THE EFFECT OF CERTAIN ANTIBIOTIC SUBSTANCES ON NEWCASTLE DISEASE VIRUS

by

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INTRODUCTION

The possibility is in sight of controlling the majority of bacterial and protozoan diseases by chemotherapeutic drugs. As a result, attention has been shifted to the control of virus diseases. Viruses are responsible for such widespread and important infections as the common cold, influenza, smallpox, poliomyelitis, yellow fever, encephalitis, and possibly cancer. Among domestic animals, foot and mouth disease, fowl pest, rinderpest, hog cholera, distemper and hard pad disease of dogs still cause enormous losses. Against some viruses it is possible to produce effective vaccines. This is true when the various virus strains that produce the disease are antigenically similar. In other cases, as in foot and mouth disease, there are several antigenically distinct strains and successful immunization thus becomes difficult since the strain causing infection may have been absent from the vaccine. Also, immunity after vaccination may continue for only a comparatively short time, as in influenza. Thus the problem of virus control may not be solved by vaccination alone.

Chemotherapeutic treatment of virus infections, however, meets with difficulties which are not present in the control of bacterial diseases. Viruses are obligatory intracellular parasites formed of nucleoproteins. When viruses parasitize a cell, they compete with the cell for metabolites essential for the formation of new nucleoproteins. The viruses use the enzymes produced by the host cells. Thus, the viruses utilize the metabolism

of the cells which they parasitize and any agent which kills the virus or interrupts virus synthesis may also damage the cell with which the virus is associated.

There are, however, mechanisms whereby virus multiplication may be subdued and research on chemotherapy of virus infections has made use of the following principles, as reviewed by Findlay (9): (1) The virus may be prevented from reaching susceptible cells, as a result of mechanical coating, suggested for the action of mucin on viruses; (2) the virus may be destroyed while it is adsorbed on the surface of the cell but has not yet penetrated into the cell substance; (3) specific virus receptors on cells may be removed by receptor-destroying enzymes such as cholera toxin; (4) certain cellular constituents necessary for virus reproduction may be inhibited or bound by added chemical compounds; or (5) when the virus is localized within certain small groups of cells, these cells may be destroyed together with the virus as in the destruction of warts.

Factors other than drug therapy and immunity may be involved in the suppression of virus infections; absence of certain food materials may reduce the extent of an infection; the injection of a non-virulent virus before a virulent one may interfere with the virulence of the latter virus. The next few years will undoubtedly see many advances in the therapy of virus infections.

It is the purpose of this thesis to present experimental results in which certain antibiotic substances extracted from growing cultures of microorganisms were tested for antiviral

activity. Newcastle disease virus was used as test organism. The microorganisms chosen for antibiotic activity were those which were available in the Department of Bacteriology, Kansas State College, and that produced large amounts of capsular material. The general plan was to screen about thirty cultures and to study in detail any that might show promise of activity.

REVIEW OF LITERATURE

Interest in Newcastle disease virus stems from various sources. Aside from its importance as the agent of a widespread disease of domestic gallinaceous birds, it may cause a conjunctivitis and possibly a generalized systemic infection in man according to Welch, et al. (30). Furthermore, since Burnet (3) demonstrated the affinities of Newcastle disease virus to the influenza-mumps group of viruses, along with its pneumonic properties for mice, it has been the subject of many basic virus studies.

Although a vaccine has been used on several million birds in the field, Upton, et al. (28) have emphasized the limitations of a vaccine against Newcastle disease. Offsprings of the immunized birds have shown some carry-over of resistance to infection. However, the study of antigenic diversity among strains of Newcastle disease virus by the serum neutralization test has indicated that the use of a single vaccine is not feasible in all situations (28). In support of this hypothesis, thirteen antigenically different strains were identified by workers at the University of Wisconsin in 1953. Thus, it may be seen that it would be difficult to meet the problem of Newcastle disease control through vaccination alone.

