that this may afford an explanation to the changes of the pH observed in the culture filtrates. A distillation apparatus for micro-Kjeldahl determination of nitrogen was obtained through the kindness of Dr. Lucia Kapica, of McGill University. The apparatus is

Figure 23



Cultural changes during growth of Penicillium cyaneofulvum in Czapek-Dox broth.

Figure 24



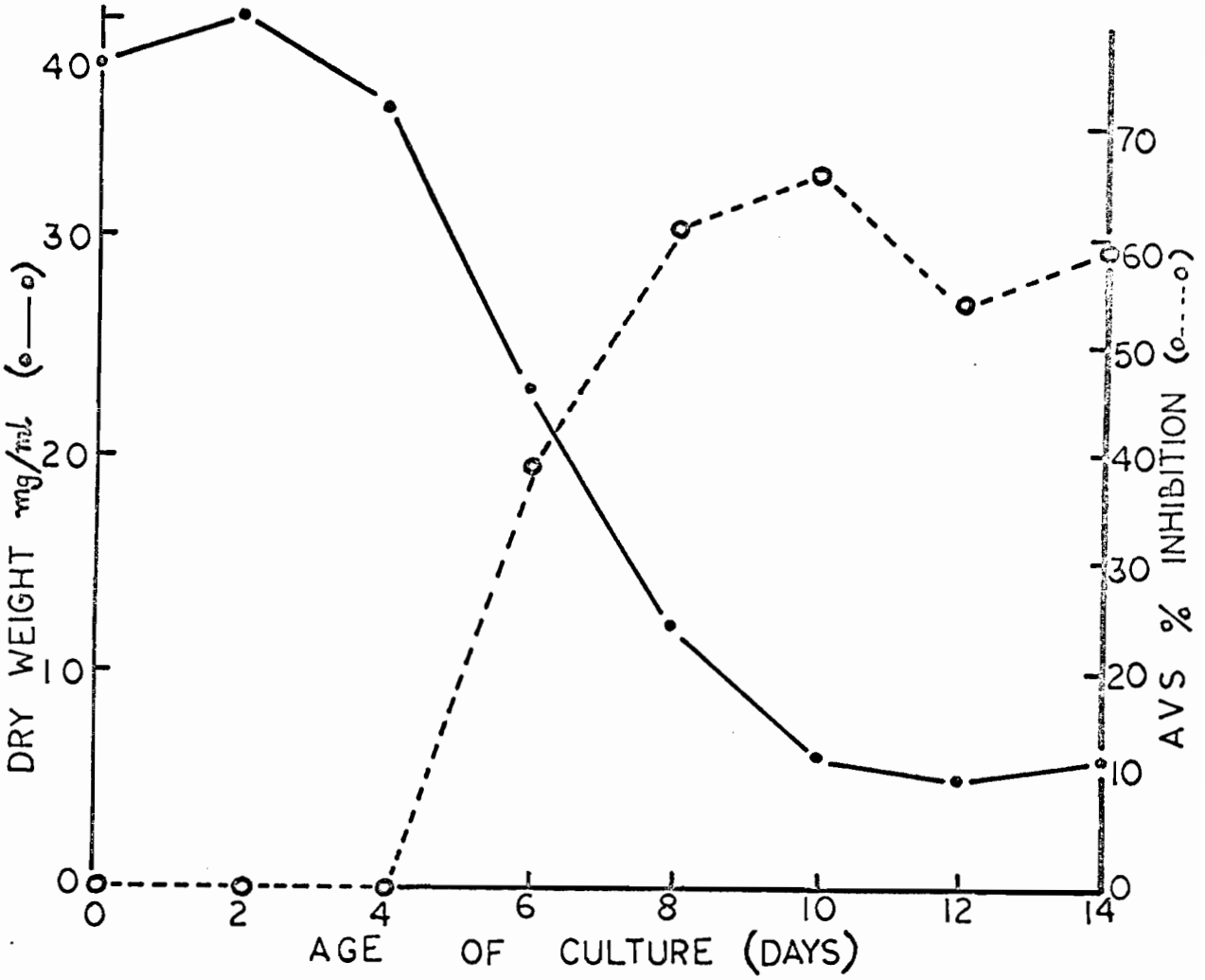
Changes in pH, ammonium nitrogen concentration and the elaboration of the antiviral substance.

essentially similar to the Markham Kjeldahl distillation apparatus described by Kabat and Mayer (1961). A 5.0 ml sample of each day's harvest was used for each titration; after making the sample alkaline with a solution of 30 per cent NaOH, the mixture was steam distilled and the generated ammonia trapped in N/70 H_2SO_4 with 3 drops of methyl red indicator added. The distillation was carried on for exactly 10 minutes; after which the unused acid was titrated with N/70 NaOH. From the amount of the standard alkali solution used, the amount of unused standard acid was calculated; this was expressed in terms of µg nitrogen per ml. of sample based on the relationship that 1 ml of the standard sulphuric acid solution is equivalent to 0.2 mg of nitrogen. The shape of the curve plotted from the results was rather curious (Fig. 24); but is in complete agreement with similar determinations for Penicillium notatum and Penicillium chrysogenum reported by Koffler et al. (1945). All the titrations were done on the same day.

The antiviral activity of 0.1 mg of each day's harvest was determined in Maitland cultures using 100 EID_{50} of influenza A virus (PR8). The results illustrated in Figures 23, 24, 25 and 26, show that no antiviral activity was demonstrated up to the fourth day of the culture, thereafter activity began to increase with the age of the culture. The negative relationship between the depletion of the medium and the elaboration of the antiviral substance is more clearly illustrated in Figure 25; while Figure 26 shows the positive correlation between pigment production and the production of the AVS. Table XV summarises all the observations made during the investigation.

