THE UNIVERSITY OF MICHIGAN

INDUSTRY PROGRAM OF THE COLLEGE OF ENGINEERING

DIFFERENTIAL CHEMICAL INHIBITION OF POLIOMYELITIS, COXSACKIE, VACCINIA, AND ECHO VIRUSES IN HELA, MONKEY KIDNEY, AND MONKEY TESTICULAR TISSUE CULTURES

Normand Robert Goulet

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in the
University of Michigan
1958

June, 1958

IP-297

Doctoral Committee:

Professor Gordon C. Brown, Chairman
Assistant Professor Kenneth W. Cochran
Professor Thomas Francis, Jr.
Resident Lecturer Pearl L. Kendrick
Assistant Professor Donald J. Merchant

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to Dr. G. C. Brown for his unusually effective and heuristic teaching, to Dr. T. Francis, Jr. for making available the facilities of the Department of Epidemiology, to Dr. K. W. Cochran for his invaluable advice and guidance, to Dr. P. L. Kendrick who imparted the value of critical evaluation, and to Dr. D. J. Merchant for his interest in my work.

Appreciation and thanks are also due to Mr. J. H. Schieble for his cooperation, time, and friendship.

To the Faculty and students of the Department of Epidemiology I give thanks for the warm welcome, advice, and assistance extended to a "visiting" New Englander.

I heartily thank the personnel of the Industry Program of the College of Engineering for their part in the preparation of this manuscript.

And without fear of being accused of extreme sentimentality, I would like to express my everlasting gratitude to my parents and to my wife who have borne the brunt of my scholarly endeavors for lo these many years.

TABLE OF CONTENTS

TABLE OF CONTENTS CONT'D

LIST OF TABLES

LIST OF TABLES CONT'D

LIST OF FIGURES

I. INTRODUCTION

Two points of prime concern to tnose studying virus inhibitors are (1) the role of "tissue toxicity" and (2) the relative ineffectiveness, *in vivo*, of inhibitors selected on the basis of tissue culture studies (Pearson, 1953; Burnet, 1955; Matthews and Smith, 1955; Tamm, 1956, Hurst and Hull, 1956).

Successful chemoprophylaxis and chemotherapy appear to be a matter of relative toxicity; i.e., the host and parasite do not seem to represent two entirely different and independent metabolic economies, and the problem is to find a substance which, as used, is not harmful to the host but which will be toxic to those portions of the host's metabolism which are essential for virus replication. The experimental demonstration in tissue culture of latent or masked viral infections (Ackermann and Kurtz, 1955; Ginsberg and Boyer, 1956; Karzon and Barron, 1957) demonstrates the propagation of virus without observable damaging effects to the host cultures, and suggests that the host and parasite may not always utilize an identical metabolic economy, and that the different economies should provide sites vulnerable to the antiviral agents without undue damage to the host.

Several theories can be expounded to explain the relative ineffectiveness of tissue culture inhibitors in the chemotherapy and chemoprophylaxis of experimental animals infected with the smaller viruses, but it must be noted that studies of the inhibition of Rickettsiae and the larger viruses in tissue culture correlate quite well with *in vivo* experiments. The high degree of specificity required of

a substance in order that it may prevent the production of virus nucleoprotein without interfering with the normal nucleoprotein synthesis of the host, suggests that some of the reported tissue culture inhibitors of the smaller viruses might be "false positives" and should not be expected to inhibit virus in the intact animal.

It appears reasonable to assume that following the multiplication process the released virus is a product of a replicating mechanism which is peculiar for each virus entity capable of growth within that host. This highly specific mechanism suggests that the reactions contributing to one virus entity differ from those contributing to other virus entities at some point in the replication process. It should be possible to inhibit one of these viruses without affecting other replicating mechanisms. Differential virus inhibition within a particular tissue culture would suggest a direct relationship between an inhibitor and one of the viruses. Since viruses can re-direct the synthesizing abilities of more than one host to produce specific virus, it should be possible to demonstrate the same direct virus-inhibitor relationship in another host, though possibly to a varying degree. Demonstration of such specific action would appear to rule out the possibility of drug-incited host injury.

A second assumption, the concurrent inhibition and growth, respectively, of two distinct viruses within the same tissue, also appears feasible. That is to say, that within a particular tissue culture, it should be possible to inhibit a virus with a compound while simultaneously allowing another virus not so affected to complete

its entire infectious cycle. This manipulation, demonstrated herein, presents conclusive evidence that the inhibition of virus multiplication can be specific and may occur without undue damage either to the host tissue or even to other reactions leading to the reproduction of another virus.

II. HISTORICAL SURVEY

Prior to 1935 positive achievements in the field of chemotherapy of infectious diseases were, by modern standards, relatively modest. Domagk's discovery of the sulfonamides radically altered the situation. Even more important than their intrinsic therapeutic value, was their effect in directing chemical effort and research into this new and hitherto inadequately explored channel. When a year or two later, Florey was able to develop an interesting laboratory phenomenon with Penicillium notatum into an outstandingly active therapeutic remedy of the lowest conceivable general toxicity, he instigated an additional vast amount of research into chemotherapeutic substances produced by living organisms. The result was an impressive degree of control over many bacterial infections achieved in a total period of little more than a single decade.

The chemotherapy of virus diseases is a more recalcitrant problem. With the exception of the largest viruses no practical means of influencing virus diseases by chemotherapy yet exists. This is perhaps not unexpected when we reflect on the differences between the bacteria, larger viruses, and the smaller viruses. The problem of chemotherapy of the diseases caused by the smaller viruses is that of selectively influencing the processes near to those essential to the life of the host without endangering its safety -- an objective once widely held to be unattainable even in the case of the relatively

independent pathogenic bacteria, but one now realized for these and for the Rickettsiae and the larger viruses. As facts accumulate, it would not seem unreasonable to harbour a restrained optimism regarding the future.

Although no substance of practical value against the virus diseases of man or domestic animals has yet emerged, inhibition of viru growth in particular complexes of host cell and virus is known. These instances have been recently reviewed by Matthews and Smith (1955) and by Hurst and Hull (1956). Although there are few, if any, reports on the inhibition of the Coxsackie or Enteric Cytopathogenic Human Orphan (ECHO) viruses, the literature is not wanting with respect to the inhibition of the viruses of poliomyetitis and vaccinia.

Several aliphatic compounds have been found to inhibit the aforementioned viruses. Thus, Thompson (1947) has shown that iodoacetate and sodium malonate prevented the multiplication of vaccinia in chick embryonic tissue. Benzaldehyde thiosemicarbazone, its p-acetamide, p-amino-p-methoxy, p-propoxy, and p-ethylsulfonyl analogues were reported as active inhibitors of vaccinia virus grown in chick embryonic tissue and in the embryonated egg. There was also a marked degree of protection with mice inoculated intracranially with the virus when benzaldehyde thiosemicarbazone was fed to the mice in diet form (Thompson, et al, 1951; Hamre, Bernstein, and Donovick, 1950; Hamre, Brownlee, and Donovick, 1951).

An ester, glycerylmonoacetate, was found to inhibit the growth of poliomyelitis virus in monkey testicular tissue (Brown, 1952).

A number of aromatic compounds have been found to interfere with the growth of viruses in their tissue culture medium. Phenols and their oxidation products, the quinones, and related compounds have been tested for their effect on poliomyelitis virus in roller-tube cultures of monkey testicular tissue. Only fifteen of these compounds were capable of inhibiting virus-induced degeneration of fibroblasts over a wide range of concentrations. Most of the inhibitors were equal in their effect against Types 1, 2, or 3 poliomyelitis virus. When tested against the three types of virus in monkey kidney cultures, some of the compounds were less effective against Type-1 virus or were not effective at all (Kramer, Robbins, and Smith, 1955; Smith, Knox, and Hollinshead, 1955; Hollinshead and Smith, 1956).

The growth of vaccinia virus in chick embryonic tissue has also been found to be inhibited by 2,4-dinitrophenol. (Thompson, 1946 and 1947).

Many of the heterocyclic compounds which have been used in the inhibition of virus growth in tissue culture are structurally related to the vitamins, components of nucleic acid, and other naturally occurring substances. Knox, Robbins, and Smith (1957) studied the possible antiviral effects of one hundred and sixty-three analogues

of the pyrimidine moieties of nucleic acid against Type-2 poliomyelitis virus in cultures of monkey testicle. Forty-three were found to be inhibitory and were further tested against the three types of poliomyelitis viruses in monkey kidney cultures. Only eight of these compounds, the pyrimidine analogues thiouracil, 5-methyl thiouracil, hypoxanthine, 2, 4, 5, 6-tetramino pyrimidine, 2-thio-4phenyl-6-oxypyrimidine, and the barbiturates pentobarbital and butethal were found to inhibit in both monkey testicular and monkey kidney cultures.

Four of these compounds had a direct in vitro virucidal action. When the chemicals were tested for their effects on the three types of poliomyelitis viruses, no apparent differences in virus susceptibility were noted in the kidney cultures, while in the testicular cultures higher concentrations were sometimes required to inhibit Type-1 virus.

All the inhibitors gave evidence of complete suppression of growth for a period up to twelve days in monkey testicular cultures, while in the kidney tissue, growth of virus was observed after a delay of twenty-four hours and then approached the titers obtained in the untreated cultures. Analogues of other basic components of nucleic acid, the purines, interfere with virus proliferation. Thus, 2, 6 - diaminopurine will inhibit poliomyelitis virus grown in monkey testicular cultures

(Brown, 1952). Gifford, Robertson, and Syverton (1954) reported that 2,6-diaminopurine did not inhibit poliomyelitis virus except at concentrations which were markedly inhibitory to the respiration of the host cells. That the mechanism of action of 2,6-diaminopurine is not clear is further demonstrated by the following observations: its inhibition of Russian spring-summer encephalitis virus in cultures of mouse and chick embryonic cells, Crocker Sarcoma 180, and Carcinoma 1025 (Friend, 1951); its injurious effects in Sarcoma 180 tissue cultues at concentrations which have no effect on the same tumor in vivo (Stock et al, 1950; Biesele et al, 1951); its prolongation of the survival time of mice infected with transplantable leukemia (Burchenal et al, 1949); its serious injurious actions on the hematopoietic system of the dog (Phillips and Thiersch, 1949), swine (Cartwright et al, 1950), and of the chick embryo (Karnofsky et al, 1949), and finally its reduction of the effective content of Kappa particles in Paramecium aurelia (Stock et al, 1951).

A number of α-haloacyl derivatives of various 5-aminopyrimidines inhibited the proliferation of vaccinia in chick embryonic tissue (Thompson et al, 1949 a,b). Halogenated analogues, for example 2,6,8-trichloropurine, were inhibitory against vaccinia virus (Thompson et al, 1950). These authors pointed out that the compounds studied were all toxic for other experimental animals, and the concentrations required to produce injury were of the same order of magnitude as those required to inhibit the multiplication of vaccinia virus. Since mice were not protected, the authors concluded that the action of the compounds was directed

primarily toward the embryonic tissue substrate.

Vitamin analogues have been used in attempts to interfere with virus proliferation. Thus, benzimidazole has proved effective as an inhibitor of poliomyelitis virus (Brown, 1952; cf. Gifford, Robertson, and Syverton, 1954) and vaccinia virus (Thompson, 1946 and 1947), and homobiotin inhibited the growth of Lansing poliomyelitis virus in monkey testicular tissue (Brown, 1952).

The enzyme inhibitors atabrine and proflavine were effective against vaccinia virus in chick embryonic tissue (Thompson, 1946). Briody and Stannard (1951) demonstrated that proflavine inhibited the growth of vaccinia on the chorio-allantoid membrane whereas the growth of mumps and Newcastle disease viruses was unaffected. The dosage of proflavine used was close to the toxic level, and the authors' dilemma appeared to be the explanation for the differential activity with respect to toxicity for the host. Proflavine is also effective against poliomyelitis virus (Brown, 1952).

Natural and synthetic amino acids and analogues of amino acids have been tested against viruses in tissue culture. Thus, the importance of methionine has been demonstrated in the biosynthesis of the Lansing strain of poliomyelitis virus in human brain and monkey testicular cultures by inhibition with ethionine (Brown and Ackermann, 1951; Brown, 1952; cf. Gifford, Robertson, and Syverton, 1954). B-2-Thienylalanine has also been found to be inhibitory to poliomyelitis virus (Brown, 1952) and to vaccinia virus (Thompson and Wilkin, 1948). Inhibition of vaccinia virus in chick embryonic tissue cultures has also been demonstrated by the use of α-amino-methane sulfonic acid, α-amino-isobutane sulfonic acid,

and α-amino-phenyl-methane sulfonic acid (Thompson, 1947).

Inhibition of the multiplication of poliomyelitis virus in HeLa cultures was demonstrated with 4-fluorophenylalanine (Ackermann, Rabson, and Kurtz, 1954). An interesting feature of this inhibition was the apparent dissociation of virus multiplication and cytopathogenic effect, with destruction of the host cells apparently unimpeded although virus multiplication was prevented. Fluorophenylalanine also inhibited the multiplication of the host cells; however, the effect upon the uninfected cell was reversible after three days, as indicated by the viability after treatment.

A few studies have been done with carbohydrate inhibitors. Robertson, Gifford, and Syverton (1956) presented evidence that exposure of HeLa cells to autoclaved solutions of D-ribose prior to infection with poliomyelitis virus reduced the rate of virus synthesis, but not the final yield. The inhibitory effect was correlated with the furfural content; furfural also inhibited virus when added twenty hours after virus inoculation.

Ginsberg and Horsfall (1949) showed that the replication of mumps virus and pneumonia virus of mice (PVM) in the embryonated egg was inhibited by the injection of small quantities of the type-specific capsular polysaccharide of Freidlanders bacillus whereas large quantities of the polysaccharide will not affect the multiplication of influenza A or B or Newcastle disease viruses. That the three viruses were capable of unrestricted multiplication whereas multiplication of either PVM or mumps was inhibited suggested that the two groups of viruses might require different host metabolic systems for their replication.

Little antibiotic activity has been reported against the smaller viruses in tissue culture. Phagopedin sigma was found to inhibit the growth of poliomyelitis in monkey testicular cultures (Brown, 1952). Hull and Lavelle (1953 and 1954) reported on the inhibition of the cytopathogenic effect of the poliomyelitis viruses in tissue cultured monkey testicular cells with a penicillium mold filtrate designated as M-8450. The authors concluded that the antibiotic exerted its effects upon the cells rather than on the virus, as pretreatment of the cells was necessary, and viable virus could be demonstrated in the undamaged treated cultures as long as five days after inoculation.

Inhibitory studies are yielding valuable information regarding the metabolic requirements of virus growth in cellular environments. Once again, however, but little of the accruing knowledge has yet led to advances toward an effective chemotherapy of virus diseases.