The Use of Antibiotics in Vaccine Preparation

Since antibiotic substances are being used in preparation of live virus vaccines to insure freedom from contamination, it is

important that the antibiotic not have antiviral activity. It has been demonstrated by Salle, et al. (24), that the antibiotic subtilin is active against Newcastle disease virus in embryonated eggs. This suggested the possibility of using antibiotics in the preparation of killed virus vaccines, and a study was undertaken to determine the immunizing ability in chickens of Newcastle virus previously inactivated with subtilin in vitro (24). Salle, et al. (24), found that the control chick embryos (unimmunized) were alive for three to five days as a result of the challenge dose, whereas the immunized chicks were alive after twenty-one days.

The Complexity of Virulence

Lai and Bang (19) suggested that the virulence of Newcastle disease virus resulted from a complex of factors, rather than a single factor which determines the outcome of an acute infection. These factors were: (1) The rate of virus multiplication peripherally; (2) the capacity of the virus to penetrate into the central nervous system (the "critical" organ in deciding the course of infection with Newcastle disease virus); (3) the rate of multiplication of the virus in the central nervous system; (4) the extent of the brain destruction per infectious unit of virus in the central nervous system; and (5) the relative antigenic properties of the virus strains and the immune response of the host.

The evidence that these factors are important in the assay of virulence of Newcastle disease virus strains has been demon-

strated by Lui and Bang (19) in the comparative analysis of two Newcastle strains, CG179 and B: (1) Following intramuscular inoculation, both strains increased at the same rate in the extraneural tissues, i. e. blood, lung, rectum, and spleen, but the CG179 strain showed an accelerated growth rate in the brain; (2) the CG179 strain multiplied more rapidly in the brain following intracerebral administration of minimal inocula; (3) recovery with the B strain was associated with a decline in virus titer; (a) in the circulating blood, (b) in the visceral organs, and (c) in the central nervous system; (4) birds infected with CG179 tended to develop neurological symptoms at lower brain titers than those infected with strain B.

These studies indicate that in the testing of antibiotics for activity against Newcastle virus the matter of complexity of factors determining virulence must be taken into consideration.

Screening Techniques for Antibiotic Activity

Techniques for screening compounds for antiviral activity include protection of chick embryos, tissue culture cells and other animal materials against viruses, bacterial hosts against phages, and inhibition of hemagglutination by certain viruses.

An important consideration in the testing of antibiotics for antiviral activity is the method of inoculation of test animals. There are two methods of chick embryo inoculation that have been used with a minimum of traumatic deaths (2). The inoculation of drugs via the allantoic cavity has been used most

frequently, but an alternate method of administering the drug through the air sac route has also been used successfully. A comparison was made by Bloom and Gordon (2) of the protective effect of equal amounts of penicillin, aureomycin, and sulfadiazine introduced by the two routes in psittacosis-infected eggs. At the twenty-four hour interval the drug concentrations following allantoic cavity injection was greater than that following air sac injection, but at forty-eight hours and thereafter they were essentially the same. Therefore, it was concluded that either route served satisfactorily to suppress the growth of the virus.

In addition to chick embryo techniques, a procedure has been developed by Gitterman and Larsen (12) for the testing of antiviral agents effective against Newcastle disease virus in tissue culture. This method served as well for the detection of toxic effects exerted by the substance. A tissue sheet of chick embryo fibroblasts was produced on a glass wall of a test tube containing a medium of horse serum and lactalbumin enzymatic hydrolysate in Earle's Balance Salts Solution. After forty-eight hours' incubation, the fibroblast cells were stained with neutral red and examined for necrosis caused by the addition of virus and for evidence of toxicity caused by the addition of the compound. Newcastle disease virus was found to be sensitive to the antibiotics streptothricin and neformicin in tissue culture.

There was one phase of testing reported in the literature by Carvajal (4) that is becoming increasingly important. This was

the work done with bacteriophage. In the search for antiphage-producing microorganisms, soil dilutions were made in media to which a bacteriophage for Bacillus cereus var. mycoides was also added simultaneously. After the plates were poured and incubated two to seven days, a cell suspension of the phage-sensitive strain of B. cereus var. mycoides was used. Streaks were made close to the microbial colonies. If the phage was not added when the soil dilutions were made, it was added to the cell suspension of B. cereus var. mycoides and then the plates streaked with this mixture in the usual way. When antiviral (antiphage) substances were produced by the microorganisms, a ring of growth of B. cereus var. mycoides developed around the colonies, while the inactive colonies (non-antiphage producers) did not prevent the lysis of B. cereus var. mycoides by its phage. In this preliminary screening, it was possible to obtain a comparatively rough estimate of antiphage production by the various microorganisms in the search for antiviral substances.