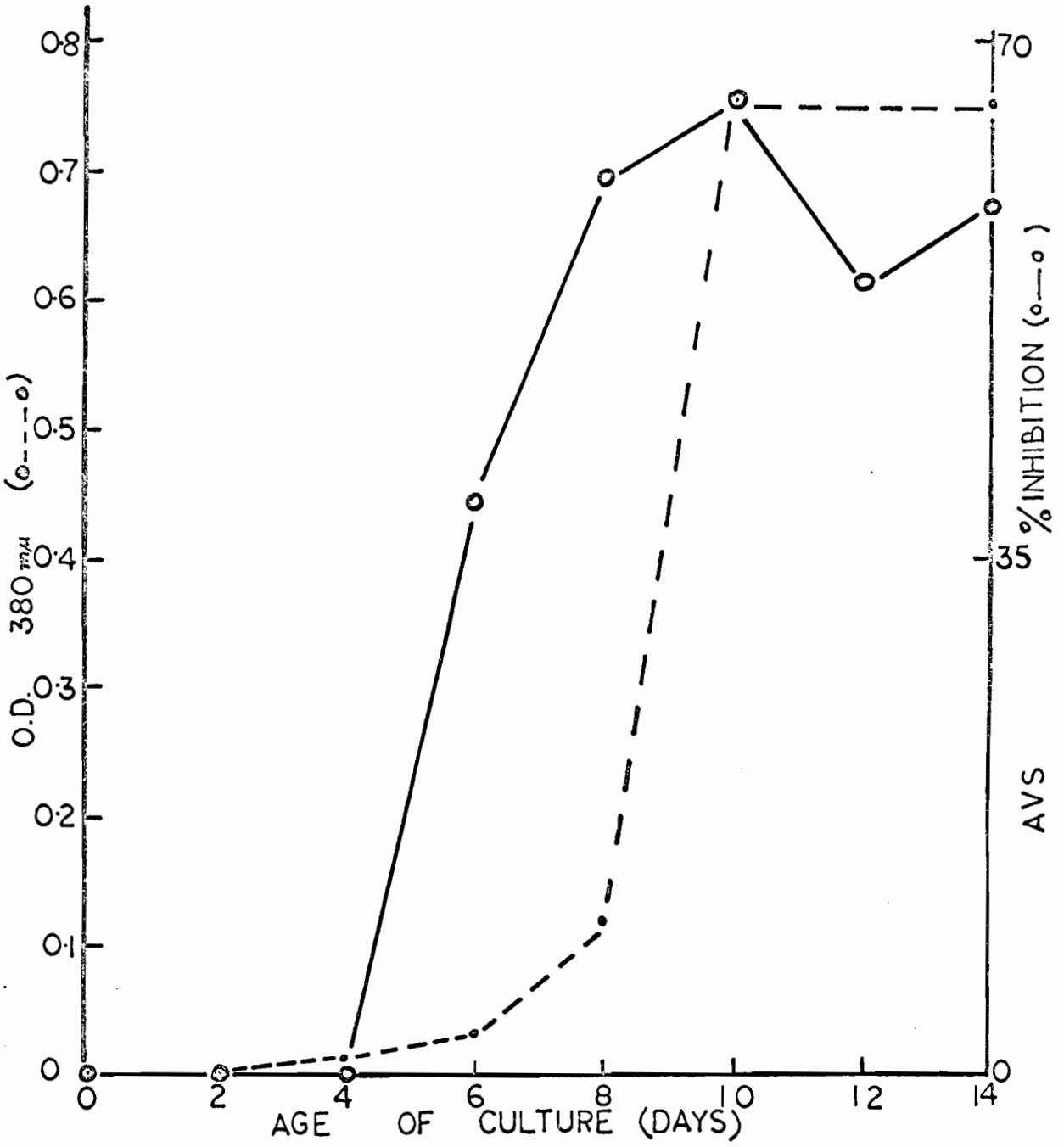
Samples from each harvest were hydrolysed and run on thin-layer chromatography plates for the detection of amino acids with the polychromatic stain of

Figure 25



Depletion of nutrients in culture medium and the temporal production of the antiviral substance.

Figure 26



Positive correlation between pigment production and the elaboration of the antiviral substance.

TABLE XV

Metabolic Changes During Growth of Penicillium cyaneo-fulvum and the Elaboration of the Antiviral Substance (Czapek-Dox Broth)

	Phase 1 0 to 4 days	Phase 2 4 to 10 days	Phase 3 10 to 14 days
Antiviral Substance	None	Linear rise	Slight fall
pH	Precipituous fall	Linear rise	Stable at or above neutrality
Sporulation	Very slight Predominantly white pellicle	Extensive Coherent mycelia felt	Very extensive Some autolysis
Pigment (Visual)	None	Deep yellow	Deep yellow with tinge of green
Pigment undil. (O. D. 380 mu) (Dil. 1/5)	0 to 0.06 (0 to 0.01)	0.05 to 2.00 (0.01 to 0.75)	2.00 to 2.00 (0.75 to 0.75)
Fluorescence	None	Fluorescent	Fluorescent
Nutrients	Used slowly	Linear depletion	Plateau
NH_3 - N	Released rapidly (4 - 6 days)	Utilised rapidly (6 - 10 days)	Released (10 - 14 days)
Buffering effect of medium	None	Buffering at pH 7.4 in later cultures	Very strong buffering effect at pH 7.4

Format adapted from Koffler et al. (1945).

Moffat and Lytle (1959). Amino acid spots were located in the culture filtrate hydrolysate beginning with the second day of growth; however, with age more clearly defined spots were demonstrated. Viewed under 366 mμ ultraviolet light complex fluorescent patterns were revealed for all harvests, with increasing complexity with age of the culture. This was most dramatic with the eighth day harvest. The patterns for the tenth, twelfth and fourteenth day samples were similar.

The cultural characteristics described were the "normal" patterns of growth of the mould in Czapek-Dox broth. In these cultures growth began on the second day after seeding of the broth, as a light white pellicle on the surface of the medium; by the tenth day virtually the entire surface of the culture was covered with extensively sporulating mycelia. In such cultures, usually, droplets of the pigmented culture fluid were seen on the surface of the mycelial felt, but the surface was not wettable possibly because of the droplets of oil that were also almost invariably seen on the surface. These observations were also recorded for Penicillium notatum (Fleming, 1929; Abraham et al. 1941). Figure 27 shows a typical normal culture and also illustrates the phenomenon described above.

On one occasion, with Batch 251165, the growth of the mould departed from these normal patterns. This appeared to be due to the use of older seed cultures, since the batch following immediately after the atypical batch, but prepared with fresh seed cultures, was normal. It seemed that for the production of typical cultures the preparation of good spore suspensions from fresh malt extract slope cultures which have not been stored at 4°C was essential.

In summary, the characteristics observed for P. cyaneo-fulvum in the

Figure 27

Typical 10-day old cultures of Penicillium cyaneo-fulvum in Czapek-Dox broth. (TOP) Upper surface. (BELOW) Under surface, showing Raschig rings.

present series of investigations approximated very closely to those reported for P. notatum (Fleming, 1929; Abraham et al., 1941; Raper et al., 1944; Koffler et al., 1945; Florey et al., 1949). However, Koffler et al. (1945) reported a peak absorbance for the yellow pigment elaborated by P. notatum at 450 mμ; while from the present studies the absorbance peaks of the yellow pigment extracted from culture filtrates of P. cyaneo-fulvum were at 280-290 mμ and at 370 mμ. This may be of significance and may indicate that the two pigments are different.

G. Comparison of the Antiviral Substance of Penicillium cyaneo-fulvum with Other Antiviral Substances of Penicillium Origin

Different antiviral agents of microbial origin were discussed in the section on Historical Review. As a summary of some of the experimental data presented it might be worth comparing at this place the antiviral substance produced by Penicillium cyaneo-fulvum with the other antiviral agents of penicillium origin that have been reported in the literature. This comparison is made in Table XVI, and shows that the antiviral substance used in the present investigation is different from the other agents reported in the literature so far.

TABLE XVI

Comparison of Antiviral Substances Elaborated by Penicillium Moulds

Penicillium species	Antiviral substance	Activity Filtrate	Activity Mycelia	Chemical Nature	Dialysability	Heat stability	Viruses inhibited	Need for Pretreatment	Mode of action	Media
P. funiculosum	Helenine	+	++	Ribonucleoprotein	-	-	Picorna Arbo	+	Intracellular ?	Complex
P. stoloniferum	Statolon M-8450 1758	++	No Report	Polyanionic-polysaccharide	-	-	Picorna Arbo	+	Intracellular ? Interferon	Complex
P. cyclopium	Cyclopin	±	++	Proteinaceous	+	-	Arbo	-	Intracellular	Complex
P. cyaneofulvum		++	++	Complex* Mixture	-	+**	Myxo Vaccinia Herpes ? Polio ?	-	Intracellular	Simple Synthetic

* Sugars, amino acids, nucleic acid bases and lipids present in semi-purified preparation.