III. MATERIALS AND METHODS

Tissue Culture Techniques

The choice of the appropriate tissue culture has been intimately related to the availability of the source materials, technical flexibility and adaptibility, and the virus spectrum of these potential hosts.

HeLa (Human Cervical Carcinoma - Gey)

These cultures, originally obtained from Dr. J. T. Syverton, were grown and maintained in as close an approximation as possible to the methods described in detail by Syverton, Scherer, and Elwood (1954).

Monkey Kidney Epithelium

The procedures employed for the growth and maintenance of these cultures were essentially similar to the quantitative methods reported by Youngner (1954 a,b), modified by the use of a lactalbumin medium introduced by Melnick (1955 b), and further modified by the substitution of horse serum for the calf serum component. Buffering of the medium was accomplished by reinforcement of the bicarbonate and phosphate with 0.01M "Tris" (tris hydroxymethyl aminomethane).

Plaque Cultures--The techniques employed in the use of plaques were essentially those reported by Dulbecco (1952) and Hsiung and Melnick (1955) modified by the incorporation of the desired concentration of drugs into the agar overlay as well as into the liquid phase.

Monkey Testicle Fibroblasts

The monkey testicular cultures were prepared by the plasma-clot roller-tube methods described in detail by Robbins, Weller, and

Enders (1952). In addition, several experiments were performed with trypsinized (0.5% trypsin, Difco, 1:250) cells grown directly on the glass surface without the aid of a supporting plasma-clot.

Antibiotics

Penicillin, streptomycin, and mycostatin were incorporated into all tissue culture media.

Penicillin (500,000 units) and streptomycin (one-half gram) were dissolved in 50 ml. Hanks balanced salt solution or Earle's saline. Mycostatin (500,000 units) was suspended in the above solution which was distributed into 1 ml. aliquots and frozen until ready for use. One ml. of the antibiotic mixture was used per 100 ml. of tissue culture medium, giving a final concentration of 100 units penicillin, 0.1 mg. streptomycin, and 100 units mycostatin.

Drugs

Selection and Preparation

The compounds used in the study were either metabolic analogues reported in the literature as inhibitory to a particular virus, experimental drugs and antibiotics which had in some cases been defined, or unknown compounds.

Most of the drugs were prepared in a routine manner. If the compound was a powder its solubility was attempted in 1) the culture medium bathing the tissue in question, 2) sterile distilled water, 3) sterile distilled water to which 1N acid or 1N base had been added, or 4) propylene glycol. The drug was then diluted so

that a final concentration of 2.5 mg/ml of culture fluid originated a two-fold dilution series. If the drug was liquid in nature, a 1/2 dilution originated the two-fold dilution series.

Toxic levels were then determined by introducing 1 ml. of each dilution of drug into replicate tube cultures.

Toxicity Criteria

The compounds were tested for toxicity in each tissue culture system in which the virus inhibitory studies were to be performed.

Microscopic Evidence--Certain gross cytological changes, such as distortion, pyknosis, granularity of the cytoplasm, swelling or bubbling of the cytoplasm, sloughing, or complete destruction of the cultures were the conditions associated with toxicity for a given tissue in culture.

Cinephotomicrographic Evidence--A limited number of selected compounds were also employed with time-lapse photomicroscopy. The toxic properties which can be determined are associated with abnormal pinocytosis, cessation of the normal undulation of the cellular membranes, cessation of Brownian movement within the cytoplasm, or inhibition of the migratory activity of the cells.

Viruses

Viruses Employed

Type-1 poliomyelitis virus (Mahoney) was employed throughout the study with the exception of one experiment wherein Type-2 poliomyelitis virus (MEF) was employed. Both strains of virus were obtained from the Connaught Laboratories, Canada.

A representative of the Coxsackie group of viruses, Type B-1 (Conn.-5), was also included in the study.

Vaccinia virus, obtained from the Michigan Department of Health, was also employed.

The study also included some of the Enteric Cytopathogenic Human Orphan (ECHO) viruses which were originally obtained from Dr. J. L. Melnick, Yale University. The viruses employed were ECHO-1 through 9, and ECHO-11 through 14.

Preparation and Identification of Virus Pools

Pools of virus were prepared in the following manner: one ml. of undiluted virus was inoculated into bottle cultures of the appropriate tissue which contained 9.0 ml. of nutrient fluid. The bottles were incubated at 37°C and observed daily for cytopathology. Upon destruction of most of the cultured cells, the fluids were pooled and centrifuged in 40 ml. conical centrifuge tubes at 2000 rpm for 15 minutes. The supernatant fluids were distributed in 1 ml. aliquots into glass tubes. The tubes were then stored at about minus 20°C. Aseptic technique was used throughout and the pools were tested for bacterial contamination by inoculation into thioglycolate and beef infusion broths.

Each virus type (except vaccinia) was identified by employing hyperimmune sera representative of the types of virus employed in the tests. For control purposes normal sera from the appropriate animal species were employed. In vitro neutralization was employed for type determinations.

Hyperimmune antipoliomyelitis serum was obtained from Dr. H. Wenner, University of Kansas. Hyperimmune antiCoxsackie serum was obtained by inoculating mice intraperitoneally with infected HeLa tissue culture fluid with bi-weekly injections for a period of four weeks. The mice were then bled, the serum pooled, and the neutralizing ability tested *in vitro*. Hyperimmune antiECHO sera were obtained by extensive intravenous and subcutaneous inoculations of young rabbits with high titered monkey kidney tissue culture antigens. The *in vitro* neutralizing titer of the rabbit sera was determined periodically. A titer of 1/1024 to 1/2048 was considered adequate.

Typing of the vaccinia pools was not attempted since the unique cytopathology caused by this virus served as the indicator of the virus used.

Titration of Viruses

Virus was estimated by determining the limiting dilution which could initiate infection in 50% of the tube cultures inoculated. Serial half-log dilutions of virus were prepared and 0.1 ml. aliquots of each dilution were added to 4 tube cultures. The cultures were incubated at 37°C and examined for cytological changes characteristic of infection each day for seven days. The 50% endpoints were calculated by the method of Reed and Muench (1938). The dilutions recorded were always the initial rather than the final dilutions. Since all the cultures contained 1 ml. of fluid, all titers were expressed as the number of tissue culture infectious doses ($TCID_{50}$) per ml. of sample.

In the earlier experiments, 10, 50, and 100 $TCID_{50}$ were employed in the studies, but later all tests were done with virus doses ranging from 32 to 320 $TCID_{50}$. Since this variation represents only one-half log over or under 100, the dosage was recorded as approximately 100 $TCID_{50}$. Results obtained with virus dosages above or below this range were discarded and the tests were repeated.

Evaluation of Virus Inhibition

The inhibitory phenomena observed included any delay in the time of appearance of cytopathology, partial inhibition of cytopathology, or complete inhibition of characteristic virus-induced changes with no evidence of virus infection.

Preliminary scoring of the inhibition was done on a plus or minus basis, these designations representing the degree of involvement of the cultures; when all the cells were destroyed the plus designation was given. Later, the effectiveness of the compounds was judged by the value obtained in calculating the therapeutic index (Kramer et al, 1955). The therapeutic index is a ratio of the maximum tolerated dosage of the compound to the minimal virus suppressive dose:

$$\frac{\text{Maximum Tolerated Dose}}{\text{Minimal Virus Suppressive Dose}} = \text{Therapeutic Index.}$$

This ratio reflects any observation of inhibition which occurs only on the first day in which the virus control cultures show complete degeneration. As an example, Table 1 represents the scoring of the cytopathology obtained with virus-infected control and drug-treated

virus-infected cultures. It can be seen from the table that on the third day the virus control cultures were completely degenerated. On that same day, the drug-treated virus-infected cultures were protected at 1/320 but not at the 1/640 drug dilution. The 50% endpoint calculated by the method of Reed and Muench (1938) is 1/442, and this value represents the minimal virus suppressive dose. The previously determined maximum tolerated dose was 1/10. The therapeutic index would then be equal to 44.2 (1/10 divided by 1/442).

The reproducibility of the evaluation was demonstrated by the repeated testing of an inhibitory compound, the antibiotic M-8450, used as a control drug in approximately twenty separate tests. The reliability of the evaluation was further demonstrated by the use of Dulbecco's plaque technique in the inhibitory studies. The therapeutic indices so obtained were essentially identical to those obtained in the tube culture studies.

IV. EXPERIMENTAL RESULTS

Studies on the Toxicity of Compounds

Preliminary to the present study, experiments were carried out to determine the toxicity of the experimental compounds for the cultured tissue (n.b., Materials and Methods). One hundred and twenty compounds have been studied in either HeLa, monkey kidney, or monkey testicular cultures. The highest non-toxic concentrations for the compounds tested in HeLa cultures can be found in Table 2.

Comparative studies have been completed for thirty-five of the aforementioned compounds which were selected mainly because of some known virus inhibitory potency. It can be seen from the data given in Table 3 that thirteen of the thirty-five compounds had different toxic levels when inoculated into various tissue cultures, and that these levels sometimes varied by as large a factor as ten or twenty-five.

It would appear that the compounds must be assayed for toxicity in each tissue before the drug-tissue complex can be employed in virus inhibitory studies.

Cinephotomicrographs

Two compounds, the antibiotic M-8450 and methionine sulfoximine, selected because of their effectiveness as virus inhibitors in HeLa cultures were employed at their maximum non-toxic concentrations in time-lapse photographic studies. These cinephotomicrographs appeared to be similar in every respect (n.b., Materials and Methods) to those obtained when normal

HeLa cultures were employed as controls. These experiments provided additional evidence that the maximum tolerated dose of M-8450 and methionine sulfoximine did not alter the morphological appearance of the HeLa cultures.

Effect of M-8450 on the Continuous Growth of HeLa Cells

Subsequent inhibitory results suggested that concentrations of M-8450 near the maximum tolerated dose might still be toxic to the cultures in a manner not readily apparent morphologically. It was of interest to determine whether the antibiotic affected the continuous growth of HeLa cells.

Accordingly, HeLa cells were cultured (n.b., Materials and Methods) in continuous contact with various concentrations of M-8450 diluted in the normal growth medium. The cultures were discarded after five weekly passages, or earlier if the results were not significantly different from the control cultures. It can be seen from Table 4 that HeLa cells exposed to 1/40, 1/80, and 1/160 dilutions of M-8450 multiplied at approximately the same rate as did the control cultures. These cultures behaved normally as indicated by their morphology, pH changes, and general cultural appearance. However, the cells exposed to a dilution of 1/10 M-8450 appeared to multiply at a slower rate. Although displaying normal pH changes, these cells were noticeably more rounded, were not in a continuous sheet, and had fewer processes than seen in the normal cultures. In a subsequent experiment, the growth of cells in a medium containing a 1/20 dilution of the antibiotic closely approximated the behavior of the control cultures.

The results obtained with 1/10 M-8450 treated cells are considered significant. If we ascribe 100% growth to the average fold-increase for the control cultures, the antibiotic inhibited 60% of the growth in the 1/10 treated cultures. It would appear from the above results that a 1/10 dilution of the antibiotic either reduces the rate of multiplication of the HeLa cells and/or selects out the M-8450 resistant cells.

An attempt was made to determine whether a 1/10 dilution of M-8450 was selecting resistant cells. If one assumes such a selection, it then appears reasonable to assume that the resistant cells may demonstrate an altered virus susceptibility. Accordingly, normal and 1/10 M-8450-treated cells from the third passaged cultures were seeded in tubes and allowed to multiply in the presence of growth medium devoid of M-8450. These tube cultures were then tested for their virus susceptibility by subjecting them to parallel titrations of poliomyelitis virus (Type-1, Mahoney). The results indicated no significant difference in virus titer. The titer in the control cells was $10^{-5.2}$, while the titer in the M-8450 treated cells was $10^{-5.0}$. As can be seen in Table 4 the M-8450 treated cultures also regained their normal reproductive activity when replaced in the normal growth medium.

Either the progenies of M-8450 treated cultures are as fully susceptible to the growth and multiplication of virus as are the normal cells or they have reverted from insusceptible to susceptible in a single generation, a premise which seems highly unlikely.

It can be concluded then, that a 1/10 dilution of M-8450 does not bring about selection of resistant cells but does reduce the rate of

multiplication of HeLa cells by virtue of its toxicity for these cells.

Comparative Inhibitory Studies in HeLa Cultures

Upon completion of the toxic endpoint determinations, the compounds were used in attempts to demonstrate differential virus inhibitory activity.

Differential Inhibition of Poliomyelitis and Coxsackie Viruses

Twenty-nine compounds were screened for their ability to inhibit the cytopathology caused by the viruses of poliomyelitis and Coxsackie. In this pilot study, the maximum non-toxic concentration of the compounds was added to three pairs of replicate tube cultures simultaneously with virus inocula containing 10, 50, and 100 tissue culture doses.

As seen in Table 5 twenty-six of the compounds tested inhibited the cytopathology attributable to poliomyelitis or Coxsackie virus infection. Six of these inhibitors showed a differential activity in that they inhibited only one of the viruses. An isocytosine, 2-amino-4-hydroxy-5,6-dimethylpyrimidine, and two compounds supplied by Abbott Laboratories, dimethyl glycine and 2-thio-4,6-diaminopyrimidine, inhibited the virus of poliomyelitis only. Hydroxylamine hydrochloride and 2-amino-4-chloro-5-methyl-6-phenylpyrimidine appeared to selectively inhibit Coxsackie but not poliomyelitis virus. The antibiotic M-8450, though it inhibited both viruses, showed differential activity in that it completely inhibited the cytopathology caused by poliomyelitis but merely delayed that caused by the Coxsackie virus.

Methionine sulfoximine and the benzimidazoles seemed to be the most active of the remaining compounds.

Just how much inhibition resulted with any of these compounds could not be ascertained at this time since a delay in the time of cytopathology was the only index of inhibition used.

Effect of Benzimidazole and Cysteic Acid on the Growth Curves of Poliomyelitis and Coxsackie Viruses

In order to determine the extent and duration of the inhibition produced by the aforementioned group of compounds, the effect of two representative compounds upon the growth curves of Type-1 poliomyelitis and Coxsackie viruses was studied. Benzimidazole and cysteic acid were selected primarily because of their effectiveness reported herein and by others (Thompson, 1946 and 1947; Brown, 1952).