It can be said, about virus infections in general, that the dearth of literature in which antiviral activity of chemical substances has been demonstrated would lead one to conclude that they are discouragingly ineffective. However, a number of compounds varying in structure have exhibited virus inhibition under specific conditions. In assessing the value of any antiviral agent one consideration must remain tantamount to its pernicious effect on the virus: the reaction of the host. Therefore, in vitro studies must also have positive verification in the

susceptible species during the course of infection with the virus under study. Since it is not always feasible that the latter condition be met, particularly if man is the primary susceptible species, a great deal of work is still in the experimental stage.

Status of Broad-Spectrum Antibiotics in Virus Infections

The antibiotics termed broad-spectrum include those effective against a wide range of bacterial infections and other infections caused by some of the larger viruses, i. e., psittacosis-lymphogranuloma group. However, this spectrum might eventually include some of the smaller viruses. In studies with terramycin, Welch, et al. (30), reported in detail the effect of this drug on the PR8 strain of influenza A virus infection in chick embryos. This study was undertaken shortly after the drug became available commercially. In vivo observations by Kneeland and Melcher (18) in the treatment of primary atypical pneumonia have shown that terramycin can alleviate the acute manifestations and shorten the febrile course of infection. Infiltration in the lung as shown by roentgen examination was little altered by this antibiotic, and the disease tended to run a more protracted course than pneumococcal lobar pneumonia.

Allen, et al. (1), assumed that the ability of aureomycin to inhibit protein synthesis might account for any virucidal activity it could exert. The findings presented indicated that aureomycin was distributed by direct adsorption to the allantoic membrane. Treatment of chick embryos infected with viruses of

the psittacosis-lymphogranuloma group with one milligram of aureomycin by the allantoic route caused an inhibition of virus growth in the allantoic membrane. The drug had no effect on "inert" virus, however, and appeared to have little effect on the adsorption of virus to host tissues. Complete inhibition of growth during the time interval corresponding to the first cycle of multiplication could be achieved only if the drug was administered within six to eight hours after virus inoculation. Partial inhibition of virus multiplication could be achieved even if the administration was delayed as late as twenty-four hours after infection. In these experiments the chief role of the antibiotic appeared to Hartman, et al. (16), to be one of the virustasis reflected in a prolongation of the latent period. The virus was able to resume its growth when a critically low level of the drug in the allantoic membrane was reached. This antibiotic has been valuable in the treatment of atypical pneumonia (8), but it has been more widely used in the treatment of the psittacosis virus infections (13).

Trachoma and lymphogranuloma venereum have been successfully treated with chloramphenicol (31). The frequency of relapse that occurs, particularly with trachoma, has been completely overcome by treatment with chloramphenicol, according to Welch, et al. (30).

In summary of the use of broad-spectrum antibiotics in virus diseases, Table 1 has been prepared. It will be noted that the antibiotic, dosage, and length of treatment in less severe cases is tabulated. It can be seen that penicillin is the most

Table 1. Summary of data regarding use of broad-spectrum antibiotics in clinical treatment of virus diseases

Disease	Antibiotic	Dosage	Length of treatment	Reference
Psittacosis	a. Penicillin	--	Of limited value	Welch, et al. (30)
	b. Aureomycin	25 mg/body wt. (Kg)		
	c. Terramycin	25 mg/body wt. (Kg)	Until pt. is afibrile	
	d. Chloramphenicol	50 mg/body wt. (Kg) (Above dosage at four to six hr. intervals)	(b, c, and d)	Wood (31)
Lymphogranuloma venereum	a. Aureomycin	1 to 5 gm daily	10 days to 2 weeks	Welch, et al. (30)
	b. Terramycin	.25 to .5 gm 4 times/day	3 to 4 weeks	
	c. Chloramphenicol	1 gm every 8 hr.	2 weeks	
Trachoma	a. Penicillin	--	Of limited value	Mitsui and Tanaka (20)
	b. Chloramphenicol	.25 gm every 3 hr.	At least 4 days	
	c. Aureomycin	.5% ointment 3 to 4 times a day	7 to 10 days	
	d. Terramycin		7 to 10 days	
Inclusion Conjunctivitis	a. Penicillin	--	Of questionable value	Welch, et al. (30)
	b. Aureomycin	--	24 hr.	
Molluscum contagiosum	Aureomycin	.25 gm twice daily	7 days	
Verrucae	a. Aureomycin	--	Only arrests spread	Welch et al. (30)
	b. Chloramphenicol	.75 gm daily	3 to 6 weeks (limited effect)	

ineffective of the commercial antibiotics, yet it has been tested against the large viruses more than any of the broad-spectrum group.