** Activity usually increased after autoclaving, 15 p.s.i., 15 mins.

V. GENERAL DISCUSSION

An antiviral substance, referred to in earlier sections of the thesis, for convenience until a more acceptable name is devised, as AVS (Cooke, 1958, 1960; Syeklocha, 1962, 1964; Cooke and Stevenson, 1965a, 1965b) has been in the present investigation, consistently extracted from culture filtrates of the mould Penicillium cyaneo-fulvum (Biourge). In an attempt to obtain the active principle in a purer form the complex McGill glucose beef heart infusion broth used in previous studies was replaced by Czapek-Dox broth. The latter is a simple synthetic medium of easily reproducible composition and containing no native protein. On a dry weight basis it contained approximately two times less solid matter than the former broth. The use of Czapek-Dox broth in conjunction with the new methods of extraction and partial purification, which involved a fractional precipitation of inactive impurities with chilled acetone in the cold, have resulted in obtaining the antiviral substance in a purer and more active form than was possible in previous studies with the culture filtrates. In earlier studies antiviral activity had been investigated only in the crude culture filtrates and semi-purified preparation of such filtrates produced by a fractional precipitation with ammonium sulphate and subsequently with ethyl alcohol. In the present investigation it was shown that the active principle could be extracted from the organisms per se, i.e. the mycelia, although not as regularly as from the culture filtrates.

A comparison of the antiviral substance with other reported antiviral agents of Penicillium origin shows that it is different and distinct. Bobyr and Ellans'ka (1965) in a recent paper reported the elaboration of an anti-tobacco mosaic virus

principle by a Russian strain of Penicillium cyaneo-fulvum but since no detailed experimental data were presented, a comparison of the antiviral principle of the McGill strain of the mould and that of the Russian strain is not possible. The antiviral substance is also distinct from "Noxiversin", a second active biological principle elaborated by the strain of P. cyaneo-fulvum used in the present investigation (Cooke, 1958; 1960). Further evidence in support of the conclusion that the antiviral agent and "Noxiversin" are separate and distinct were provided in the current studies, by the use of Czapek-Dox broth for production and the use of acetone for extracting the active principle. Diena (1954) reported that "Noxiversin" was not produced by the mould in Czapek-Dox broth and the biological activity was destroyed by organic solvents (Diena, 1956).

The Modified Maitland-type tissue cultures again proved to be the ideal system for testing the biological activity of the AVS. In routine biological tests 100 EID_{50} of influenza A virus, PR8 strain, were used; 0.2 mg or 0.25 mg produced better than 80 per cent inhibition of the virus. However, a similar inhibitory activity was demonstrated against higher doses of the virus and the spectrum of activity was extended to include other members of the myxovirus group, such as influenza B virus, Lee strain, and Newcastle disease virus; all of these viruses were grown in Maitland-type tissue cultures. Influenza A virus (PR8) was selected as an index measurement to compare with previous studies, since all the detailed investigations in the earlier studies were conducted with this virus (Cooke, 1960; Syeklocha, 1962, 1964; Cooke and Stevenson, 1965a, 1965b).

One of the main obstacles in the study of antiviral agents is that most of the measures that are effective in treating viral infections also compromise the

integrity of the host cells to varying degrees, because of the absolute dependence of viral replication upon the host's physiological mechanisms. It is therefore of paramount importance that the dose of an antiviral substance used in a biological system should be below the toxic limit. In the present studies all the doses of the antiviral substance used were carefully checked for toxicity; this was facilitated by the new methods of screening any particular batch for activity, which established a profile of inhibitory activity by testing the extract in different doses. Potency tests were also conducted in Maitland cultures whenever the substance was to be used for a new biological test, as a check on the preparations.

Although very effective in suppressing the multiplication of myxoviruses in in vitro studies, the antiviral substance was completely ineffective against the same viruses in ovo. Because of the sharp contrast between these results and those obtained in earlier studies (Cooke, 1960) several modifications were introduced in the in ovo experiments as a means of ensuring the manifestation of possible inhibitory activity in eggs. For instance, the routine virus dose was reduced 10-fold and tested against doses of the agent which were 10-fold higher than those used in routine Maitland tissue cultures; tests were also conducted in the amniotic sac of 10-day old embryonated eggs as a means of avoiding a possible dilution of the active principle by the allantoic fluid, which in 10-day old embryonated hen's eggs has been estimated to be about 5 ml (McLimans, et al., 1957). The corresponding volume of the amniotic fluid was estimated at 2 ml (David-West, 1962). However, in none of these modifications was any inhibition of virus multiplication demonstrated. Previous in ovo studies with mumps virus were hampered by the low potency of the test virus (Cooke, 1960; Syeklocha, 1962), but of those eggs which received both virus and antiviral

substance there was complete inhibition of virus multiplication (Cooke and Stevenson, 1965b). In the present studies a high-titred mumps virus pool was obtained by passing the virus alternately in the amniotic sac and the allantoic sac of 7-day old embryonated hen's eggs and incubating the eggs for 7 days at 35°C. Attempts to demonstrate inhibitory activity of the substance against this virus in eggs were unsuccessful. Maitland tissue cultures were not used with mumps virus because even in the eggs, mumps virus multiplied very slowly and required more exacting conditions than the other myxoviruses used. It was felt that the chorioallantoic membrane used in the Maitland cultures may not stand up to the lengthy incubation that the virus would require. The lack of activity in intact embryonated eggs may be due to destruction of the active substance in the embryonic sacs.

Since the antiviral principle had shown the greatest activity against the multiplication of the RNA-containing myxoviruses in tissue cultures it was decided to try its effect against another RNA-containing animal virus representing a different virus group. Poliovirus, belonging to the picornavirus group, was selected for study partly because it is easily amenable to the production of cytopathogenic effect and the development of easily discernable plaques in tissue culture cell monolayers, and partly because it has been used in previous studies with the antiviral agent prepared by other methods (Cooke, 1960). In preliminary studies with Sabin type 1 poliovirus there were indications that the substance did not suppress the development of poliovirus plaques in Rhesus monkey kidney cell monolayers nor was cytopathogenic effect in the same cell system inhibited. The results on cytopathogenic effect contrasted with those of Cooke (1960) and Cooke and Stevenson (1965b) who reported the suppression of cytopathogenic effect of the Mahoney strain of type 1 poliovirus. However, the present

observations were not pursued in detail owing to the technical limitations of not obtaining complete cell monolayers as well as the fact that the monkey kidney cells were not as readily available as was desired. It is suggested that it would be worth while to investigate in greater depth the anti-poliovirus activity of the agent using the plaque suppression technique. If it is possible to demonstrate activity by this technique, the poliovirus system may prove better than the influenza virus system for quantitative studies, since influenza viruses are not as amenable to the plaque technique as polioviruses.

Two DNA-containing animal viruses, vaccinia virus and herpes simplex virus were practically insusceptible to the inhibitory action of the antiviral substance in in ovo systems. There was no significant suppression of pock formation on the chorioallantoic membrane by either virus. Vaccinia virus was studied in greater detail; with this virus some inhibition was demonstrated if the agent and virus were first interacted in Maitland tissue cultures analogous to the routine test used for influenza virus studies, before titrating the virus contents of the tissues in embryonated eggs for pock formation. No pocks were formed in vitro on the chorio-allantoic membrane pieces. This is in accord with similar observations made by Overman and Tamm (1957), and it was concluded that these observations afforded strong evidence in support of the suggestion that host reactions involving leucocytes and the vascular system may play an important role in pock formation in vivo. In direct pock suppression experiments on the chorioallantoic membrane it appeared that the antiviral substance selected smaller, secondary or satellite pock-forming variants, since in the absence of the substance, predominantly large necrotic pocks were formed. This would imply that the virus population was heterogeneous. The

ability to produce these small pocks seemed to be stable, since the pock size was retained in two subcultures in the absence of the substance.