In the first experiment, three parallel bottle cultures of seven-day old HeLa cells were treated with normal maintenance containing 0.1 mg/ml of benzimidazole, and maintenance containing 1.0 mg/ml of cysteic acid. One hundred tissue culture doses of poliomyelitis virus per ml of culture fluid were immediately added to each bottle. The bottles were incubated at 37°C for two hours. The cells were then washed five times (to remove virus) with normal maintenance medium and the appropriate maintenance solutions were reintroduced into the bottles. Samples of the last wash were saved for titration of virus content. The bottles were further incubated at 37°C and samples were taken from each bottle at two hour intervals. The volume was equated after each sampling by replenishment with the appropriate maintenance solution. The experiment

was terminated when there was maximum degeneration in the control culture. The above experiment was subsequently repeated against Coxsackie virus.

The samples taken in both experiments were titrated (n.b., Materials and Methods) and the results are presented graphically in Figures 1 and 2. It can be seen from these growth curves that the control virus cannot be detected before eight hours. There is a slight delay in the appearance of virus in the drug-treated cultures. Maximum titers are attained at forty-six hours in the case of poliomyelitis infection, and at thirty-eight hours with Coxsackie virus. The drugs retard the appearance of infective virus during the entire growth cycle and the final titers in the drug-treated cultures differ from the control by as much as one to two logs.

Selected Inhibitors

Since the use of compounds at or near the maximum tolerated dose does not indicate relative degrees of effectiveness, and since the time involved in growth curve studies of large numbers of compounds is prohibitive, an extension of the former technique was employed in an attempt to quantitate the relative efficacy of the compounds.

The HeLa cultures were now routinely pre-treated with the compounds for 24 hours at 37°C prior to infection with one hundred tissue culture doses of virus. Table 2 lists the minimal suppressive concentrations for the viruses of poliomyelitis, Coxsackie, and vaccinia. The inhibition resulting with each compound-virus complex was then

evaluated by the use of the therapeutic index (n.b., Materials and Methods).

Through these techniques certain compounds have emerged as having potential significance, and they are listed in Table 6. The antibiotic M-8450 completely inhibits the virus of poliomyelitis with no evidence of virus infection appearing in dilutions of the antibiotic up to and including 1/160. Coxsackie virus infection, however, is merely delayed for twenty-four to seventy-two hours with all inhibitory dilutions. The antibiotic does not seem to have a significant effect on vaccinia infection.

Two other antibiotics, Helenine and M5-11496, were selected because of their known inhibitory activity against poliomyelitis infection in monkeys and mice (Cochran and Francis, 1956, Cochran, 1956). It can be seen that Helenine was active against poliomyelitis virus only if the cultures had been pre-treated for forty-eight hours. Similar activity against Coxsackie virus was obtained with pre-treatment of either twenty-four or forty-eight hours. M5-11496 appeared to have some activity against the virus of poliomyelitis. Neither compound inhibited the cytopathology of vaccinia virus.

Methionine sulfoximine completely inhibited the cytopathology of all three viruses at the maximum tolerated dose of 0.5 mg/ml while its effect at lower concentrations was merely dilatory.

Benzaldehyde thiosemicarbazone appeared to be a more specific inhibitor of vaccinia virus than of the other two agents.

Diazouracil, another compound which was inhibitory for poliomyelitis in mice (Cochran, 1957), failed in repeated attempts to inhibit this virus in tissue culture.

In the preliminary experiments the benzimidazole derivatives seemed to be good inhibitors when employed near the maximum tolerated dose. However, calculation of the therapeutic indices revealed only small amounts of activity against these viruses. One derivative, 5, 6-dichloro-1-D-ribosylbenzimidazole, appeared to be specific in its inhibition of poliomyelitis virus.

Many of the compounds having therapeutic indices of two, four, or eight are not shown in Table 6. The author considers these low indices as probably reflecting a non-specific inhibitory phenomenon. This refinement of the data would leave but four significant inhibitors in the HeLa tissue culture system, M-8450, methionine sulfoximine, benzaldehyde thiosemicarbazone, and 5,6-dichloro-1-D-ribosylbenzimidazole.

Studies on the Activity of the Antibiotic M-8450--Since M-8450 was the only compound to completely inhibit the cytopathology of poliomyelitis over a wide range of concentrations, it was adjudged most promising, and concurrent studies were initiated to elucidate its mode of action.

Analytical Centrifugation

Since the antibiotic preparation was a crude filtrate of the mold Penicillium stoloniferum, it was of interest to determine if the active principle could be separated by ultracentrifugation.

The biophysical preparation of th filtrate fractions was performed by Dr. R. Hartman, Department of Epidemiology, University of

Michigan. Ten ml of the filtrate were subjected to a gravitational force of 150,000g in a Spinco analytical centrifuge (Model E) for ten hours. Examination of the Schlieren photographs revealed that the sedimenting component of the filtrate appeared to separate as a single boundary which was composed of two closely associated peaks. In the present experiment the filtrate was centrifuged until the material represented by the two peaks was sedimented into a single pellet. The supernatant was removed from the analytical cell and saved for virus studies. The pellet was washed with one ml of culture fluid and saved, and then reconstituted with another ml of culture fluid. A control tube containing the crude filtrate was kept at room temperature throughout the centrifugation and reconstitution. The samples were then tested for toxicity in HeLa cultures and then assayed for inhibitory activity against Type-1 poliomyelitis virus.

It can be seen from the indices given in Table 7 that the inhibitory principle in the filtrate was clearly associated with the pellet fraction. It also appeared that the control sample left at room temperature lost some activity. Since reconstitution of the pellet with one ml represented a ten-fold concentrate of the starting material, considerable activity was lost in the centrifugation and reconstituting processes. It was also of interest that the concentrate was no more toxic to the cultures than was the original filtrate.

Since centrifugation for ten hours at 150,000g was sufficient to separate the active principle present in the crude M-8450 filtrate, it can be concluded that the inhibitory principle is probably a large molecule or is at least associated with a large molecule.

Effect of Removing the Antibiotic from Treated Cultures

Hull and Lavelle (1953 and 1954) reported that the protection afforded monkey testicular cultures by M-8450 could not be removed by extensive washing of the cultures. An experiment was designed to confirm these findings in HeLa cells.

Tube cultures were pre-treated with a 1/20 dilution of M-8450 for twenty-four hours at 37°C. The cultures were then washed five times with Hanks balanced salt solution. One ml of maintenance solution was added and one hundred tissue culture doses of either poliomyelitis or Coxsackie virus were inoculated into the tubes. Control cultures were similarly pre-treated with hyperimmune antipoliomyelitis monkey serum (1/80) and antiCoxsackie mouse serum (1/40). These cultures were also washed and infected with the appropriate viruses. Normal cultures, and M-8450 treated cultures in which the antibiotic was allowed to remain throughout the experiment, were also included as controls.

The results are presented in Table 8. It can be seen that treating the cultures with a 1/20 dilution of M-8450 protected them from the cytopathology caused by poliomyelitis virus and delayed infection with the Coxsackie virus, whether the antibiotic was removed or not. Exposure to hyperimmune sera did not so protect the cultures. Since pre-treatment of the cultures was necessary and since the effect could not be removed by extensive washing, it is quite likely that the antibiotic exerted its activity by being incorporated into the cellular metabolism of the host cells, although a surface phenomenon cannot be precluded.

Effect on Adsorption and Attachment of Virus

Further attempts to characterize the activity of M-8450 and to test the hypothesis of intracellular action of this antibiotic are presented in the following experiment.

Three five-day bottle cultures of HeLa cells containing 9.0 ml of maintenance solution were incubated for twenty-four hours at 37°C. One of these bottles contained 1/10 M-8450. A fourth bottle containing normal maintenance solution (no cells) was included to determine the virus thermal inactivation factor in the experiment. Poliomyelitis (Type-1) virus having a titer of $10^{-6.0}$ was diluted to $10^{-2.0}$ and one ml of this dilution was added to each bottle giving a final dilution of $10^{-3.0}$. The bottles were returned to 37°C, and one ml samples were taken from each bottle at sixty minutes, two hours and thirty minutes, and four hours after infection. The samples were then titered and the plaque-forming ability determined in monkey kidney cultures (n.b., Materials and Methods). These results are given in Table 9.

It can be seen that there was a one-half log drop in the titer of the incubator control sample after four hours, probably due to thermal inactivation. The results obtained with the remaining samples represent virus that has not been inactivated nor adsorbed. No significant difference could be found between the virus controls and the M-8450 treated sample. In comparing the four hour titer of the incubator control with those of the virus controls and the M-8450 treated sample, it can be seen that approximately one log of virus was adsorbed both in the virus controls and in the M-8450 treated. This adsorption occurred within the first hour. Thereafter, the amount or rate of adsorption appeared

to be very low. It can be concluded then, that this lack of effect on attachment and adsorption of virus militates against a surface action by M-8450.

Effect of Medium

The possibility of finding a metabolite which could reverse the virus inhibitory property of M-8450 was considered. Since the active principle has not been defined chemically, modification of the inhibitory effect might be feasible by altering some component in the medium.

The medium employed in this study was composed of Hanks balanced salt solution, sodium bicarbonate, glucose, horse serum, and a complex of amino acids, vitamins, and nucleic acids (n.b., Materials and Methods). The amino acid-vitamin-nucleic acid components were altered by grouping the required compounds into separate stock solutions (A through E) and increasing the volumes of each individual stock solution while correspondingly decreasing the volume of the final maintenance medium. This, in essence, increased the concentration of each component of that particular stock solution.

Stock A consisted of glycine, histidine, cystine, and parenamine. Stock B consisted of glycerol, sodium pyruvate, and sodium acetate. Stock C consisted of succinic and malic acids. Stock D contained adenine sulfate, guanine, xanthine, uracil, thymine, and cytosine. Stock E consisted of monobasic potassium phosphate, thiamine, nicotinamide, calcium pantothenate, pyridoxal hydrochloride, pyridoxamine dihydrochloride, ribose, riboflavine, inositol, biotin, folic acid, and

p-amino benzoic acid.

The toxic limit produced by increasing the relative volume of each individual stock solution in the final medium was determined, and the highest non-toxic combinations were then used in attempts to reverse the inhibition by M-8450. The therapeutic index for each combination is given in Table 10. It can be seen that all but stock solution B could be increased to 50% of the final medium without the appearance of toxic effects. Increasing the relative volume of any one stock solution reversed the inhibitory effect of M-8450.

Eagle et al (1956) demonstrated that HeLa cells could be maintained in a medium consisting of Hanks balanced salt solution, glucose, and glutamine, and that the virus of poliomyelitis could be grown to expected titers in this medium. Similar experiments were performed and these findings confirmed. It was then of interest to see if the reciprocal relation was true; that is, whether the virus inhibition by M-8450 was greater in a simpler medium. It can be seen in Table 11 that the therapeutic index of M-8450 in Eagle's medium was approximately five times higher than that found when Syverton's maintenance was employed.

Attempts to Recover Antigen from Treated Cultures

It appeared pertinent to determine whether virus could be recovered from the M-8450-treated poliomyelitis infected cultures where no evidence of infection was observed, and whether virus was produced by the M-8450-treated, Coxsackie-infected cultures which did exhibit a delayed cytopathology.

Cultures were treated overnight at 37°C with a 1/10 dilution of M-8450. One group was infected with one hundred tissue culture doses of Type-2 poliomyelitis virus (MEF), and another group with one hundred tissue culture doses of Coxsackie virus. Twenty-four hours after infection, the fluids were removed from the cultures and frozen. Fresh drug-free maintenance medium was added to all the cultures. This process was repeated at twenty-four hour intervals until cytopathology was complete or until the end of the experiment (6th day). Virus recovery was attempted by inoculating 0.5 ml of the samples into replicate HeLa cultures.

It can be seen from Table 12 that when the M-8450-treated cultures were challenged with poliomyelitis virus there was no evidence of cytopathology nor could virus be isolated from the fluid medium. However, when the M-8450-treated cultures were challenged with Coxsackie virus, the virus was detectable in the medium as early as twenty-four hours after infection and cytopathology was complete at seventy-two hours.

Since an altered (non-cytopathogenic) virus might not be detected with the isolation method employed, attempts to detect incomplete or inactive virus were made. Pools were made from the M-8450-treated poliomyelitis-infected, poliomyelitis virus, and M-8450 control culture fluids. These pools were inoculated into mice by introducing three weekly inoculations (0.5 ml) of each pool intraperitoneally. The mice were bled on the fourth week, and the sera obtained were run in a tissue culture neutralization test at undiluted, 1/4, 1/8, and 1/16 increments.

The results of the neutralization test are given in Table 13. Fluids from the virus control cultures produced antibody detectable at a dilution of 1/16 when inoculated into mice, whereas the fluids from the M-8450-treated poliomyelitis infected cultures did not induce the formation of detectable antibody. These results indicate that either no detectable antibody-producing virus is produced by the virus-infected drug-treated cultures, or that if a form of incomplete virus is produced, it does not have the ability to stimulate the formation of antibody that would neutralize the original virus. It is possible that the fluids contain incomplete virus which does produce antibody, but that this antibody is so altered that it will not neutralize the complete form of virus.

In an attempt to further investigate whether incomplete virus was produced, the fluids from the M-8450-treated poliomyelitis-infected cultures were used as antigen in a complement fixation test. The complement fixing ability of the mouse sera, obtained by inoculating these fluids into mice, were also determined. The results of the complement fixation tests were also negative. No antigen could be detected in the culture fluids, nor could complement fixing antibody be detected in the mouse antisera.

Differential Inhibition in Monkey Kidney Epithelial Cultures

Effectiveness of HeLa System Virus Inhibitors

Since it was necessary to determine whether certain compounds which had shown virus inhibitory activity in the HeLa culture system would act independently of a specific host tissue, they were tested against the same viruses in cultures of monkey kidney epithelial tissue. The study

was also expanded by including fourteen other viruses, the Enteric Cytopathogenic Human Orphan (ECHO) viruses (n.b., Materials and Methods).

The maximum non-toxic and the minimal inhibitory concentrations for the four significant HeLa system virus inhibitors are given in Table 14. The pertinent therapeutic indices for these compounds can be found in Table 15.

It can be seen from the indices in Table 15 that the antibiotic M-8450 inhibited the viruses of poliomyelitis and Coxsackie. The antibiotic did not completely protect the cultures against either virus, but merely delayed the cytopathology for twenty-four hours. Some low level activity against most of the ECHO viruses was also obtained (n.b., Table 14).

Methionine sulfoximine, a potent inhibitor of poliomyelitis, Coxsackie, and vaccinia viruses in the HeLa system, did not inhibit any of the viruses when assayed in monkey kidney cultures.

Benzaldehyde thiosemicarbazone appeared to be specific in its inhibition of vaccinia virus. Another inhibitor in the HeLa system, 5,6-dichloro-1-D-ribosylbenzimidazole, failed to inhibit any of the viruses tested.

Effectiveness of other Experimental Compounds

Several benzimidazole derivatives, and a number of water soluble plant extracts which have been shown to have anti-tumor activity (Lucas et al, 1957) were similarly processed in monkey kidney cultures[1].