Status of Narrow-Spectrum Antibiotics

Many virus workers have been dauntless in their efforts to find substances that effectively inhibit these parasites. As a result of their efforts many compounds have been extracted that show specificity in antiviral activity. The antiviral spectrum is limited, however, to the one or two viruses that are susceptible to the action of the antibiotic. Semliki Forest virus, for instance, was isolated by Smithburn and Haddow (See Thompson and Lavender, 27) from Uganda mosquitoes and is quite fatal for mice. Although the virus has not been recovered from man, the antibodies for it have been demonstrated in human serum specimens collected in Africa and in Asia. Powell and Culbertson (21) have reported the successful isolation of a penicillium mold filtrate, designated serially as M5-8450 (hereafter referred to as 8450), which showed chemoprophylactic action against Semliki Forest and MM strain of encephalomyelitis virus in white mice. They found that the antibiotic protected the mice receiving intraperitoneal injections of arbitrary amounts of the antibiotic, whereas untreated controls died within two weeks. Subsequent work by Thompson and Lavender (27) has netted more convincing results in that protective action against 100 LD_{50} or more was obtained by injecting mice intraperitoneally with 8450. The

injection of filtrate must antedate the subcutaneous injection of virus by twenty-four hours. The route of inoculation is stressed here because other combinations of routes met with little or no inhibition of the virus. Efforts to establish antiviral immunity were without success as well as *in vitro* neutralization of the virus with 8450.

Antibiotic 8450 not only protects mice against Semliki Forest virus and the MM strain of encephalomyelitis virus, but against the Columbia SK strain of equine encephalomyelitis and the MEF-1 strain of poliomyelitis virus, according to Powell and Culbertson (21). Young cotton rats and seven-day-old hamsters were used for serial passage of the MEF-1 virus preliminary to its use in chemotherapy tests in albino Swiss mice. Before hamster passage, MEF-1 virus was virulent peripherically for suckling mice but was almost inocuous peripherally for Swiss mice. However, during hamster passage, a considerable degree of virulence developed for mice. This appeared to Powell and Culbertson (21) to offer a superior test method in suckling mice whereby 8450 could be tested against poliomyelitis virus before resorting to tests in monkeys. Both virus and filtrate, given intraperitoneally resulted in demonstrable chemoprophylaxis against MEF-1. Intraperitoneally injected virus was inhibited by intravenously injected filtrate. Suitable dosage for inhibition proved to be the same for MEF-1 and the other viruses studied, namely .05 to .25 milliliters of 8450 injected twenty-four hours before the virus.

In further work with mold filtrates the antibiotic helenine was isolated by Shope (25) from Penicillium mold species funiculosum on the Island of Guam in 1945 and maintained for study in the United States. It was found to be effective in protecting mice from the lethal action of swine influenza virus and it exerted a favorable therapeutic effect upon Semliki Forest virus in white mice. The therapeutic efficacy of helenine seemed somewhat peculiar, however, in that a level of dosage existed above which increasing the amount did not improve the therapeutic results. Below this level there was a gradual tapering off of protection as diminished doses of the material were administered--a plateau effect. As a consequence of this situation, infected animals failed to respond more favorably to multiple injections of helenine than to single injections when the dose fell within the plateau zone of maximum effect.

Helenine exerted its maximum effect when given within the first ten hours after viral infection but influenced detectably the course of virus infection when treatment was delayed for twenty-four hours (25). It was not effective against massive doses of virus and gave the best therapeutic results when used in animals infected with from ten to 1000 fatal doses of virus.