The antiviral substance neither inhibited the induction of a cytopathogenic effect in monkey kidney cell cultures and chick embryo fibroblast cell cultures by vaccinia virus, nor did it suppress the formation of plaques by the virus in monolayer cultures of chick embryo fibroblast cells. These results are in accord with those of Syeklocha (1964), who also failed to demonstrate inhibitory activity against vaccinia virus CPE in HeLa cell cultures and who also found no suppression of plaque formation by the virus in chick embryo fibroblast cell cultures.

The general picture that emerges from these studies of the biological action of the antiviral substance is that it has a selective in vitro inhibitory action only against members of the myxovirus group, among the viruses tested so far. The DNA-containing animal viruses so far tested are for all practical purposes, insensitive to the inhibitor. With poliovirus the results obtained in the limited preliminary studies contradicted those of previous studies with the virus. Further investigations are obviously indicated in this respect.

In in vivo experiments in mice it was shown that a single 2.8 mg dose of the semipurified substance was capable of suppressing the fatal effects of 6,400 haemagglutinating units of intranasally instilled influenza A virus (PR8). The best results were obtained when the AVS was also given intranasally immediately after the virus inoculation. In such experiments, a four-day delay in the onset of death of the treated groups as well as a sparing effect of 40 per cent was observed. A delaying effect of three days and a sparing effect of 30 per cent were also observed when the antiviral substance was inoculated intraperitoneally immediately following the intra-

nasal instillation of the virus. However, when the intraperitoneally inoculated AVS preceded virus inoculation by 1 hour there was no effect other than a 24-hour delay in the onset of death of the AVS-treated group. On the other hand, when the substance was given 1 hour after virus inoculation there was also only a 24-hour delay in the onset of death and only a 10 per cent sparing effect. A possible interpretation that would embrace these observations is that the active principle was rapidly metabolised in the peritoneal cavity of the mouse and that for maximum activity a direct contact of the AVS and the target zone of infection in the respiratory epithelium was essential during the early stages of the infective processes.

The significant differences between the present in vivo studies and those reported previously should be emphasized. Generally in these studies a prior in vitro incubation of virus and substance at room temperature for about 30 minutes followed by the inoculation of the mixture appeared to be essential for the demonstration of inhibition of the infectivity of the virus (Cooke, 1960; Cooke and Stevenson, 1965b). In further work intraperitoneal treatment with several doses of the substance was tried with significant reduction in mortality rates (Syeklocha, 1962). Unfortunately activity was not uniformly demonstrated in mice (Syeklocha, 1964. In all of these earlier in vivo experiments the results are not as clearcut as those reported in the present investigation. This may be due,at least in part, to the greater efficacy of the antiviral agent prepared by the acetone method of partial purification; this aspect of the problem should be investigated in greater detail.

There was no correlation between the gross state of health of the mice, the degree of lung consolidation and survival. Therefore, these criteria could not be used in the final analysis of the activity of the antiviral substance. Moreover, the

substance by itself caused some lung consolidation in the control groups. Lung suspensions prepared after the fourteen days of observation of the mice, were not infective in embryonated eggs, while the titre of haemagglutination-inhibition antibodies was high for both the treated groups and the control mice. These data may be explained in the following terms: one, that the lung lesions may have been due largely to intoxication rather than a consequence of virus multiplication; intoxication without virus multiplication has been well documented in various myxovirus systems (Smorodintseff and Ostrovskaya, 1937; Henle and Henle, 1944; Sugg, 1949; Sugg, 1950; Ginsberg, 1951; Ogasawara et al., 1959). Two, the viruses may have been neutralized by specific antibodies. Taylor (1941) showed that following the infection of mice with influenza virus specific antibodies developed very rapidly. This may also explain the recovery of some sickly mice with time during the course of the observation. The high haemagglutination-inhibition titre demonstrated for both the infected and the control lung suspensions may be due to a combination of both specific haemagglutination-inhibition antibodies and nonspecific haemagglutination inhibitors present in the suspensions. Further in vivo studies in mice are being conducted in the Department.

By exhibiting a very satisfactory in vivo activity in influenza-infected mice, the antiviral substance seems to have satisfied the "crucial test" demanded of any proven antiviral agent by Staehelin (1959). However, this optimism may be dampened by the fact that it is a long road yet from effective laboratory in vivo tests to the ultimate goal of clinical use.

A marginal effect of the suppression of vaccinial lesions on rabbit skin was also demonstrated. However, attempts to obtain more definitive results by

increasing the dose of antiviral substance were hampered by the erythema caused by high doses of the agent. In this system, it seemed that in order to obtain the best results the doses of virus and the antiviral agent should be critically balanced. The marginal effect in the rabbit may also correlate with similar effects on the chorioallantoic membrane of embryonated eggs.

In previous studies with bacteriophage (Syeklocha, 1964) the substance was found to be ineffective against T2 coliphage and a B. subtilis bacteriophage. However, since there has been consistent antiviral activity against the RNA-containing myxoviruses, two bacteriophages that more closely approximate animal viruses than the T2 series were selected; the RNA-containing bacteriophage f2 and the single-stranded DNA phage ØX174 were chosen. In several repeated experiments the substance was found to be ineffective in suppressing plaque formation of these bacteriophages. The failure to demonstrate any antiphage activity may be due, at least in part, to inability of the active principle to diffuse through the cell walls of bacteria, which are, of course, quite different in chemical composition from those of animal cells.

The experiments on the mode of action of the antiviral substance were performed in order to gain an insight into the possible ways by which the substance elicited its antiviral activity. In designing these experiments the normal sequential phases of viral replication were taken into consideration and it was hypothesized that the antiviral substance might prevent virus multiplication by interfering with one or more of the essential steps in the synthesis of new virus particles. The possibility of a direct inactivation of virus particles by the substance was also considered. In the growth curve experiments it was shown that the agent extended the

latent phase of virus multiplication by 6 to 9 hours. This would suggest a target of action situated between virus adsorption and virus maturation. The data obtained from the growth curve does not permit a delineation of more than one discrete cycle in the multiplication of the test virus (influenza A virus). This may be due to the fact that influenza virus, unlike poliovirus, is not released from infected cells in a "burst", but in "trickles" extending over a relatively protracted period of time (Cairns, 1952; Tamm, et al., 1953a). It was shown by the experiment on minimal time for effective adsorption of influenza virus to chorioallantoic membranes that even as early as two minutes after virus inoculation, sufficient virus had become irreversibly adsorbed to the membranes to cause infection. This compares with the results of Hirst (1942; 1943) who also reported a very rapid adsorption of influenza virus to cells; about 97 per cent became adsorbed to red blood cells within nine minutes of interaction and about 99 per cent was adsorbed to the cells of the respiratory tract epithelium. Dales and Choppin (1962) showed by electron microscopy that within ten minutes of infection, chorioallantoic membrane pieces of 10-day old eggs irreversibly adsorbed influenza virus particles. Lanni and Lanni (1955) showed by kinetic experiments that nearly every collison between influenza virus and its substrate was followed by a combination. From this information experiments were conducted in which the AVS was added to Maitland cultures after infecting the chorioallantoic membrane with virus and allowing sufficient time for virus adsorption and penetration. It was shown from these experiments that the antiviral substance was effective even when added to the culture one hour after virus infection. Furthermore, it was also shown that its effects could be reversed by washing the chorioallantoic membrane pieces after interacting the membranes, virus and antiviral substance for

one hour. These results taken together suggest that the agent was not preventing virus adsorption or penetration into cells. This conclusion is also consistent with the demonstration that the active substance did not possess haemagglutinating or haemagglutination-inhibition activity, since these phenomena are connected with adsorptive properties of virus and inhibitor and demand certain steric considerations and complementary receptor sites.