Differential inhibitory activity has been demonstrated against some of the ECHO viruses. Thus, the mushroom extracts M-2 from Calvatia

[1] The plant extracts were obtained from Dr. E.H. Lucas, Mich. State Univ.

maxima, M-4 from Boletus edulis, and a species of Cattleya orchid, M-14, inhibited some of the ECHO viruses. It can be seen from the indices in Table 15 that the M-2 fraction appeared to induce significant inhibition of ECHO-4,9, and 11 viruses. The M-4 fraction was slightly active against ECHO-7 and 8 viruses. The M-14 extract appeared to be specific in its action against ECHO-2 virus. It can be seen from Table 16 that these and other plant extracts did not appear to inhibit significantly any other ECHO virus, nor did they demonstrate activity against the viruses of poliomyelitis, Coxsackie, or vaccinia.

Studies on the Activity of the Plant Extract M-2

Extent of ECHO-4 and 11 Inhibition - The previous studies showed that the M-2 extract was outstanding in its ability to inhibit ECHO-4 and 11 viruses. Therefore, an experiment was designed to determine 1) how much virus could be inhibited, 2) the necessity of pre-treatment and, 3) whether infection could precede drug treatment and still be influenced by this extract.

Cultures were pre-treated with two-fold dilutions of M-2 for twenty-four hours at 37°C, following which 0.1 ml of various dilutions of virus were added. It can be seen from Table 17 that the M-2 fraction inhibited as much as 10,000 tissue culture doses of ECHO-4 and 3,200 tissue culture doses of ECHO-11. Dilutions of 1/120 and 1/240 of the M-2 fraction completely protected the cultures from the cytopathology caused by 100 tissue culture doses of either virus, or from that caused by 1000 tissue culture doses of ECHO-4 virus.

To two other groups of cultures, 1/120, 1/240, 1/480, and 1/960 dilutions of M-2 were added at the time of, and one hour following, infection with ECHO-4 virus. As can be seen from Table 18 it was possible to infect the cultures at the time of drug addition or one hour prior to the addition of drug and still demonstrate complete inhibitory activity with the more concentrated dilutions of the drug.

The desirability of a twenty-four hour pre-treatment rather than the later addition of M-2 was seen when the therapeutic indices for M-2 were compared (Table 19). The M-2 fraction decreased in effectiveness when 1) a virus inoculum larger than 1000 tissue culture doses was introduced or 2) when the cultures were drug-treated at the time of virus introduction or one hour after infection.

IN VITRO Effect Upon ECHO-4 and 11 Viruses

In considering the influence of M-2 on the course of virus infection, its action in vitro upon these viruses was studied.

Three one ml portions of ECHO-4 infected tissue culture fluid were placed into separate test tubes. To one of these, one ml of M-2 (1/20 final dilution) was added. One ml of normal maintenance solution was added to the other two tubes. One of the latter was stored at 4°C for twenty-four hours while the remaining two tubes incubated at 37°C for the same time. All three samples were then titrated for virus content (n.b., Materials and Methods). The titers of the control tubes incubated at 4°C and 37°C and of the M-2 treated sample incubated at 37°C were found to be $10^{-5.0}$, $10^{-5.0}$, and $10^{-4.5}$ respectively. The same procedure was followed for ECHO-11 virus, and

the titers obtained were $10^{-4.0}$, $10^{-3.5}$, and $10^{-3.5}$ respectively.

It is clear that the maximum concentration of M-2 which was used in prior experiments has no pronounced in vitro effect upon the stability of the viruses. Hence, the effect of M-2 directly upon the virus is not a consideration in interpreting its influence on virus mulitplication.

Concurrent Inhibition and Growth of Viruses

Since the M-2 fraction has been shown to inhibit the cytopathology of considerable amounts of ECHO-4 and 11 viruses, and since no other virus tested is so inhibited (with the possible exception of ECHO-9), evidence of virus inhibition in the absence of any cell damage which would interfere with the multiplication of other viruses within the same tissue should now be demonstrable.

Cultures were pre-treated with 1/120, 1/240, 1/480, and 1/960 dilutions of M-2 for twenty-four hours at 37°C. The cultures were then divided into five groups. Three of the groups were exposed to 100 tissue culture doses of ECHO-4 virus and two groups were exposed to 100 tissue culture doses of ECHO-11 virus. Forty-eight hours later and twenty-four hours prior to complete cytopathology of the virus controls, the fluids were removed, the cultures washed once, and fresh drug was added to all the cultures. At this time, large doses of either ECHO-4 (10,000 tissue culture doses), ECHO-11 (3200 tissue culture doses), or ECHO-1 (100,000 tissue culture doses)virus were added to one of the three groups of cultures previously exposed to ECHO-4 or 11 virus. Table 20 presents the experiment in outline form and the results obtained. It can be seen from the

results obtained with the drug control cultures in group 6 that all the dilutions of M-2 tested protected the cultures against the cytopathology of 100 tissue culture doses of either ECHO-4 or 11 viruses for 72 hours after the initial virus exposure. There was also complete protection with the lower concentrations of M-2 against a repeated exposure even when larger doses of ECHO-4 or 11 viruses were employed. However, no protection was afforded against re-exposure to a large dose of ECHO-1 virus, which had been shown in previous experiments not to be inhibited by the M-2 fraction.

When smaller quantities of M-2 (1/480 and 1/960) were employed, cytopathology was prevented on the first exposure to virus, but not when larger doses of virus were added on re-exposure. In all these instances the virus which finally broke through the inhibitory mechanisms was recovered and typed, and always corresponded to the second virus.

An additional experiment was designed to complement the above findings. A 1/200 dilution of M-2 was used to protect the cultures. The cultures were exposed to 100 tissue culture doses of ECHO-11 virus, and forty-eight hours later were re-exposed to 0.1 ml of a 10^{-1} dilution of ECHO-1,2,3,4,5,6,7, and 11 viruses, as well as poliomyelitis, Coxsackie, and vaccinia viruses. These viruses represented challenge doses of 100 to 100,000 tissue culture doses, depending upon the original titer of the virus pools. In this experiment the fluids were not changed prior to the second virus exposure.

As can be seen in Table 21, essentially identical results were obtained. Cytopathology was prevented when the cultures were

re-exposed to ECHO-4 or 11 virus, but was complete when the cultures were re-exposed to a virus not affected by the M-2 fraction.

These experiments strongly suggest that the influence of M-2 upon cellular reactions is associated with the replication of ECHO-4 and 11 viruses specifically, and does not alter the metabolic processes required for the replication of other viruses not affected by the M-2 fraction.

Effect on the Antibody Response of Mice Inoculated with ECHO-4 and 11 Viruses

Since the ECHO viruses do not produce known characteristic disease signs in laboratory animals (Melnick, 1955a), attempts to demonstrate the efficacy of the M-2 fraction in vivo might be possible if a measurable antibody response to ECHO virus inoculation could be suppressed.

In experiments not described herein, it was demonstrated that three intraperitoneal inoculations of ECHO virus were sufficient to produce detectable antibody levels in the sera of white mice. Accordingly, three weekly inoculations (0.5 ml) of undiluted monkey kidney tissue culture antigen were given to two groups of mice. One group received ECHO-4 virus and the other group received ECHO-11 virus. A 1/4 dilution of M-2, given at 0.1 ml/gm of body weight, was inoculated intraperitoneally on the day before antigen was given, at the same time as antigen inoculation, and the day following antigen inoculation. This triad of drug inoculations was repeated each time that virus was to be given, i.e., a total of three drug inoculations per week for three weeks. A group of drug-control mice received the full complement of drug inoculations without receiving antigen.

At the end of the fourth week all the mice were bled, and the sera diluted in two-fold increments (1/4 through 1/128) and assayed for antibody content in a monkey kidney tissue culture neutralization test.

It can be seen from Table 22 that the M-2 fraction does inhibit the formation of antibody in mice inoculated with ECHO-4 or 11 viruses.

Inhibition of Poliomyelitis and Coxsackie Viruses by M-8450 In Monkey Testicular Cultures

Inhibition of poliomyelitis virus by M-8450 in plasma-clot roller-tube cultures of monkey testicles has been reported by Hull and Lavelle (1953 and 1954). In view of the inhibitory patterns established by this antibiotic in HeLa and monkey kidney cultures, it was of interest to determine whether the antibiotic would inhibit both poliomyelitis and Coxsackie viruses in cultures of monkey testicle grown without a supporting plasma-clot (n.b., Materials and Methods).

The cultures were pre-treated for twenty-four hours at 37°C with a 1/10 and 1/20 dilution of M-8450. Virus inocula containing 1770, 177, and 17 tissue culture doses of poliomyelitis and 1000, 100, and 10 tissue culture doses of Coxsackie virus were added to the cultures, and the cultures were observed for characteristic viral pathology.

Pre-treatment of the cultures with 1/10 M-8450 afforded complete protection to the cultures against the maximum quantities of both viruses employed. Complete protection was also obtained against the same quantities of poliomyelitis virus when a 1/20 dilution of the antibiotic was employed.

DISCUSSION

Evidence compiled in this investigation indicated that many compounds were capable of inhibiting virus in tissue culture at or near the maximum tolerated dose. When the effectiveness of the compounds was judged by the therapeutic index, i.e., according to the ratio of the maximum tolerated dosage of the compound to the minimal virus suppressive dose, most of these inhibitors had a therapeutic index of two, four, or eight. In view of the high degree of specificity required of a compound in order that it be considered a specific virus inhibitor, it seemed logical to assume that these low indices represented inhibition of virus by virtue of toxicity which was directed against the host cells but which was not detectable morphologically. Exclusion of the compounds demonstrating therapeutic indices of two, four, or eight reduced the number of virus inhibitors to those compounds having therapeutic indices greater than eight.

When the HeLa culture system was employed in the virus studies, only four compounds out of ninety-five assayed had an index greater than eight. Methionine sulfoximine appeared to inhibit all three viruses employed in these cultures (poliomyelitis, Coxsackie, and vaccinia). The antibiotic M-8450 inhibited the viruses of poliomyelitis and Coxsackie. Benzaldehyde thiosemicarbazone and 5,6-dichloro-1-ribosylbenzimidazole appeared to be differential in their action and inhibited vaccinia and poliomyelitis viruses respectively.

When monkey kidney cultures were employed to evaluate these four inhibitors, it was found that only M-8450 and benzaldehyde thiosemicarbazone retained their specific inhibitory patterns. Methionine sulfoximine and 5,6-dichloro-1-D-ribosylbenzimidazole did not inhibit any of the viruses employed (ECHO viruses included). The inability of compounds to act independently of a specific host tissue has also been recently reported by Knox, Robbins, and Smith (1957).

The only substance tested in monkey testicular cultures, M-8450, again inhibited the poliomyelitis and Coxsackie viruses. Considerable variation was observed in the degree of inhibition when assays were attempted in the various culture systems. Thus, M-8450 completely inhibited both poliomyelitis and Coxsackie viruses in monkey testicular cultures. In the HeLa system, the antibiotic completely inhibited poliomyelitis virus but merely delayed the Coxsackie virus infection for forty-eight to seventy-two hours. When monkey kidney cultures were employed the cytopathology of both viruses was delayed for twenty-four hours, and neither virus could be completely inhibited.

Although there was variation in the degree of inhibition, the significant point, however, is that two substances, M-8450 and benzaldehyde thiosemicarbazone, specifically inhibited virus in the culture systems employed. The specific action of M-8450 against poliomyelitis and Coxsackie viruses supports the <u>in vivo</u> experiments reported by Powell and Culbertson (1953) and by Cochran, Brown, and Francis (1954). The specificity of benzaldehyde thiosemicarbazone for vaccinia virus replication is in agreement with the reports of the effectiveness of the compound in another tissue

culture system, in chick embryos, and in mice (Hamre, Bernstein, and Donovick, 1950; Hamre, Brownlee, and Donovick, 1951; Thompson, Price, and Minton, 1951).

That methionine sulfoximine, a potent inhibitor of all three viruses in the HeLa system, is tissue specific rather than virus specific is demonstrated by the unlimited growth of these same viruses in the presence of the compound in monkey kidney cultures. It is possible that methionine sulfoximine has a toxic affinity for the HeLa cell (the concept of differential tissue toxicity affinity has been experimentally demonstrated by Hsu, Robins, and Cheng in 1956). Another indication that the HeLa culture is more sensitive to the action of methionine sulfoximine is the observation that the compound is at least five times more toxic to the HeLa cultures than it is to the monkey kidney cultures. The inability of methionine sulfoximine to ultimately alter the infection of mice with poliomyelitis virus (Ainslie, 1952) can be cited as additional evidence of the non-specific behavior of methionine sulfoximine as an inhibitor of poliomyelitis virus.

Another demonstration of differential inhibition of viruses was seen in monkey kidney culture assays with the ECHO agents. The extracts from the mushrooms Calvatia maxima (M-2) and Boletus edulis (M-4), and from a species of Cattleya orchid (M-14) were found to have therapeutic indices greater than eight against some but not all of the ECHO viruses. The specificity of the ECHO virus inhibition with these extracts, though not confirmed in other tissue cultures systems nor by animal studies, appeared to be significant in that many other virus entities employed were not so

inhibited. It would be desirable to assay these extracts in at least one other tissue culture system.

The specific nature of one of these plant extracts, M-2, has been conclusively demonstrated however, by the concurrent inhibition and growth, respectively, of two distinct viruses in the same monkey kidney culture. The M-2 extract has been shown to inhibit either ECHO-4 or 11 virus while allowing the completion of an entire infection cycle produced by another one of several ECHO viruses (as well as poliomyelitis Coxsackie, and vaccinia) within the same tissue. An in vivo experiment has shown that the M-2 fraction also inhibits the formation of antibodies in mice inoculated with ECHO-4 or 11 viruses. These experiments strongly suggest that the influence of M-2 upon the cellular reactions is associated with the replication of ECHO-4 and 11 viruses, and does not alter the metabolic processes required for the replication of the other viruses employed within the same tissue. One can deduce, with less certainty however, that ECHO-4 and 11 viruses utilize similar pathways in their replicating processes since both viruses are individually inhibited by the M-2 fraction and since both viruses can be concurrently inhibited within the same culture.

SUMMARY AND CONCLUSIONS

One hundred and twenty compounds were assayed either in HeLa, monkey kidney, or monkey testicular cultures for their inhibitory capacities against poliomyelitis, Coxsackie, vaccinia, and fourteen ECHO viruses.

In comparing the virus inhibitory abilities of these compounds in the three tissue culture systems, it has been possible to demonstrate the differential and specific virus inhibitory nature of several of the compounds. The demonstration of such specific action appears to rule out the influence of drug-incited host injury as an explanation for the observed inhibition.