The mode of action of helenine against SK virus appeared to be inhibition of multiplication of the virus or temporary interference with the neuroinvasiveness of the virus. However, with Semliki Forest virus this was carried even a step further for helenine destroyed the virus frequently in such a manner as to prevent the development of any viral immunity by the cured host.

In further support of Shope's views (25) on the effectiveness of helenine in SK virus infection other therapeutic data were presented. The virus-neutralizing serum was obtained from swine recovered from intracerebral infection with SK virus. It was found that anti-SK virus serum, given forty-eight hours after infection, exerted no sparing effect despite the fact that from other experiments, such serum, given three to six hours after infection, was completely protective against the effects of SK virus. Helenine, on the other hand, gave moderate protection when administered three and twenty-four hours after infection. But a still better therapeutic effect was achieved in the group of mice treated with both helenine and anti-SK serum. The action of the helenine in this experiment was adjudged as that of a virustatic agent which prolonged the period during which specific virus-neutralizing serum could exert its virucidal activity. These studies demonstrate the trend of experimentation with fungal antibiotics.

Effect of Type-Specific Substances on Virus Multiplication

The designation, "type-specific" substances, refers to one specific component of bacterial origin which has been characterized in bacteriology for purposes other than antibiotic activity.

Polysaccharides. Certain viral infections may be modified by injection of suitable polysaccharides even though infection has been well established. Multiplication of pneumonia virus of mice (PVM) or mumps virus of mice has been inhibited by small

quantities of Friedländer bacillus type B polysaccharide in experiments by Ginsberg and Horsfall (11). Inhibitory activity is exhibited even when the carbohydrate is given four days after inoculation of either virus. Evidence points to the fact that the polysaccharide inhibits multiplication of mumps or pneumonia virus of mice by its ability to block an intracellular metabolic system of the host on which these viruses depend for multiplication (11, 16). The polysaccharide does not inactivate or demonstably alter either of the viruses as such, nor does the carbohydrate block the virus receptors of susceptible host cells or prevent the cells from adsorbing either virus.

The type-specific polysaccharides of Friedländer bacilli do not inhibit the multiplication of influenza A, influenza B, or Newcastle disease virus, according to Ginsberg and Horsfall (11). The fact that these three viruses are capable of unrestricted multiplication in the presence of large quantities of polysaccharide, whereas multiplication of either mumps virus or pneumonia virus of mice is inhibited by very small quantities of polysaccharide suggested to these workers that the two groups of viruses must require different host metabolic systems for multiplication. The extent of the pneumonic process appeared to be a function of the degree of viral multiplication--the greater the inhibition of multiplication, the less extensive was the pneumonia and the more probable was the recovery of animals treated with the polysaccharide. Effective therapy in mice with pneumonia virus of mice (PVM) was obtained with a single intra-

nasal injection of .02 milligram of the substance two or three days after virus inoculation. Treated animals recovered completely from the viral infection which was, in control animals, uniformly fatal.

The question might well be proposed: What is the mode of action of the polysaccharide material produced by microorganisms? The polysaccharide produced inhibition if given in the first two-thirds of the latent period of the multiplication cycle--within ten hours--but was ineffective if given at twelve hours or later. This suggested to Ginsberg and Horsfall (11) that the polysaccharide inhibited some relatively late step in the formation of pneumonia virus of mice.

Mucoproteins. Groupe and his workers (14) have studied the effect of the microbial product APM, prepared from culture filtrates of Achromobacter sp. 134, in suppressing nontransmissible pneumonia in mice induced by Newcastle disease virus. Large numbers of mice were inoculated intranasally with Newcastle disease virus. One-half of these received daily subcutaneous injections of 1.0 milligrams of APM beginning one hour after inoculation of the virus. Twelve to fifteen mice from each of the two groups were sacrificed daily for six days and on the ninth day after inoculation a determination was made of the lesion score. This was expressed as percentage pneumonia, and average weight of the lungs. It was found that pneumonia was definitely modified by subcutaneous injections of APM but only during the period of rapid extension of the lesion. APM did not increase in

effectiveness with an increase in inoculum, nor was an increased protectiveness afforded by a decrease in virus inoculum. Therefore, it was concluded that the suppressive effect of APM on pneumonia induced by Newcastle disease virus could be the result of competition between APM and virus for a cellular constituent.