The antiviral substance did not appear to be blocking virus release from the infected cells since at no time was the intracellular virus titre higher than the corresponding titre in the culture fluid, at the peak of the activity of the substance. The effect of the agent on influenza virus elution from red blood cells was also studied; there was no demonstrable effect on the influenza virus enzyme, neuraminidase. Although the exact function of this enzyme remains obscure, its action on red blood cells has been constantly used as a model for virus release from cells. The experts are split on the question of the magnitude of the importance that should be attached to the virus enzyme in the infective processes. However, there seems to be little disagreement that the enzyme plays some role in the overall infective process. There are two major schools of thought on the issue; the one sponsored by Hirst (1943) maintained that the enzyme is necessary for virus penetration, while the other sponsored by Fazekas de St. Groth (1948a) postulates that viruses penetrate host cells by a process of "viropexis", which implies a passive uptake by the cells. Support for the former hypothesis has come from Rubin (1957), Rubin et al., (1957), Kathan (1965); Ackermann and Maassab (1954b) held the view that the enzyme is necessary for virus release. This view is, however, analogous to those expressed by Hirst (1943)

since the same substrate materials have to be destroyed in both penetration and release of the virus particles. Recently, Hoffmann et al. (1965) studied the effect of Amantadine on influenza virus release from red blood cells so as to acquire clues as to whether the drug is stopping virus release from cells. Dales and Choppin (1962) have provided evidence from electron microscopy in support of "viropexis". Schlesinger and Karr (1956) implicated the enzyme in the overall infective process. Fazekas de St. Groth (1950) reported that the enzyme is necessary for the destruction of mucus substrate in the respiratory tract; this would facilitate interaction between virus and host cells (Cohen, 1963). Hirst (1965) suggested that the virus enzyme might represent a "counterattack against a body defense".

The literature is very scanty on antiviral agents that act by blocking virus release from cells. The only compound that has been implicated to act by this mechanism is alpha-amino-para-methoxyphenylmethane sulfonic acid (Ackermann and Maassab, 1954a; 1954b).

The incubation of an inhibitory dose of the antiviral substance and influenza A virus at room temperature for 24 hours did not inactivate the virus, as revealed by egg infectivity tests. This indicates that the substance does not act by directly inactivating the virus particles.

A pretreatment of the membranes with nontoxic doses of AVS for six hours at 35°C revealed that, upon infecting the treated membranes in fresh culture medium, the susceptibility of the membranes to virus infection was significantly lowered. This would imply that the substance may act on the chorioallantoic membranes. Although the doses of the substance used were not toxic by the toxicity tests used throughout the present investigations, it is suggested that it would be of interest in future

studies to resort to such biochemical tests as oxygen consumption and carbon dioxide production as additional tests for toxicity.

Weighing the evidence presented, the suggestion is made that the antiviral substance may act, possibly, through an intracellular physiological disturbance imposed on the host cells, the exact nature of which is not immediately clear. However, such an interference would ultimately reflect itself in the suppression of virus multiplication. This concept seems attractive in view of the fact that the whole process of virus replication is so intimately dependent on the physiological integrity of the susceptible host's cells. In the whole animal the disturbance might be repaired in the course of time, but in the Maitland-type tissue cultures, where there is no cell multiplication no repair of the damage could be expected.

In its intracellular locus of activity, the possibility of interferon induction cannot be completely ruled out, although pretreatment of the membranes was not essential for the demonstration of activity. It is of interest in this regard that positive tests for both DNA and RNA were given by the substance, although only the RNA seems concerned with activity.

There was no evidence in the present series of investigations to suggest that the antiviral substance may be acting by stopping virus adsorption or release (Syeklocha, 1964).

In the dose-response curve there was virtually no gradual decrease in activity with increasing dose of the agent but rather there was a sudden drop in inhibitory activity at certain dose levels. This sudden drop in activity might indicate that the optimal active amount of the agent was acting within a narrow critical range,

similar to the effects observed by Shope (1953b) for helenine. A second possible explanation might be that the active principle is present only in small amounts and operating in a certain critical concentration which was difficult to reduce progressively to lower doses. Thus the result was akin to an all-or-none reaction.

Chemical analysis of the semi-purified antiviral extract demonstrated that it is a complex mixture of sugars, amino acids, nucleic acid bases and lipids. Sugars were determined by the anthrone reaction, which measures total hexoses (Fairbairn, 1953); hexose was also determined by a modified diphenylamine reagent (Segovia et al., 1965), which does not react with nucleic acid sugars. The presence of pentoses and hexoses was also demonstrated by thin-layer chromatography. Protein was determined by the Folin phenol reagent (Lowry et al., 1951) and in two-dimensional thin-layer chromatograms at least eight amino acids were demonstrated to be present in the extract. None of the aromatic amino acids were spotted in the present studies and in those reported by Syeklocha (1964). This may be due to destruction during the acid hydrolysis; tyrosine and tryptophan are known to be labile under these conditions (Duggan and Udenfriend, 1956; Neurath, 1963; Stahl, 1965). However, the presence of tyrosine and tryptophan was suggested by the Folin phenol reaction, which measures chiefly the tyrosine and tryptophan contents of protein (Kabat and Mayer, 1961). A further colorimetric test (Tauber, 1949) also demonstrated the presence of tryptophan. The exact role, if any, of these amino acids in the overall biological activity of the antiviral substance is obscure.

Deoxyribonucleic acid was determined by the diphenylamine method (Burton, 1956) and ribonucleic acid by the orcinol reaction (Dische, 1960). The presence of nucleic acid components was also suggested by the hyperchromic effect

observed in the ultraviolet absorption spectrum of the substance on heating the substance at 98°C for three minutes and taking readings within five minutes in a Beckman DU spectrophotometer. Hyperchromicity is a characteristic phenomenon of double-helix nucleic acid (Kunitz, 1950) as well as dinucleotides and dinucleosides (Sinsheimer, 1954; Privat de Garilhe, 1956) and it is a result of the cleavage of internucleotide or internucleoside bonds.

The ultraviolet light absorption peak of 270 mμ, as well as the value of 0.97 for the 280 mμ / 260 mμ ratio, also suggested a mixture of proteins and nucleic acid components. It is of interest that the absorbance peak was more emphasized in preparations that were filtered through a Seitz filter and since in the process some of the yellow pigment present in the preparation was removed, it appeared that certain components of the pigment were interfering with the readings. The yellow pigment is produced by the mould during growth and served as a good index of the state of growth of the mould in Czapek-Dox broth.