The results of the present investigation indicate the following with respect to the compounds employed:

1. The antibiotic M-8450 is a specific inhibitor of poliomyelitis and Coxsackie virus infection. The antibiotic will significantly inhibit both viruses when assayed in HeLa, monkey kidney, or monkey testicular cultures.

2. Benzaldehyde thiosemicarbazone is a specific inhibitor of vaccinia virus in HeLa and monkey kidney cultures.

3. Methionine sulfoximine, a potent inhibitor of poliomyelitis, Coxsackie, and vaccinia in HeLa cells, failed to restrict the growth of these (and also fourteen ECHO viruses) viruses when assayed in the monkey kidney culture system.

4. Although considered a significant inhibitor of poliomyelitis virus in the HeLa cultures, 5,6-dichloro-1-D-ribosylbenzimidazole also

failed to inhibit this virus in the monkey kidney cultures.

5. Assays of plant extracts against ECHO virus infection in monkey kidney cultures demonstrated the differential specificity of these substances; the fraction M-2 from Calvatia maxima inhibited ECHO-4, and 11 viruses; the fraction M-4 from Boletus edulis inhibited ECHO-7 and 8 viruses; and the fraction M-14 from Cattleya inhibited ECHO-2 virus.

6. The specificity of ECHO-4 and 11 virus inhibition by the M-2 fraction from Calvatia maxima was conclusively demonstrated by the concurrent inhibition and growth, respectively, of two distinct viruses within the same tissue -- growth was accomplished by a virus which was not affected by the M-2 fraction.

7. The in vivo activity of the M-2 fraction was demonstrated by the inhibition of antibody production in mice inoculated with ECHO-4 and 11 viruses.

TABLE 1

HYPOTHETICAL RESULTS OF CYTOPATHOLOGY OF VIRUS CONTROL AND DRUG-TREATED CULTURES

CULTURES	CYTOPATHOLOGY** (Time in Days) 1	2	3	4	5	6	7
100 Tissue Culture Doses of Virus	-	-	+				
	-	-	+				
100 Tissue Culture Doses of Virus plus 1/10 Drug	-	-	-	-	-	-	-
	-	-	-	-	-	-	-
100 Tissue Culture Doses of Virus plus 1/20 Drug	-	-	-	-	-	-	-
	-	-	-	-	-	-	-
100 Tissue Culture Doses of Virus plus 1/40 Drug	-	-	-	-	-	-	-
	-	-	-	-	-	-	-
100 Tissue Culture Doses of Virus plus 1/80 Drug	-	-	-	-	-	-	-
	-	-	-	-	-	-	-
100 Tissue Culture Doses of Virus plus 1/160 Drug	-	-	-	-	-	-	-
	-	-	-	-	-	-	-
100 Tissue Culture Doses of Virus plus 1/320 Drug	-	-	-p*	-p	+		
	-	-	-p	+			
100 Tissue Culture Doses of Virus plus 1/640 Drug	-	-	+				
	-	-	+				
100 Tissue Culture Doses of Virus plus 1/1280	-	-	+				
	-	-	+				

** The designation (+) is given when all the cells are off the glass; a (-) designation indicates protection of the cultures.

* The designation (-p) indicates an occasional virus plaque.

APPENDIX

TABLE 2

COMPOUNDS ASSAYED FOR INHIBITION OF ONE HUNDRED TISSUE CULTURE DOSES OF POLIOMYELITIS, COXSACKIE, AND VACCINIA VIRUSES IN HELA CULTURES

COMPOUNDS	HIGHEST NON-TOXIC CONC. (mg/ml)*	LOWEST INHIBITORY CONCENTRATION**		
		Polio.	Cox.	Vacc.
Abbott #1713, β-methyl-N-dimethyl indole	0.1	> 0.1	> 0.1	
Abbott #3665, p-amino-acetophenone	0.1	0.05	0.05	
Abbott #4462***	0.1	0.05	0.05	
Abbott #4271, dimethyl glycine	2.5	1.0	> 2.5	
Abbott #6238, 2-thio-4, 6-diaminopyrimidine	0.1	0.05	> 0.1	
Amycetin	0.025	> 0.025	> 0.025	> 0.025
Anisomycin	0.006	> 0.006	> 0.006	> 0.006
8-Azaguanine	0.1	> 0.1	> 0.1	> 0.1
8-Azauracil	0.625	> 0.625	> 0.625	> 0.625
1-β-D-Glucopyranosyl-2-ethyl-5-methylbenzimidazole	>2.5	> 2.5	> 2.5	
5-Chlorobenzimidazole HCl	0.1	0.012	0.1	0.025
1-β-D-Glucopyranosyl-5-methylbenzimidazole	> 2.5	> 2.5	> 2.5	> 2.5

* Highest concentration tested was 2.5mg ml
** All cultures pre-treated 24 hours at 37 C
*** Identity of the compound restricted

TABLE 2--Continued

COMPOUNDS	HIGHEST NON-TOXIC CONC. (mg/ml)	LOWEST INHIBITORY CONCENTRATION		
		Polio	Cox.	Vacc.
5-Methyl-2-isopropyl benzimidazole HCl	0.1	> 0.1	> 0.1	> 0.1
5,6-Dichlorobenzimidazole	0.05	> 0.05	> 0.05	> 0.05
5,6-Dichloro-1-D-ribosyl benzimidazole	0.1	0.0015	0.05	0.05
1-β-D-Ribopyranosyl-5-6-dimethyl benzimidazole	1.0	0.25	0.5	0.5
1,2-Deoxy-D-ribopyranosyl-5,6-dimethyl benzimidazole	1.0	0.25	1.0	> 1.0
1-α-D-Arabopyranosyl-5,6-dimethyl benzimidazole	> 2.5	1.0	1.0	
Benzimidazole(Eastman Kodak)	0.1	0.01	> 0.1	
Benzimidazole (British Drug Houses, Ltr, London)	0.5	0.01	0.1	
5,6-Dimethyl benzimidazole	0.1	> 0.1	> 0.1	> 0.1
2-Ethyl-5-methylbenzimidazole	0.1	> 0.1	> 0.1	> 0.1
2,5-Dimethyl benzimidazole	0.1	> 0.1	> 0.1	> 0.1
2-Benzimidazolethiol	0.1	0.05	0.05	
5-Amino-2-benzimidazolethiol	0.1	0.05	0.05	
2-Aminobenzimidazole	0.1	> 0.1	> 0.1	> 0.1
6-Methoxy-4-nitrobenzimidazole	0.031	>0.031	> 0.031	> 0.031
4-Methoxy-6-nitrobenzimidazole	0.024	> 0.024	> 0.024	> 0.024
Benzaldehyde thiosemicarbazone	0.0057	0.0014	0.0028	0.00023
Crotoxin	1.0	> 1.0	> 1.0	> 1.0
Cysteic Acid	1.0	0.1	0.1	
2,4-Dinitrophenol	0.05	> 0.05	> 0.05	> 0.05

TABLE 2--Continued

COMPOUNDS	HIGHEST NON-TOXIC CONC. (mg/ml)	LOWEST INHIBITORY CONCENTRATION		
		Polio.	Cox.	Vacc.
2,2-Diphenyl-3-hydroxy-propyl dicarbamate	0.1	0.05	0.05	
2-n-Butyl-2-ethyl-1-3-propanediol dicarbamate	0.01	> 0.01	> 0.01	> 0.01
2,2-Diethyl-1-3-propanediol dicarbamate	0.5	0.5	0.5	
2,6-Diaminopurine sulfate	0.5	0.1	0.1	
Ethionine	0.5	> 0.5	> 0.5	
Erythromycin	0.1	> 0.1	> 0.1	
Florida(B17-FSC)	0.625	> 0.625	> 0.625	> 0.625
Florida(A14-FSC)	0.625	> 0.078	0.078	0.156
Gallic acid	0.25	0.12	0.12	0.12
Helenine	10.0*	10.0*	80.0*	10.0*
Helenine**	10.0*	80.0*	80.0*	10.0*
Hydroxylamine hydrochloride	0.1	> 0.1	0.01	
Hydrazine dihydrochloride	0.1	0.01	0.01	
Isopropionine	1.0	0.5	> 1.0	
Malononitrile	0.156	> 0.156	> 0.156	> 0.156
Mephenesin	0.1	0.01	0.01	
Methionine sulfoximine	0.5	0.0017	0.0075	0.0025
Meprobamate	0.5	> 0.5	> 0.5	> 0.5
M-8450	10.0*	495.0*	422.0*	42.0*

*Reciprocal of the dilution
**48-hour pre-incubation

TABLE 2--Continued

COMPOUNDS	HIGHEST NON-TOXIC CONC. (mg/ml)	LOWEST INHIBITORY CONCENTRATION		
		Polio	Cox.	Vacc.
M5-11496	0.125	0.015	0.06	0.06
Neomycin	1.0	> 1.0	> 1.0	>1.0
Nepara #1245, quinolinic acid in water	> 2.5	> 2.5	> 2.5	> 2.5
Nepara #1618, commercial amine fraction	0.5	> 0.5	0.25	> 0.5
Nepara #1716, butyl isonicotinate	1.0	> 1.0	> d.0	0.5
Nepara #1766, N-(n-dodecyl-N-methyl-N'-N'-dimethyl)-ethylene diamine-monoundecyl-enate	0.05	> 0.05	> 0.05	> 0.05
Nepara #1779, 2-nitro-4-pyridinecarboxylic acid	> 2.5	> 2.5	> 2.5	> 2.5
Nepara #1928, hydroquinone glucose	> 2.5	> 2.5	> 2.5	> 2.5
Nepara #1931, diazouracil	0.1	0.1	0.05	0.1
Nepara #2336, bioflavonoid complex (from lemon)	1.0	> 1.0	> 1.0	> 1.0
Nepara #2416, Naringin	0.5	> 0.5	0.1	> 0.5
p-Aminophenol	0.01	>0.01	> 0.01	
propylene glycol	1%	1%	1%	1%
5-Hydroxy-1-methyl tetrazolopyrimidine	> 2.5	> 2.5	> 2.5	
Puromycin	0.0002	> 0.0002	> 0.0002	> 0.0002
Pyridacil derivative	0.039	> 0.039	> 0.039	> 0.039
2-Amino-4-hydroxy-5-methyl 6-phenyl pyrimidine	0.05	> 0.05	> 0.05	> 0.05

TABLE 2--Continued

COMPOUNDS	HIGHEST NON-TOXIC CONC. (mg/ml)	LOWEST INHIBITORY CONCENTRATION		
		Polio	Cox.	Vacc.
2-Amino-4-hydroxy-5-propyl-6-methyl pyrimidine	0.5	> 0.5	> 0.5	> 0.5
2-Amino-4-hydroxy-5-(α-methyl isobutane)-6-methyl pyrimidine	0.01	> 0.01	> 0.01	> 0.01
2,4-Amino-5-phenyl-6-methyl pyrimidine	0.1	0.1	0.1	> 0.1
2-Amino-4-methoxy-5-methyl-6-phenyl pyrimidine	0.05	> 0.05	> 0.05	> 0.05
2-Amino-4-chloro-5-methyl-6-phenyl pyrimidine	0.01	> 0.01	0.01	> 0.01
2, 4-Amino-5-methyl-6-phenyl pyrimidine	0.1	> 0.1	> 0.1	
2-Thio-4-hydroxy-5-methyl-6-phenyl pyrimidine	0.1	> 0.1	> 0.1	> 0.1
2,4-Hydroxy-5-methyl-6-phenyl pyrimidine	0.5	> 0.5	> 0.5	> 0.5
2-Amino-4-hydroxy-5-propyl-6-phenyl pyrimidine	0.05	> 0.05	> 0.05	> 0.05
2-Amino-4-hydroxy-5-n-butane-6-phenyl pyrimidine	0.05	> 0.05	> 0.05	> 0.05
2-Hydroxy-4-amino-5-methyl-6-phenyl pyrimidine	0.5	0.1	0.1	
2-Amino-4-hydroxy-5,6-dimethyl pyrimidine	0.5	0.1	> 0.5	
2-Methyl-4-hydroxy-5-methyl-6-phenyl pyrimidine	0.1	0.1	0.1	
2-Amino-4-hydroxy-6-furan pyrimidine	0.1	> 0.1	> 0.1	> 0.1
2-Amino-4-hydroxy-5-methyl-6-thiophene pyrimidine	0.05	> 0.05	> 0.05	> 0.05

TABLE 2--Continued

COMPOUNDS	HIGHEST NON-TOXIC CONC. (mg/ml)	LOWEST INHIBITORY CONCENTRATION		
		Polio.	Cox.	Vacc.
2-Amino-4-hydroxy-5-methyl-6-furan pyrimidine	0.1	> 0.1	> 0.1	> 0.1
2-Amino-4-hydroxy-5-methyl-6-pyrrole pyrimidine	0.5	> 0.5	> 0.5	> 0.5
2-Amino-4-hydroxy-5-methyl-6-pyridine pyrimidine	0.5	> 0.5	> 0.5	> 0.5
Semicarbazide hydrochloride	2.5	> 2.5	> 2.5	
Thiosemicarbazide	1.0	> 1.0	> 1.0	0.5
α-Amino-phenyl-ethane-sulfonic acid	0.1	> 0.1	> 0.1	> 0.1
α-Amino-phenyl-methane sulfonic acid	0.5	0.1	> 0.5	
Thiouracil	1.0	0.5	0.5	
6-n-propyl-2-thioracil	0.5	0.1	> 0.5	
Thorozine	0.0005	> 0.0005	> 0.0005	> 0.0005
Tolseran	0.1	> 0.1	> 0.1	> 0.1
6-Uracil-n-methyl sulfone	0.0097	> 0.0097	> 0.0097	> 0.0097

TABLE 3

COMPARATIVE TOXICITY OF COMPOUNDS
IN TISSUE CULTURE

COMPOUNDS*	HIGHEST NON-TOXIC CONC. (MG/Ml) HELA CULTURES	MONKEY TESTICLE CULTURES **	MONKEY KIDNEY CULTURES
Abbott #1713, β-Methyl-N-dimethyl indole	0.1	0.5	
Abbott #4462***	0.1	> 2.5	
Abbott #3665, β-amino acetophenone	0.1		0.1
Abbott #4271, dimethyl glycine	> 2.5		> 2.5
Abbott #6238, 2-thio-4,6-diamino pyrymidine	0.1		0.1
8-azaguanine	0.1	0.5	
1-β-D-Glucopyranosyl-2-ethyl-5-methyl benzimidazole	> 2.5		> 2.5
5-Chlorobenzimidazole HCl	0.1		> 0.1
1-β-D-Glucopyranosyl-5-methyl benzimidazole	> 2.5		> 2.5
5-Methyl-2-isopropyl benzimidazole HCl	0.1		> 0.1
5,6-Dichlorobenzimidazole	0.1		0.01
5,6-Dichloro-1-D-ribosyl benzimidazole	0.1		0.1
1-β-D-ribopyranosyl-5,6-dimethyl benzimidazole	1.0		1.0
1,2-Deoxy-D-ribopyranosyl-5,6-dimethyl benzimidazole	1.0		1.0
1-α-D-arabopyranosyl-5,6-dimethyl berzimidazole	> 2.5		1.0

*Underlined compounds indicate those for which different toxic levels have been found.
**Plasma-clot roller tubes.
***Identity of compound restricted.

TABLE 3--Continued

COMPOUNDS	HIGHEST NON-TOXIC CON. (MG/ML) HELA CULTURES	MONKEY TESTICLE CULTURES	MONKEY KIDNEY CULTURES
Benzimidazole (Eastman Kodak)	0.1		0.1
Benzimidazole (British Drug Houses, Ltr, London)	0.5		
5,6-Dimethyl benzimidazole	0.1	0.1	0.1
2-Ethyl-5-methyl benzimidazole	0.1	0.1	
2,5-Dimethyl benzimidazole	0.1	0.1	
2-Benzimidazolethiol	0.1		0.1
5-Amino-2-benzimidazolethiol	0.1		0.1
2-Amino benzimidazole	0.1	0.1	
4-Methoxy-6-nitrobenzimidazole	0.024		0.039
6-Methoxy-4-nitrobenzimidazole	0.031		0.031
Benzaldehyde thiosemicarbazone	0.005		0.039
Cysteic acid	1.0		> 2.5
Helenine	10*		10*
Hydroxylamine HCl	0.1	0.05	> 0.1
Hydrazine dihydrochloride	0.1	0.1	> 0.1
Mephenesin	0.1		0.1
Methionine sulfoximine	0.5		> 2.5
Meprobamate	0.5		0.1
M-8450	10*	10**	10*
Semicarbazide hydrochloride	>2.5	> 2.5	> 2.5

*Reciprocal of the dilution.
**Reciprocal of the dilution; cultures grown without plasma-clot.

TABLE 4

EFFECT OF M-8450 ON THE MULTIPLICATION OF HELA CELLS IN CONTINUOUS CULTURE

Dilution of M-8450 in Growth Medium	Fold Increase per Weekly Passage					Average Fold Increase *	Growth (%)
	1st	2nd	3rd	4th	5th		
Control**	3.68	6.0	5.2	4.9	5.5	5.05	100
1/10	1.88	2.1	1.9→2.1		1.86	1.96	40
1/40	3.51	5.1	↘ 4.9**			4.3	86
1/80	4.1	4.0				4.05	80
1/160	5.4	5.4				5.4	108

*Arithmetic average
**M-8450 withheld from the medium

TABLE 5

COMPOUNDS DEMONSTRATING INHIBITORY ACTIVITY AGAINST THE VIRUSES OF POLIOMYELITIS AND COXSACKIE GROWN IN HELA CULTURES

COMPOUNDS (MG/Ml)	VIRUSES *** 100TCID$_{50}$		50TCID$_{50}$		10TCID$_{50}$	
	Polio	Cox	Polio	Cox	Polio	Cox
Abbott #3665, β-amino aceto henone	-	-	-	-	-	-
*Abbott #4462, 0.1	-	-	-	-	-	-
Abbott #6238, 2-Thio-4,6-diamino pyrimidine 0.1	-	+	-	+	-	+
Abbott #4271, dimethyl glycine, 2.5	-	+	-	-	-	+
Benzimidazole, 0.1	-	-	-	-	-	-
2-Benzimidazolethio, 0.1	-	-	-	-	-	-
5-Amino-2-benzimidazole-thiol 0.1	-	-	-	-	-	-
1-α-D-arabopyranosyl-5,6-dimethyl benzimidazole 0.1	-	-	-	-	-	-
1-β-D-ribo yranosyl-5,6-dimethyl benzimidazole, 0.1	+	+	+	+	+	+
1-2-Deoxy-D-ribopyrano-syl-5,6-dimethyl benzimidazole, 1 0	-	-	-	-	-	-
5-Chlorobenzimidazole Hcl, 0 1	-	-	-	-	-	-
5,6-Dichloro-1-D-ribosyl-benzimidazole, 0.1	-	-	-	-	-	-
b Cysteic acid, 1.0	-	-	-	-	-	-
Erythromycin,).1	+	+	+	+	+	+
Hydroxylamine HCl, 0.1	+	+?	+	-	+?	-
Hydrazine dihydrochlod ride, 0.1	-	-	-	-	-	-
M-8450, 10**	-	-	-	-	-	-
Methionine sulfoximine,0.1	-	-	-	-	-	-
Mephenesin, 0.1	-	-	-	-	-	-
Milltown, 0.1	+	+	+	+	+	+
Diazouracil, 0.1	-	-	-	-	-	-
2,2-Diethyl-1,3-propane-diol dicarbamate, 0.5	-	-	-	-	-	-
2,2-Diphenyl-3-hydroxy propyl dicarbamate	-	-	-	-	-	-
2-Amino-4-chloro-5-methyl 6-phenyl yrimidine 0.01	+	-	+	-	+	-
2,4-Amino-5-methyl-6-henyl yrimidine, 0.1	-	-	-	-	-	-
2-Methyl-4-hydroxy-5-methyl-6-phenyl pyrimidine, 0.1	-	-	-	-	-	-
2-Hydroxy-4-amino-5-methyl-6-phenyl pyrimidine, 0.1	-	-	-	-	-	-
2-Amino-4-hydroxy-5,6 dimethyl pyrimidine 0.1	-	+	-	+	-	+
Semicarbazide HCl, 2.5	+?	-	-	-	+?	-

*Identity of the compound restricted.
**Reciprocal of the dilution.
***The (-) (+) designation represents the protection and degeneration of the cultures

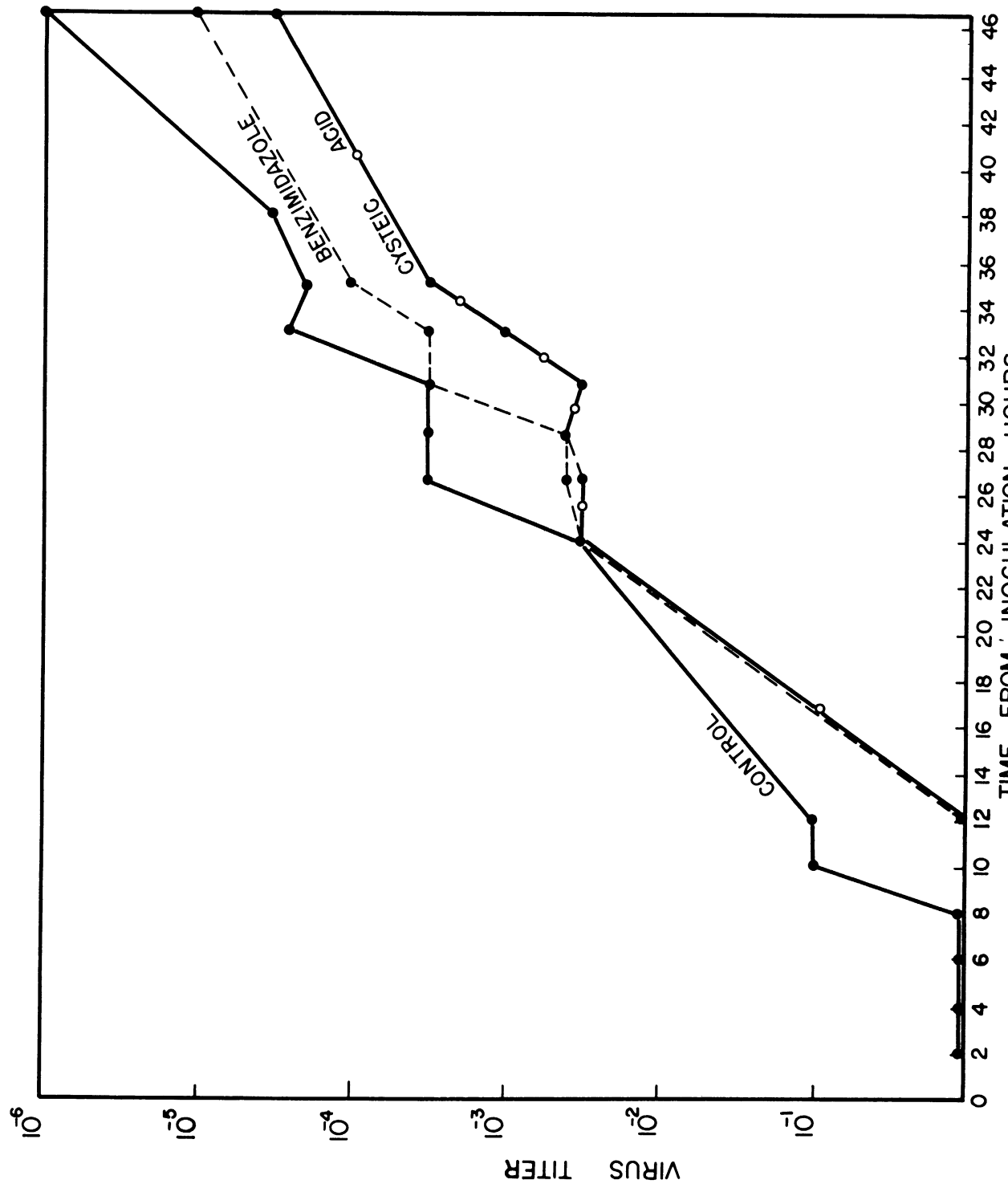

Figure 1. Effect of Benzimidazole (0.1 mg/ml) and Cyteic Acid (1.0 mg/ml) on Poliomyelitis (Mahoney) virus Grown in HeLa Cultures.

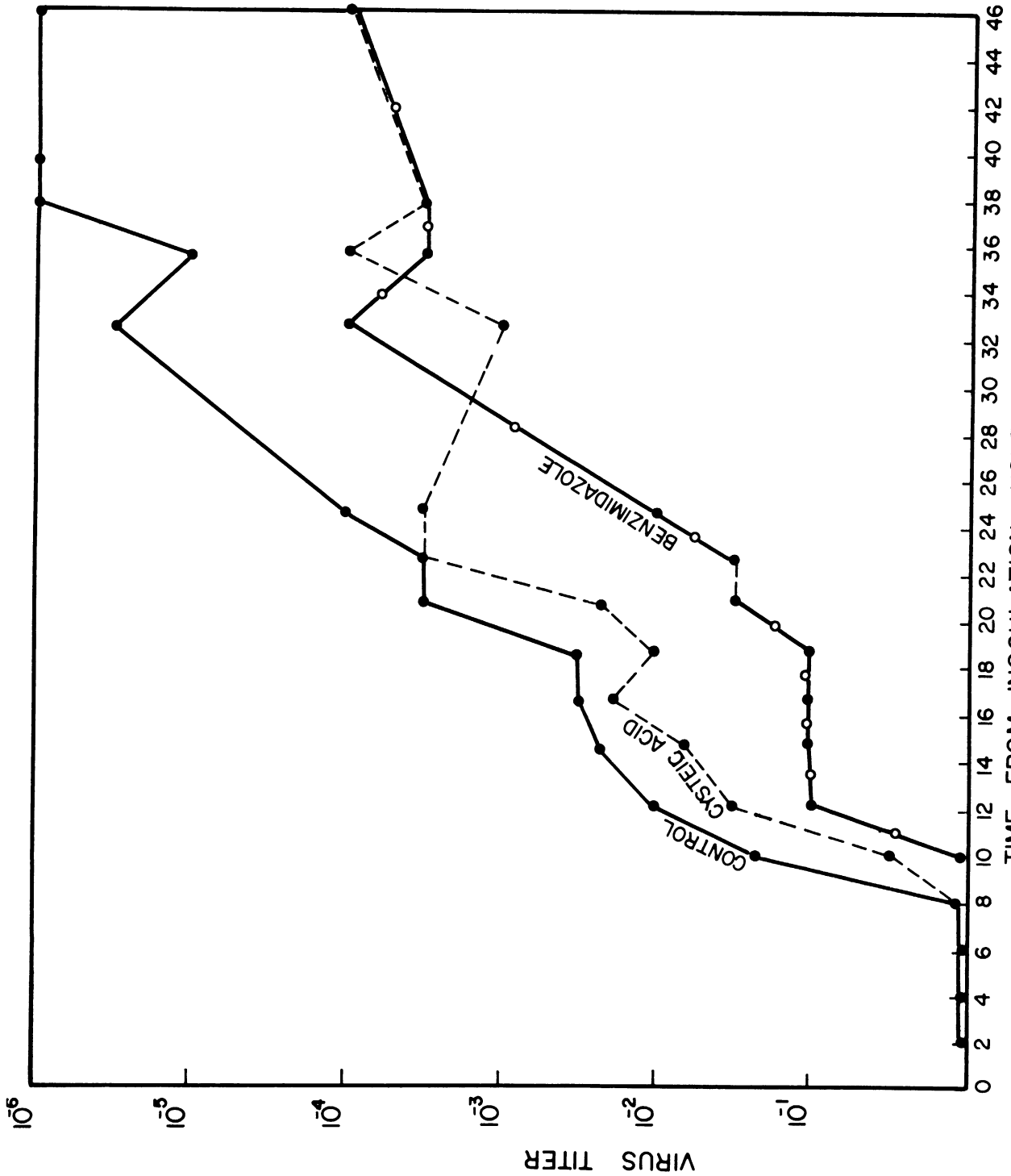

Figure 2. Effect of Benzimidazole (0.1 mg/ml) and Cysteic Acid (1.0 mg/ml) on Coxsackie (Conn. 5) Virus Grown in HeLa Cultures.

TABLE 6

THERAPEUTIC INDICES* OF SELECTED VIRUS INHIBITORS IN HELA CULTURES

COMPOUNDS	VIRUSES		
	Poliomyelitis	Coxsackie	Vaccinia
M-8450	49.5	42.2	4.2
Helenine	1.0	8.0	1.0
Helenine **	8.0	8.0	1.0
Methionine sulfoximine	294.0	66.5	200.0
M5-11496	8.3	2.0	2.0
Benzaldehyde thiosemicarbazone	4.0	2.0	24.0
Diazouracil	1.0	2.0	1.0
A14-(FSC)	8.0	8.0	4.0
5,6-Dichloro-1-D-ribosyl benzimidazole	66.5	2.0	2.0

*Therapeutic Index = Maximum tolerated dose/minimal suppresive dose.
**48-Hour pre-treatment of cultures at 37°C.