Lipid was extracted with n-butanol and analysis showed that most of the lipid material was present in the form of phospholipids.

The deeply fluorescent material detected in all the chromatograms, but which failed to stain with ninhydrin, benzidine and aniline hydrogen phthalate reagents, demands future investigation. Because the material fluoresced more deeply at 366 mμ ultraviolet light, a preliminary study was initiated in an attempt to locate alkaloids with the iodoplatinate spray for alkaloids (Stahl, 1965). Two white spots were demonstrated and suggested the presence of alkaloids (Stahl, 1965). However, the nature of the fluorescent material was still not revealed, since even after the alkaloid spray not all of the material was developed.

Treatment of the antiviral extract with trichloroacetic acid in the cold failed to precipitate the substance. This observation along with the methods of partial purification, which involved a fractional precipitation with 8 volumes of acetone, would suggest that relatively larger macromolecules were removed during the extraction and purification methods. It is possible that the material contained nucleotides, nucleosides, smaller peptides as well as carbohydrate components. The undialysability of the active principle may be explained in two ways; either the activity is a property of relatively large molecules or the active principle may be associated with carrier molecules of relatively large size. It is obvious from the discussion that the use of the terms "small" and "large" in these studies are only relative.

The active principle appeared to be a stable substance since it withstood autoclaving, repeated freezing and thawing in dry ice- 70 per alcohol mixture and was stable at 4°C for at least eight months. These observations taken together suggest that the active principle is unlikely to be protein, which is known to be labile under these physical conditions.

Attempts to define the exact nature of the active principle by enzyme inactivation did not provide conclusive results; with the exception of RNAase all the other enzymes tested yielded virtually negative results. It appears from the enzyme inactivation studies that the amino acids, deoxyribonucleic acid and lipid components of the antiviral extract do not play significant part in the active principle.

In summary, the following generalizations seemed valid: the antiviral extract would appear to be more complex than has been previously suggested (Cooke, 1960; Syeklocha, 1964); the nature of the compounds involved in the biolo-

gical activity still remain obscure other than an indication of the possible participation of ribonucleic acid components. However, protein, deoxyribonucleic acid and lipid do not appear to be essential components of the active principle. Finally, since the extract is still in a semi-purified form the possibility of interference by other substances in the colorimetric determinations cannot be dismissed and although the diphenylamine reaction for DNA (Burton, 1956) was reported to be without serious interference (Hutchison and Munro, 1961), it is suggested that the tests conducted established relative values of the substances investigated.

Mycologists argue about the relationship between Penicillium notatum and Penicillium cyaneo-fulvum (Raper and Thom, 1949). The results of the detailed studies and careful observations of the growth characteristics of P. cyaneo-fulvum in Czapek-Dox broth seem to point to certain similarities as well as differences between the two moulds. There was an initial drop in pH of the culture medium which may be due to the accumulation of organic acids and the final rise in pH may be due to the utilization of these acids as well as the liberation of ammonia, basic nitrogen from the mycelia and sodium ions following the reduction of nitrates. These cultural changes approximated those reported for Penicillium notatum and Penicillium chrysogenum (Fleming, 1929; Abraham et al., 1941; Raper et al., 1944; Koffler et al., 1945; Florey et al., 1949). Although there was a positive correlation between the accumulation of pigment in the culture fluid and the rise of antiviral activity these two properties did not appear to be related biologically. However, the production of the pigment is a good index of the growth conditions of the mould. The pigment was dialysable and was detected earlier than the antiviral activity. The production of the yellow pigment is characteristic of the P.notatum-chrysogenum group (Raper et al., 1944;

Koffler et al., 1945; Foster, 1949) but it is not important for the antibiotic activity of penicillin (Koffler et al., 1945; Florey et al., 1949). The peak of absorbance of the yellow pigment from the P. notatum-chrysogenum group was reported at 450 mμ (Koffler et al., 1945). However, the peaks of absorbance of the pigment extracted from P. cyaneo-fulvum (Biourge) in the present studies were at 280 mμ- 290 mμ and at 370 mμ. This may reflect species differences.

Evidences for de novo synthesis of the antiviral principle were provided by the negative correlation between the depletion of the culture medium during the growth of the mould and the rise of antiviral activity as well as the fact that no activity was demonstrated until after the fourth day of incubation of the culture.

VI. SUMMARY

1. An antiviral principle elaborated by Penicillium cyaneo-fulvum and which in previous studies was produced in a complex glucose beef heart infusion broth and prepared from such culture filtrates by fractional precipitation of impurities with ammonium sulphate and ethyl alcohol, has been successfully produced in simple synthetic Czapek-Dox broth and partially purified by fractional precipitation of impurities from the culture filtrates with chilled acetone. The antiviral substance inhibited the multiplication of influenza A virus (PR8), influenza B virus (Lee) and Newcastle disease virus in modified Maitland-type tissue cultures prepared from the chorioallantoic membrane of embryonated egg. However, the substance was ineffective against these viruses as well as mumps virus in in ovo tests. The production of pocks by vaccinia virus and herpes simplex virus on chorioallantoic membranes was not inhibited; with the former virus the substance appeared to select for the production of small secondary pocks instead. On the rabbit skin a marginal activity against vaccinial lesions was demonstrated. In mice infected by intranasal instillation of influenza A virus (PR8) a marked delaying effect and reduction of mortality rate were observed; the best effects were obtained when the antiviral substance was also given by the intranasal route, immediately after virus inoculation. No anti-phage activity was demonstrated against RNA-containing phage (f2) and single-stranded DNA phage (ØX174).

2. Studies of the growth cycle of influenza virus in modified Maitland cultures indicated that the antiviral substance prolonged the latent phase by 6 to 9 hours and detailed studies on the mode of action of the substance indicated that the substance neither stopped virus adsorption nor prevented the release of newly formed virus from cells. It is also not viricidal and the evidence points to an intracellular

site of action.

3. Chemical determinations conducted with the semi-purified preparation demonstrated the presence of sugars, amino acids, nucleic acid bases, lipids, largely phospholipids and possibly alkaloids. On thin-layer chromatography the presence of pentoses and hexoses was shown; eight amino acids were detected on chromatograms prepared with acid hydrolyzate of the substance. No aromatic amino acids were detected by chromatography. However, colorimetric tests demonstrated the presence of tyrosine and tryptophan. The ultraviolet absorption spectrum of the substance showed peak absorbance at 270 mμ. The activity was reduced by RNAase treatment but not significantly by DNAase, trypsin or lipase.

4. The active principle is not dialysable; it is heat stable; it withstands several cycles of freezing and thawing in dry ice - alcohol mixture and it is stable after lyophilization. It is not precipitated by tricholoroacetic acid in the cold.

VII. CONTRIBUTION TO KNOWLEDGE OR CLAIMS TO ORIGINALITY

1. In the present investigation, the antiviral principle elaborated by Penicillium cyaneo-fulvum Biourge has been consistently produced in Czapek-Dox broth. This is a simple synthetic medium of easily reproducible composition in contrast to the complex McGill glucose beef heart infusion broth which was used in previous studies of the antiviral substance. This is the first time a simple synthetic medium has been used in the detailed study of an antiviral substance of microbial origin.

2. A new and simpler method of extraction and partial purification of the antiviral substance has been introduced and this in conjunction with the introduction of the simple synthetic medium has resulted in a better and more consistent production as well as obtaining the active principle in a purer and more active form.