TABLE 7

THERAPEUTIC INDICES* OF FRACTIONS OF ULTRA-CENTRIFUGED M-8450 TESTED AGAINST ONE HUNDRED TISSUE CULTURE DOSES OF POLIOMYELITIS VIRUS IN HELA CULTURES

SAMPLE	THERAPEUTIC INDEX
4°C Control	44.7**
Room Temperature Control	11.3
Supernatant	1.4
Pellet Wash	1.4
Reconstituted Pellet	32.0

*Therapeutic index = Maximum tolerated dose/minimal suppressive dose.
**This figure represents the mode of the results obtained in other similar tissue culture assays.

TABLE 8

PROTECTION OF M-8450 TREATED AND WASHED HELA CULTURES FROM THE CYTOPATHOLOGY OF POLIOMYELITIS AND COXSACKIE VIRUSES

TREATMENT	CYTOPATHOLOGY*
Poliomyelitis virus	+
Coxsackie virus	+
M-8450 plus poliomyelitis virus	-
M-8450 plus Coxsackie virus	-
M-8450, washed, plus poliomyelitis virus	-
M-8450, washed, plus Coxsackie virus	-
Polio.antiserum, washed, plus polio. virus	+
Cox. antiserum, washed, plus Cox. virus	+

*The (-) (+) designations represent the protection and degeneration of the cultures.

TABLE 9

EFFECT OF 1/10 M-8450 ON POLIOMYELITIS VIRUS ADSORPTION AND ATTACHMENT ONTO HELA CULTURES

TIME AFTER INFECTION	POLIOMYELITIS INFECTED CULTURES				
	VIRUS TITER (NEG. LOG.) IN MONKEY KIDNEY TUBE CULTURES			NUMBER OF PLAQUES PER MONKEY KIDNEY BOTTLE	
	Virus Control	M-8450 Treated	37°C* Control	Virus Control	M-8450 Treated
60 minutes	1.66,2.25	1.83	-	22,40	29
2 hours 30 min.	1.66,1.66	1.66	-	21,19	19
4 hours	1.5, 1.5	1.5	2.5	20,28	19

*Incubator control without cells.

TABLE 10

EFFECT OF MEDIUM UPON THE INHIBITION BY M-8450 OF ONE HUNDRED TISSUE CULTURE DOSES OF POLIOMYELITIS VIRUS IN HELA CELLS

STOCK SOLUTIONS *	HIGHEST NON-TOXIC CONCENTRATION OF STOCK SOLUTIONS	THERAPEUTIC INDEX **
A	50%	12.0
B	15%	11.0
C	50%	8.0
D	50%	4.0
E	50%	4.0
Control Medium***	100%	44.7****

*Composition of stock solutions given in text.
**Therapeutic index = maximum tolerated dose/ minimal suppressive dose.
***Complete Syverton maintenance medium.
****This figure represents the mode of the results obtained in other similar tissue culture assays.

TABLE 11

EFFECT OF EAGLES'S MEDIUM UPON THE INHIBITION BY M-8450 OF POLIOMYELITIS VIRUS IN HELA CULTURES

MAINTENANCE MEDIUM	TISSUE CULTURE DOSE OF POLIOMYLITIS VIRUS	THERAPEUTIC INDEX*
Eagle's	320	330.5
Syverton's	178	64.0

*Therapeutic index = maximum tolerated dose/ minimal suppressive dose.

TABLE 12

CYTOPATHOLOGY AND VIRUS ISOLATIONS FROM M-8450-TREATED VIRUS-INFECTED HELA CULTURES

HOURS AFTER INFECTION	POLIOMYELITIS INFECTED CULTURES				COXSACKIE INFECTED CULTURES			
	Controls		M-8450 Treated		Controls		M-8450 Treated	
	I*	C**	I	C	I	C	I	C
24	nd	+?***	-	-	nd	+?	+	-
38	nd	+	-	-	nd	+	+	+?
72			-	-			nd	+
96			-	-				
120			-	-				
144			-	-				

*Virus isolation attempts in Hela cultures
**Cytopathology
***The designation (+) represents degeneration of the cultures or a positive virus isolation. The designation (-) represents no evidence of degeneration or a failure to isolate virus.
nd = not done.

TABLE 13

ANTIGENICITY OF M-8450-TREATED, POLIOMYELITIS-INFECTED CULTURE FLUIDS IN MICE

SERUM DILUTIONS	MOUSE ANTISERA PRODUCED AGAINST		
	Virus Control Culture Fluids	Drug-Treated Culture Fluids	Drug-Treated, Virus-Infected Culture Fluids
Undiluted	-*	+	+
1/4	-	+	+
1/8	-	+	+
1/16	-	+	+

*The designations (-) and (+) respectively represent protection and degeneration of cultures in a tissue culture neutralization test.

TABLE 14

LOWEST INHIBITORY CONCENTRATION (MG/Ml) OF COMPOUNDS ASSAYED FOR INHIBITION OF ONE HUNDRED TISSUE CULTURE DOSES OF VIRUS IN MONKEY KIDNEY CULTURES

COMPOUNDS**	Highest Toxic Conc. MG Ml	VIRUSES EMPLOYED															
		Echo 1	Echo 2	Echo 3	Echo 4	Echo 5	Echo 6	Echo 7	Echo 8	Echo 9	Echo 11	Echo 12	Echo 13	Echo 14	Polio Type 1	Cox B-1	Vacc.
M-8450*	10***	<10	60	60	60	30	60	60	80	80	60	<10	30	20	320*	120*	<10
Methionine Sulfoximine	2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
Benzaldehyde Thiosemicarbazone*	.039	>.039	>.039	>.039	>.039	>.039	>.039	>.039	>.039	>.039	>.039	> 039	> .039	>.039	>.039	>.039	>0017*
5,6-Dichloro-1-D-Ribosyl benzimidazole	0.1			.025	>0.1		>0.1	>0.1	.0125			0.05			>0.1		
4-Methoxy-6-nitrobenzimidazole	.039	>.039	>.039	> 039	.0065	>.039	.0097	>.039	>.039	>.039	.019	>.039	>.039	.0095	>.039	>.039	.019
6-Methoxy-4-nitrobenzimazole	.031	>.031	.0075		.0037	>0.31		>.031		0013	.015	>.031	>.031		>.031		>.031
1,2-Deoxy-D-Ribopyranosyl 5,6-dimethyl benzimidazole	1.0			.125	.187		0.5	0.5	.125								
1-β-D-Ribopyranosyl 5,6-dimethyl benzimidazole	1.0			>1.0	.125		>1.0	.375	.125								
1-α-D-Arabopyranosyl 5,6-dimethyl benzimidazole	1.0			>1.0	.125		>1.0	>1.0	.125								

*Compounds considered significantly inhibitory by virtue of large therapeutic index.
**Cultures pre-treated 24 hours at 37°C. ***Reciprocal of the dilution.

TABLE 15

THERAPEUTIC INDICES* OF SELECTED VIRUS INHIBITORS IN MONKEY KIDNEY CULTURES

COMPOUND	VIRUSES								
	Polio.	Cox.	Vacc.	Echo-2	Echo-4	Echo-7	Echo-8	Echo-9	Echo-11
M-8450	32.0	12.0	<1.0	6.0	6.0	6.0	8.0	8.0	6.0
Methionine sulfoximine	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Benzaldehyde thiosemicarbazone	<1.0	<1.0	22.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
5,6-Dichloro-1-D-ribosyl benzimidazole	2.0	-	-	-	<1.0	<1.0	8.0	-	-
Plant extract, M-2	<1.0	<1.0	<1.0	<1.0	28.8	4.0	<1.0	12.0	28.0
Plant extract, M-4	2.0	-	<1.0	4.0	4.0	8.0	16.0	3.0	3.0
Plant extract, M-14	<1.0	<1.0	<1.0	48.0	<1.0	<1.0	4.0	<1.0	<1.0

*Therapeutic index = Maximum tolerated dose/minimal suppressive dose.

TABLE 16

RECIPROCAL OF THE LOWEST INHIBITORY DILUTION OF PLANT EXTRACTS
ASSAYED FOR VIRUS INHIBITORY PROPERTIES IN MONKEY KIDNEY CULTURES

PLANT EXTRACT FRACTIONS **	LOWEST NON-TOXIC DILUTION	VIRUSES EMPLOYED															
		Echo 1	Echo 2	Echo 3	Echo 4	Echo 5	Echo 6	Echo 7	Echo 8	Echo 9	Echo 11	Echo 12	Echo 13	Echo 14	Polio Type 1	Cox. B-1	Vacc.
M 1	80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	320	< 80	-	< 80	< 80	< 80
M 2*	100	<100	<100	<100	2880*	<100	<100	400	<100	1200*	2880*	<100	600	<100	<100	<100	<100
M 3	120	<120	<120	<120	<120	<120	<120	240	<120	480	<120	<120	480	<120	240	<120	<120
M 4*	120	480	480	720	480	240	<120	960*	1920*	360	360	240	240	-	240	-	<120
M 5	10	< 10	20	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	-	< 10	-	< 10
M 6	120	<120	<120	360	<120	<120	<120	720	720	<120	<120	<120	720	-	<120	<120	<120
M 7	100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	-	200	<100	<100
M 8	100	200	<100	<100	<100	<100	<100	<100	<100	<100	<100	600	<100	-	<100	<100	<100
M 9	200	<200	<200	<200	<200	<200	600	<200	<200	<200	<200	600	<200	-	<200	<200	400
M10	100	400	<100	<100	<100	<100	<100	<100	<100	<100	600	400	<100	-	-	-	-
M11	100	400	<100	<100	<100	<100	200	<100	<100	300	<100	<100	<100	-	<100	<100	<100
M12	100	<100	<100	<100	<100	<100	<100	<100	<100	300	<100	<100	200	-	<100	<100	<100
M13	100	<100	<100	<100	-	<100	<100	<100	-	-	300	-	-	-	<100	<100	<100
M14*	10	< 10	480*	< 10	< 10	< 10	< 10	< 10	40	< 10	< 10	< 10	< 10	-	< 10	< 10	< 10
M15	160	<160	<160	<160	<160	<160	<160	<160	<160	<160	<160	<160	<160	-	<160	<160	<160
M16	160	320	<160	<160	320	<160	320	320	320	<160	<160	320	640	-	320	320	320
M18	200	-	600	-	200	-	-	-	-00	1200	200	800	800	-	200	200	800
M19	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M20-25	160	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Compounds considered significantly inhibitory by virtue of large therapeutic index.
**All cultures pre-treated 24 hours at 37°C.

TABLE 17

INHIBITION OF CYTOPATHOLOGY* OF M-2-TREATED MONKEY KIDNEY CULTURES INFECTED WITH ECHO-4 AND 11 VIRUSES

VIRUS INOCULATED	$TCID_{50}$	TIME AFTER INFECTION (HOURS)	VIRUS CONTROLS (NORMAL MEDIUM)	24-HOUR PRETREATMENT WITH M-2											
				1/120		1/240		1/480		1/960		1/1920		1/3840	
Echo-4	10,000	48	+ + + +	-	-	-	-	-	-	-	-	+	+	+	+
		72		-	-	+	-	+	+	+	+				
		96		+	+	-	+								
	1,000	48	+ + + +	-	-	-	-	-	-	-	-	-	-	+	+
		72		-	-	-	-	-	-	+	+	+	+		
		96		-	-	-	-	+	+						
	100	48	- - - -	-	-	-	-	-	-	-	-	-	-		
		72	+ + + +	-	-	-	-	-	-	-	-	-	-		
		96		-	-	-	-	-	+	+	+	+	+		
Echo-11	3,200	48	+ +	-	-	-	-	-	-	-	+	+	+	+	+
		72		-	-	+	-	+	+	+					
		96		+	+	-	+								
	1,000	48	+ + + +	-	-	-	-	-	-	-	-	-	-	+	+
		72		-	-	-	-	-	+	+	+	+	+		
		96		+	+	+	+	+							
	100	48	- - - -	-	-	-	-	-	-	-	-	-	-	+	+
		72	+ + + +	-	-	-	-	-	-	-	-	-	-		
		96		-	-	-	-	-	+	+	+	+	+		

*The designations (+) (-) represent the degree of involvement of the cultures. When all the cells are off the glass the (+) designation is given.

TABLE 18

INHIBITION OF CYTOPATHOLOGY* OF MONKEY KIDNEY CULTURES TREATED WITH M-2 AT TIME OF ECHO-4 INFECTION AND ONE HOUR LATER

DRUG (M-2)	VIRUS (100 $TCID_{50}$	TIME AFTER INFECTION (HOURS)	VIRUS CONTROLS (NORMAL MEDIUM)	DILUTIONS OF M-2							
				1/120		1/240		1/480		1/960	
With virus	Echo-4	48	- - - -	-	-	-	-	-	-	-	-
		72	+ + + +	-	-	-	-	-	-	-	-
		96		-	-	-	+	+	+	+	+
1 hour after virus	Echo-4	48	- - - -	-	-	-	-	-	-	-	-
		72	+ + + +	-	-	-	-	+	+	+	+
		96		-	-	+	+				

*The designations (+) (-) represent the degree of involvement of the cultures When all the cells are off the glass the (+) designation is given.

TABLE 19

THERAPEUTIC INDICES* FOR M-2 TREATED MONKEY KIDNEY CULTURES

ECHO VIRUS	TITER (Neg. Log.)	PRETREATMENT 24 HOURS BEFORE VIRUS				Drug with virus	1 hr. after virus	Virus Controls
		10,000 TCD_{50}	3200 TCD_{50}	1000 TCD_{50}	100 TCD50	100 TCD_{50}	100 TCD_{50}	100 TCD50
#4	5.0	14.4	-	28.8	28.8	14.4	3.6	< 1.0
#11	4.5	-	9.6	28.8	28.8	-	-	< 1.0

*Therapeutic index = maximum tolerated dose/minimal suppressive dose.

TABLE 20

INHIBITION OF CYTOPATHOLOGY*** OF M-2 TREATED MONKEY KIDNEY CULTURES AFTER DOUBLE VIRUS EXPOSURES

GROUP #	CULTURES PRETREATED 24 HOURS WITH M-2	FIRST EXPOSURE VIRUS (100 $TCID_{50}$) *	CULTURES RE-EXPOSED TO VIRUS	CYTOPATHOLOGY IN HRS AFTER FIRST VIRUS EXPOSURE 72	96	120	TYPE OF ECHO VIRUS RECOVERED
1	1/120	Echo-4	Echo-4 (10,000 $TCID_{50}$)	-	-	-	nd**
	1/240	"		-	+		nd
	1/480	"		-	+		4
	1/960	"		+			4
2	1/120	Echo-4	Echo-11 (3200 $TCID_{50}$)	-	-	-	nd
	1/240	"		-	-	-	nd
	1/480	"		-	+		11
	1/960	"		+			11
3	1/120	Echo-4	Echo-1 (100,000 $TCID_{50}$)	+			1
	1/240	"		+			1
	1/480	"		+			1
	1/960	"		+			1
4	1/120	Echo-11	Echo-4 (10,000 $TCID_{50}$)	-	-	-	nd
	1/240	"		-	+		4
	1/480	"		-	+		4
	1/960	"		+			4
5	1/120	Echo 11	Echo-11 (3200 $TCID_{50}$)	-	-	-	nd
	1/240	"		-	-	-	nd
	1/480	"		-	+		nd
	1/960	"		+			11
6 Drug Control Cultures	1/120	Echo 4 or 11		-	-	-	nd
	1/240			-	-	+	nd
	1/480	"		-	+		nd
	1/960	"		-	+		nd
Virus Controls		Echo 4 or 11		+			nd

*Cultures washed once and fresh M-2 added.

**Not done.

***The designations (-) (+) represent protection and degeneration of the cultures.

TABLE 21

CYTOPATHOLOGY** OF M-2 TREATED MONKEY KIDNEY CULTURES AFTER DOUBLE VIRUS EXPOSURES

GROUP	24-HOUR PRETREATMENT WITH M-2	FIRST EXPOSURE VIRUS (100 $TCID_{50}$)	SECOND EXPOSURE VIRUS (0.1 ml of 10^{-1} dil.)	CYTOPATHOLOGY IN HOURS AFTER FIRST VIRUS EXPOSURE		
				72	96	120
1	1/200	Echo-11	Echo 1	+		
2	1/200	"	Echo 2	+		
3	1/200	"	Echo 3	+		
4	1/200	"	Echo 4	-	-	-
5	1/200	"	Echo 5	+		
6	1/200	"	Echo 6	+		
7	1/200	"	Echo 7	+		
8	1/200	"	Echo 11	-	-	-p*
9	1/200	"	Polio	+		
10	1/200	"	Coxsackie	+		
11	1/200	"	Vaccinia	+		
Untreated controls		"		+		

*Evidence of occasional virus plaques
**The designations (-) (+) represent the protection and degeneration of the cultures

TABLE 22

RECIPROCAL OF THE ANTIBODY TITERS* OF SERA OBTAINED FROM MICE INOCULATED WITH ECHO-4 AND 11 VIRUSES AND TREATED WITH M-2 FRACTION

TYPE OF VIRUS INOCULATED	VIRUS CONTROL MICE	M-2 TREATED CONTROL MICE	M-2 TREATED VIRUS-INOCULATED MICE
Echo-4	16		6
Echo-11	64	4	12

*Obtained in monkey kidney tissue culture neutralization test.

BIBLIOGRAPHY

Ackermann, W. W. and Kurtz, H., 1955, Observations Concerning a Persisting Infection of HeLa Cells with Poliomyelitis Virus. J. Exp. Med., 102: 555-565.

Ackermann, W. W., Rabson, A., and Kurtz, H., 1954, Growth Characteristics of Poliomyelitis Virus in HeLa Cell Cultures: Lack of Parallelism in Cellular Injury and Virus Increase. J. Exp. Med., 100: 437-450.

Ainslie, J. D., 1952, Growth Curve of the Lansing Strain of Poliomyelitis Virus in Mice; the Effect of Sodium Monofluoracetate and Methionine Sulfoximine on the Early Phase of Growth of the Virus. J. Exp. Med., 95: 9-18.

Biesele, J. J., Berger, R. E., Wilson, A. Y., Hitchings, G. H. and Elion, G. B., 1951, Studies on 2,6-Diaminopurine and Related Substances in Cultures of Embryonic Sarcomatous Rodent Tissues. Cancer, 4: 186-197.

Briody, B. A. and Stannard, C., 1951, The Effect of Proflavine on the Growth of Viruses in the Chick Embryo. J. Imm., 67: 423-430.

Brown, G. C., 1952, The Influence of Chemicals on the Propagation of Poliomyelitis Virus in Tissue Culture. J. Imm., 69: 441-450.

Brown, G. C. and Ackermann, W. W., 1951, Effect of DL-Ethionine on Poliomyelitis Virus Growth in Tissue Culture. Proc. Soc. Exp. Biol. and Med., 77: 367-369.

Burchenal, J. H., Bendick, A., Brown, G. B., Elion, G. B., Hitchings, G. H., Rhoads, C. P. and Stock, C.C., 1949, Preliminary Studies on the Effect of 2,6-Diaminopurine on Transplanted Mouse Leukemia. Cancer, 2: 119-120.

Burnet, F. M., 1955, Principles of Animal Virology, Academic Press, Inc., N.Y.

Cartwright, G. E., Palmer, J. G., Hitchings, G. H., Elion, G. B., Gunn, F. D. and Wintrobe, M. M., 1950, Studies on the Effect of 2,6-Diaminopurine on the Blood and Bone Marrow of Swine. J. Lab. and Clin. Med., 35: 518-527.

Cochran, K. W., 1956, Personal communication.

Cochran, K. W., 1957, Chemoprophylaxis with Diazouracil of Poliomyelitis in Mice. Science, 126: 1115-1116.

Cochran, K. W., Brown, G. C. and Francis, T. F., 1954, Antiviral Action of a Mold Filtrate on Experimental Poliomyelitis in Cynomolgus Monkeys. Proc. Soc. Exp. Biol. and Med., 85: 104-106.

Cochran, K. W. and Francis, T. F., 1956, Antiviral Action of Helenine on Experimental Poliomyelitis. Proc. Soc. Exp. Biol. and Med., 92: 230-232.

Dulbecco, R., 1952, Production of Plaques in Monolayer Tissue Cultures by Single Particles of an Animal Virus. Proc. Nat'l Acad. Sci., 38: 747-752.

Eagle, H., Oyama, V. I., Levy, M., Horton, C. L. and Fleischman, R., 1956, The Growth Response of Mammalian Cells in Tissue Culture to L-Glutamine and L-Glutamic Acid. J. Biol. Chem., 218: 607-616.

Friend, C. C., 1951, Effect of 2,6-Diaminopurine on Russian Spring-Summer Encephalitis Infection in Tissue Culture. Proc. Soc. Exp. Biol. and Med., 78: 150-153.

Gifford, G. E., Robertson, H. E. and Syverton, J. T., 1954, Application of Manometric Methods to Testing Chemical Agents in vitro for Interference with Poliomyelitis Virus Synthesis. Proc. Soc. Exp. Biol. ad Med., 85: 515-522.

Ginsberg, H. S. and Boyer, G. S., 1956, Masked Viral Infection of HeLa Cell Cultures. Fed. Proc., 15: 589.

Ginsberg, H. S. and Horsfall, F. L., 1949, Concurrent Infection with Influenza Virus and Mumps Virus or Pneumonia Virus of Mice (PVM) as Bearing on the Inhibition of Virus Multiplication by Bacterial Polysaccharides. J. Exp. Med., 89: 37-52.

Hamre, D., Bernstein, J. and Donovick, R., 1950, Activity of p-Aminobenzaldehyde, 3-Thiosemicarbazone on Vaccinia Virus in the Chick Embryo and in the Mouse. Proc. Soc. Exp. Biol. and Med., 73: 275-278.

Hamre, D., Brownlee, K. A. and Donovick, R., 1951, Studies on the Chemotherapy of Vaccinia. a. The Activity of Some Thiosemicarbazones. J. Imm., 67: 305-312.

Hollinshead, A. C. and Smith, P. C., 1956, Relative Effectiveness of Certain Inhibitory Chemicals on the Three Types of Poliomyelitis Virus. J. Phar. and Exp. Therap., 117: 97-100.

Hull, R. N. and Lavelle, J. M., 1953, Inhibition of Cytopathogenic Effect of Poliomyelitis Viruses in Tissue Culture by Antibiotic M-8450. Proc. Soc. Exp. Biol. and Med., 83: 787-790.

Hull, R. N. and Lavelle, J. M., 1954, Further Studies on the Inhibition of the Cytopathogenic Effect of Poliomyelitis Viruses by Antibiotic M-8450 in Tissue Culture. Ann. N. Y. Acad. Sci. 58: 1188-1194.

Hsiung, G. D. and Melnick, J. L., 1955, Plaque Formation with Poliomyelitis, Coxsackie and Orphan (ECHO) Viruses in Bottle Cultures of Monkey Epithelial Cells. Virology, 1: 533-535.

Hsu, T. C., Robins, R. K. and Cheng, C. C., 1956, Studies on 4-Amino-pyrazolo (3,4-d)-pyrimidine: Antineoplastic Action in vitro. Science, 123: 848-849.

Hurst, E. W. and Hull, R., 1956, Approaches to the Chemotherapy of Virus Diseases, Pharmacological Review. 8: 199-263.

Karnofsky, D. A., Patterson, P. A. and Ridgway, L. P., 1949, Effect of Folic Acid, 4-Amino Folic Acids and Related Substances on Growth of Chick Embryo. Proc. Soc. Exp. Biol. and Med., 71: 447-452.

Karzon, D. T. and Barron, A., 1957, Personal communication.

Knox, C. H., Robbins, M. L. and Smith, P. K., 1957, Influence of Pyrimidine Analogues and Related Compounds on the Propagation of Poliomyelitis Virus in Tissue Culture. J. Pharmacol. and Exp. Ther., 119: 495-505.

Kramer, P. E., Robbins, M. L. and Smith, P. K., 1955, Phenolic Compounds as Chemotherapeutic Agents against Poliomyelitis Virus in Tissue Culture. P. Phar. and Exp. Ther., 113: 262-271.

Lucas, E. H., Ringler, R. L., Byerrum, R. U., Stevens, J. A., Clarke, D. A. and Stock, C. C., 1957, Tumor Inhibition in Boletus edulis and other Holobasidiomycetes. Antibiotics and Chemotherapy, 7: 1-4.

Matthews, R. E. F. and Smith, J. D., 1955, The Chemotherapy of Viruses, Advances in Virus Research, Academic Press, New York, N. Y.

Melnick, J. L., 1955a, Enteric Cytopathogenic Human Orphan (ECHO) Viruses. Science, 122: 1181-1182.

Melnick, J. L., 1955b, Tissue Culture Techniques and Their Application to Original Isolation, Growth, and Assay of Poliomyelitis and Orphan Viruses. Ann. New York Acad. Sci., 61: 674-773.

Pearson, H. E., 1953, Biochemical Aspects of Viral Growth, Annual Review of Microbiology, 7: 179-195.

Phillips, F. S. and Thiersch, J. B., 1949, Actions of 2,6-Diamino-purine in Mice, Rats, and Dogs. Fed. Proc., 8: 325.

Powell, H. M. and Culbertson, C. G., 1953, Action of an Antiviral Mold Filtrate Against MEF-1 Poliomyelitis Virus in Mice. Proc. Soc. Exp. Biol. and Med. 83: 161-163.

Reed, L. J. and Muench, M., 1938, Simple Method of Estimating Fifty Percent Endpoints. Am. J. Hyg., 27: 493-497.

Robbins, F. C., Weller, T. H. and Enders, J. F., 1952, Studies on the Cultivation of Poliomyelitis Viruses in Tissue Culture. 2. The Propagation of the Poliomyelitis Viruses in Roller-Tube Cultures of Various Human Tissues. J. Imm., 69: 673-604.

Robertson, H. E., Gifford, G. E. and Syverton, J. T., 1956, Effect of Furfural and Some Simple Furan Compounds on the Reproduction of Poliovirus in HeLa Cells. Am. J. Hyg., 64: 149-157.

Smith, P. K., Knox, C. H. and Hollinshead, A. C., 1955, Inhibitory Effect of Certain Chemicals on the Three Types of Poliomyelitis Virus in Tissue Culture. Fed. Proc., 14: 387.

Stock, C. C., Biesele, J. J., Burchemal, J. H., Karnofsky, D. A., Moore, A. E. and Sugiura, K., 1950, Folic Acid Analogs and Experimental Tumors. Ann. New York Acad. Sci., 52: 1360-1377.

Stock, C. C., Jacobson, W. and Williamson, M., 1951, An Influence of 2,6-Diaminopurine upon the Content of Kappa in Paramecium aurelia, Variety 4. Proc. Soc. Exp. Biol. and Med., 78:874-876.

Syverton, J. T., Scherer, W. F. and Elwood, P. M., 1954, Studies on the Propagation in vitro of Poliomyelitis Viruses. 5. The Application of Strain HeLa Human Epithelial Cells for Isolation and Typing. J. Lab. and Clin. Med., 43: 286-302.

Tamm, I., 1956, Selective Chemical Inhibition of Influenza B Virus Multiplication. J. Bact., 72: 42-53.

Thompson, R. L., 1946, The Effect of Enzyme Inhibitors, Nutriles and Substitution Compounds on the Growth of Vaccinia Virus. J. Bact., 51: 629-630.

Thompson, R. L., 1947, The Effect of Metabolites, Metabolic Antagonists and Enzyme Inhibitors on the Growth of the Vaccinia Virus in Maitland Type of Tissue Culture. J. Imm., 55: 345-352.

Thompson, R. L., Price, M. L. and Minton, S. A., 1951, Protection of Mice Against Vaccinia Virus by Administration of Benzaldehyde Thiosemicarbazone. Proc. Soc. Exp. Biol. and Med., 78: 11-13.

Thompson, R. L., Price, M. L., Minton, S. A., Elion, G. B. and Hitchings, G. H., 1950, Effects of Purine Derivatives and Analogues on Multiplication of the Vaccinia Virus. J. Imm., 65: 529-534.

Thompson, R. L. and Wilkin, M. L., 1948, Inhibition of Growth of the Vaccinia Virus by β-2-Thienylalanine and Its Reversal by Phenylalanine. Proc. Soc. Exp. Biol. and Med., 68: 434-436.

Thompson, R. L., Wilkin, M. L., Hitchings, G. H., Elion, G. B., Falco, E. A. and Russell, P. B., 1949a, The Effects of Antagonists on the Multiplication of Vaccinia Virus in vitro. Science, 110:454.

Thompson, R. L., Wilkin, M. L., Hitchings, G. H. and Russell, P. B., 1949b, The Virostatic and Virucidal Action of α Haloacylamides on Vaccinia Virus in vitro. Proc. Soc. Exp. Biol. and Med., 72: 169-171.

Youngner, J. S., 1954a, Monolayer Tissue Cultures. 1. Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells. Proc. Socd Exp. Biol. and Med., 85: 202-205.

Youngner, J. S., 1954b, Monolayer Tissue Cultures. 3. Propagation of Poliomyelitis Viruses in Cultures of Trypsin-Dispersed Monkey Kidney Cells. J. Imm., 73: 392-396.