3. A new method of screening the culture filtrate extracts for antiviral activity was introduced. This established a profile of activity for each batch processed and facilitated the elimination of toxic doses in subsequent biological tests. Furthermore, by determining the dry weight before any biological test was conducted with the extracts, it was possible to compare the different batches directly at the same dose levels. This was not the case in previous studies carried out with this agent. For the first time a dose-response curve was plotted with the antiviral substance.

4. Additional toxicity tests in eggs were also used as further checks on the results of the profile of activity.

5. A modified method for preparing the chorioallantoic membrane fragments for

the modified Maitland tissue cultures was used and it proved to be simpler and more economical in time than the methods used in earlier studies.

6. Detailed studies and careful observations of the growth characteristics of the mould in Czapek-Dox broth afforded evidences for a de novo synthesis of the antiviral principle. A possible biochemical difference between Penicillium cyaneo-fulvum and Penicillium notatum was also observed in the nature of the pigment produced by the two moulds in fluid medium.

7. The antiviral substance was in previous studies suggested to be a carbohydrate-amino acid complex. The present studies, however, demonstrated that the antiviral extract is more complex than was suggested earlier. It was shown by different chemical determinations, to be a complex mixture of sugars, amino acids, nucleic acid bases, lipids and possibly alkaloids in its semi-purified form. For the first time chemical tests were conducted for the detection of nucleic acid components, lipids and alkaloids and it is in the present studies that the presence of aromatic amino acids was first demonstrated. A definite ultraviolet absorption peak was demonstrated at 270 mμ; only a slight increase in absorbance between 260 mμ and 280 mμ was observed in previous studies. Chemical inactivation studies indicated that of all the enzymes tried only RNAase caused a significant reduction in the biological activity of the substance. The results with this enzyme were negative in previous studies.

8. The spectrum of activity of the antiviral substance was extended to include influenza B virus, Lee strain, and Newcastle disease virus in modified Maitland cultures.

9. More detailed in ovo tests with influenza A virus (PR8), influenza B virus (Lee), Newcastle disease virus and mumps virus demonstrated that the antiviral substance was inactive in eggs. In the present investigation, a high-titred mumps virus pool was obtained by alternate amniotic sac and allantoic sac passages of the virus in 7-day old eggs. In previous studies, the results with this virus were not conclusive because of the difficulty in obtaining a potent virus pool.

10. In mice infected intranasally with influenza virus, the present preparations of the antiviral substance have produced more clearcut inhibitory effects than were possible in previous studies and confirmed delaying effect and overall lowering of mortality rate established in some previous experiments.

11. For the first time the antiviral activity of the substance was tested in rabbits; a marginal effect against vaccinia virus infection in rabbit skin was demonstrated. In in ovo tests with the virus the active substance appeared to select for the production of small secondary pocks. A second DNA-containing animal virus, herpes simplex virus, was for the first time used in these studies; as in the case with vaccinia virus the active principle did not inhibit pock formation by herpes simplex virus in chorioallantoic membrane.

12. The active material possessed no anti-phage activity when tested against phage f2 and phage ØX174. These phages had not been tested before.

13. In previous studies it was suggested that the substance might act by inhibiting virus adsorption to cells and preventing the release of newly formed virus from cells. However, in the extended studies on the mode of action carried out in the present investigation evidence has been acquired which suggests that the

substance neither prevents virus adsorption nor blocks virus release. It is not directly viricidal and an intracellular site of action has been implicated.

14. The successful production of the active antiviral principle in Czapek-Dox broth and its partial purification with acetone have provided additional evidence to show that the antiviral substance is distinct from the toxin neutralizing substance "Noxiversin", produced by the mould since "Noxiversin" is not produced by the mould in Czapek-Dox broth and since the toxin neutralizing principle is destroyed by organic solvents.

15. Stahl (1965) stated that amino acids on thin-layer silica gel chromatograms do not fluoresce under ultraviolet light. However, in the present study it was observed that most of the amino acid standards as well as the acid hydrolyzates of the antiviral substance did fluoresce under the light, at both 260 mμ and 366 mμ.

APPENDIX

1. McGill Beef Heart Infusion Broth

Fresh beef heart, fat-free, minced	450 gm.
Distilled water	1,000 ml.

Heat at 75°C to 80°C for 1½ hours

Syphon off meat infusion

Add 1% proteose peptone

Add 1% stock salt solution (stock: 25% NaCl, 2% KCl and 1% $CaCl_2$)

Adjust to pH 8.4 with 10N NaOH

Heat at 100°C for 30 minutes

Filter through paper pulp

Adjust pH to 7.2 with 1N HCl

Heat at 100°C for 30 minutes

Cool and filter through paper pulp

Check pH for 7.2. Bottle and sterilize at 120°C for 20 minutes.

2. McGill Glucose Beef Heart Infusion Broth

One per cent sterile glucose is added to sterile McGill beef heart infusion broth (pH 7.2).

3. Malt Extract Agar Slopes

Malt extract	20.0 gm
Neopeptone	1.0 gm
Cerelose	20.0 gm
Distilled water	1000 ml

Dissolve

Add agar	20.0 gm

Dissolve, dispense into 6 inch x 5/8 inch test tubes

Autoclave at 120°C for 20 minutes.

4. Czapek-Dox Broth

Distilled water	1000 ml
Saccharose	30.0 gm
$NaNO_3$	3.0 gm
K_2HPO_4	1.0 gm
$MgSO_4 . 7H_2O$	0.5 gm
KCl	0.5 gm
$FeSO_4 . 7H_2O$	0.01 gm

Adjust pH to 7.2

Autoclave at 120°C , 15 p.s.i. for 15 minutes.

Note: The components are dissolved in the above order.

The pH on preparation is about 8.0.

Upon adding the ferrous salt slight cloudiness is produced.

Some light whitish precipitate settles out after autoclaving but this does not interfere with the use of the medium.

5. Glucosol Medium for Modified Maitland Cultures

Solution A:	Na_2HPO_4	7.105 gm
	KH_2PO_4	2.269 gm
	Phenol red	20.0 mgm
	Distilled water	1000 ml
	pH 7.3	

Solution B:	NaCl	8.0 gm
	$CaCl_2$	0.2 gm
	$MgCl_2 . 6H_2O$	0.5 gm
	Glucose	1.0 gm
	Distilled water	1000 ml

pH 6.2

Solutions A and B are prepared separately

Dispense each in 100 ml portions

Autoclave at 120°C, 15 p.s.i. for 15 minutes

Before use mix A and B in equal proportions

Add 100 units penicillin and 100 µg streptomycin per ml.

6. Trypticase Soy Broth (B.B.L.)

Trypticase	17.0 gm
Phytone	3.0 gm
NaCl	5.0 gm
K_2HPO_4	2.5 gm
Dextrose	2.5 gm
Distilled water	1000 ml

pH 7.2

Dispense into tubes; autoclave at 120°C, 15 p.s.i. for 15 minutes.

7. Medium 199

Procured from Microbiological Associates, Bethesda , U.S.A.

Before use it is constituted with calf serum and antibiotics

(100 units penicillin and 100 µg streptomycin per ml.)

8. Hanks' Balanced Salt Solution 10X Concentrated

Solution A:	NaCl	80.0 gm
	KCl	4.0 gm
	$MgSO_4 . 7H_2O$	1.0 gm
	$MgCl_2 . 6 H_2O$	1.0 gm
	$CaCl_2$	1.4 gm
	Glucose	10.0 gm
	Distilled water	800 ml
Solution B:	Na_2HPO_4	0.6 gm
	KH_2PO_4	0.6 gm
	Distilled water	200 ml

Autoclave A and B separately at 120°C , 15 p.s.i. for 15 minutes

Mix by adding B to A, when cool; final pH 7.0 - 7.2.

9. Earle's Balanced Salt Solution 10X Concentrated

Solution A:	NaCl	68.0 gm
	KCl	4.0 gm
	$MgSO_4 . 7H_2O$	2.0 gm
	$NaH_2PO_4 . H_2O$	1.25 gm
	Glucose	10.0 gm
	Distilled water	800 ml
Solution B:	$CaCl_2$	2.0 gm
	Distilled water	200 ml

Autoclave A and B separately at 120°C, 15 p.s.i. for 15 minutes

Mix by adding B to A, when cool; final pH 7.0 - 7.2.

10. Agar Overlay Medium for Tissue Culture

Medium A:	Sterile distilled water	60 ml
	Earle's 10X concentrated	18 ml
	Calf serum	3.6 ml
	$NaHCO_3$ (7.5%)	5.4 ml
	Neutral red (1:1,000)	0.2 ml
	Penicillin	100 units per ml
	Streptomycin	100 µg per ml
	Warm to 37°C before use.	
Medium B:	Agar (Difco-Noble)	2.7 gm
	Distilled water	90 ml
	Autoclave at 120°C, 15 p.s.i. for 15 minutes	
	Maintain at 43°C before use	
Medium C:	Mix A and B by pouring A to B.	
	Use immediately. After bottles are overlaid they are covered from light.	

11. Phosphate Buffered Saline (Dulbecco and Vogt)

Solution A:	NaCl	8.0 gm
	KCl	2.0 gm
	$CaCl_2$ (anhydrous)	0.1 gm
	$MgCl_2 \cdot 6H_2O$	0.1 gm
	Distilled water	800 ml

Solution B: Na_2HPO_4 1.15 gm

KH_2PO_4 0.2 gm

Distilled water 200 ml

Autoclave separately at 120°C, 15 p.s.i. for 15 minutes.

Mix by adding B to A, when cool; final pH 7.2.

12. Alsever's Solution

Glucose	20.5 gm
Sodium citrate	8.0 gm
Sodium chloride	4.2 gm
Citric acid	0.55 gm
Distilled water	1000 ml

Autoclave at 120°C, 15 p.s.i. for 15 minutes.

13. Phosphate Buffer for Bacteriophage Experiments

$Na_2HPO_4 . 12 H_2O$	16.0 gm
K_2HPO_4	2.96 gm
Distilled water	1000 ml

Autoclave at 120°C, 15 p.s.i. for 15 minutes.

pH 7.2 - 7.4.

14. Medium 3XD for Bacteriophage Experiments

KH_2PO_4	4.5 gm
Na_2HPO_4	10.5 gm
NH_4Cl	3.0 gm
$MgSO_4 . 7H_2O$	0.3 gm
Casamino acids (Difco)	15.0 gm

Glycerol	30.0 gm
1 N $CaCl_2$ Solution	0.3 ml
1 % Gelatin Solution	30.0 ml
Distilled water	1000 ml

Adjust pH to 7.2; autoclave at 120°C, 15 p.s.i. for 15 minutes.

A whitish precipitate forms; decant medium aseptically before use.

15. Growth Medium for Bacteriophage f2 (Loeb and Zinder, 1961)

Bacto-tryptone	10.0 gm
Yeast Extract	1.0 gm
Glucose	1.0 gm
NaCl	8.0 gm
Distilled water	1000 ml

To the basal medium add *M*/500 $CaCl_2$

Autoclave at 120°C, 15 p.s.i. for 15 minutes. pH 7.

16. Borate Buffer for Thin-Layer Chromatography (Jacin and Mishkin, 1965)

0.02 M boric acid solution (pH 5.9)	100 ml
0.02 M sodium tetraborate solution (pH 9.3)	3.0 ml

pH 8.0.

17. Polychromatic Stain (Moffat and Lytle, 1959)

Solution 1: Ninhydrin	0.1 gm
Absolute ethanol	50.0 ml

Mix thoroughly

Glacial acetic acid	10.0 ml
Collidine	2.0 ml

Mix thoroughly

Solution 2:	$Cu(NO_3)_2 . 3H_2O$	1.0 gm
	Absolute ethanol	100.0 ml

Just before use mix 50 ml of Solution 1 and 3.0 ml of Solution 2.

18. Aniline Hydrogen Phthalate Stain (Partridge, 1949)

Aniline	0.93 gm
Phthalic acid	1.66 gm
Butanol (water-saturated)	100 ml

19. Benzidine Stain (Horrocks, 1949).

Benzidine	0.5 gm
Glacial acetic acid	20.0 ml
Absolute ethanol	80.0 ml

20. Iodoplatinate Stain (Stahl, 1965)

10% Platinum chloride	3.0 ml
Distilled water	97.0 ml
6% aqueous KI	100.0 ml

Store in brown bottle.

21. May-Grünwald Giemsa Stain (Negroni, 1964)

Rinse coverslip in Hanks' balanced salt solution

Fix in absolute methyl alcohol for 10 minutes

Stain in May-Grunwald stain for 5 minutes

Stain in Giemsa (1/10) for 10 minutes

Rinse in distilled water

Dehydrate, 3 changes of acetone

Dehydrate, 3 changes of acetone-xylol (1:1: v/v)

Clear in xylol. Mount in permount.

22. Anthrone Reagent (Fairbairn, 1953)

Anthrone	1.0 gm
Sulphuric acid solution*	1000 ml (Cooled)
* Concentrated sulphuric acid	760.0 ml
Distilled water	240.0 ml

23. Modified Diphenylamine Reagent for Hexoses (Segovia et al., 1965)

Diphenylamine	1.33 gm
Glacial acetic acid	100.0 ml
Concentrated HCl	50.0 ml

24. Diphenylamine Reagent for Deoxyribonucleic acid (Burton, 1956)

Diphenylamine (2X recrystallized)	1.5 gm
Glacial acetic acid	100.0 ml
Concentrated H_2SO_4	1.5 ml

Add 0.1 ml aqueous acetaldehyde (16 mg/ml) to 20 ml DPA reagent.

25. Crystal Violet Stock Solution

Crystal violet	100.0 gm
Phenol crystals	200.0 gm
Absolute ethanol	1000 ml

Dilute 1:10 before use.

26. Nigrosin Vital Stain

Nigrosin	0.3 gm
Hanks' Balanced Salt Solution	100.0 ml

27. Picric Acid Solution

Saturated solution in distilled water.

28. Barium Chloride Test for Sulphate

Dialyzate	5.0 ml
HCl 1N	0.5 ml
$BaCl_2$ solution (2%)	5.0 ml

29. ACETEST Tablets

Procured from Ames Company of Canada Ltd., Toronto.

30. Embryonated Hens' Eggs

Eggs were obtained from Couvoir de Pont-Viau, Pont-Viau, Quebec.

31. Mice and Rabbits

Mice and rabbits were supplied by Romain Robidoux, Saint-Constant, Laprairie, Quebec.

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