By another series of experiments, Groupe et al. (14) have stimulated interest in the inhibitory effect of a mucoprotein substance isolated from the capsule-forming species Pseudomonas viscosa -122. The isolated substance was called viscosin and although showing no activity against Newcastle disease, the antibiotic exerted a marked protective effect in embryonating eggs subsequently infected with infectious bronchitis virus allantoically. In addition, a slight but detectable suppression was observed on the growth of influenza A virus infection in mice.

Receptor-Destroying Enzyme. One of the first major biological contributions to the understanding and interpretation of results obtained from studies on influenza virus multiplication was brought about through the use of receptor-destroying enzyme (RDE). This purified soluble enzyme was produced by cultures of Vibrio cholera (16). The enzyme imitated, nearly to the last detail, the activity of the enzyme supposed to be present at the virus surface. It stabilized red cells in the same manner as did the virus, making the cells unavailable for further agglutination when pre-treated with RDE. It was to be expected that studies with RDE would eventually be extended to infection-inhibition phenomenon. Thus, experiments by Powell and Culbertson (21),

using the PR-8 and LEE strains of influenza A and B viruses respectively, have shown that receptor destroying enzyme is capable of protecting mice from the neurotoxic effect of influenza. Paradoxical as it may seem, partially purified RDE, heated under conditions that destroyed its capacity to render chicken erythrocytes unagglutinable, exhibited the inhibition of these influenza strains. This statement should be supplemented with a brief description of the methods used by these workers. Toxic quantities of influenza A virus were prepared in the allantoic cavity of chick embryos and injected intracerebrally into mice. RDE was heated to a temperature of 120°C for thirty minutes and physiological saline used as a diluent in all experiments in which RDE was employed. A one per cent solution of partially purified RDE in saline containing 0.1 per cent $CaCl_2$ was divided into two equal parts. One-half served as the unheated control solution, the other half as the heated portion mentioned above. The protective activities of the control and heated solutions for mice were compared. Although the hemagglutination-inhibition titer of partially purified RDE was reduced from 1/2048 to 0, it was concluded that heat denatured RDE was either capable of protecting mice against influenza infection or that it contains a heat stable impurity that is protective.

Antibiotics Effective against Bacteriophage

It is interesting to note that many of the experiments conducted in assay of antiphage substances have been the result of

work done at the Botanical Garden, New York (15). The genus Streptomyces has been most productive in supplying many of the antibiotics effective against bacteria and the large viruses, i. e. terramycin, aureomycin, etc. It is from this genus that activity has been obtained against the bacterial viruses. Phagolessin A58 has been found to be moderately active against a number of the bacterial viruses (16). This was evidenced when fifty out of sixty phages tested exhibited sensitivity to a greater or lesser degree. The modus operandi of phagolessin, according to Cohen (6) seemed to be its ability to bring about direct phagocidal action and thereby caused irreversible inactivation of free phage particles after relatively brief contact. Cholera phage C is the standard phage for the demonstration of antibiotic sensitivity in a dilution of 1:40,000 containing 40,000 units per milliliter of phagolessin. However, subsequent experimentation by Hall and Asheshov (15), using the seven coliphages of the T system, acting on their common host Escherichia coli strain B, have revealed that only T_1, T_3, and T_7 proved to be sensitive to the antibiotic.

Comparing the properties of ultraviolet or X-ray inactivated phage with those inactivated by phagolessin revealed the following similarities and differences:

> (1) All three types of inactivated phage (T_1, T_3, and T_7) retain the ability to kill sensitive bacteria; (2) all three have the ability mutually to exclude an unrelated phage; (3) these properties mentioned above are lost at different rates by T_3 and T_7 and hence may be used to separate them.

Hall and Asheshov (15) found another unique feature to be apparent in the behavior of the three T phages sensitive to

phagolessin. T_1 loses its infectivity most rapidly, but loses its killing ability more slowly than T_3 and retains its ability to exclude other phages for a longer time than either T_3 or T_7. What has just been said regarding the activity of phagolessin has not taken into consideration the effect it has on the host cell E. coli B. An actively growing broth culture of the host, E. coli B., was diluted into several nutrient broth tubes containing varying amounts of phagolessin and incubated at 37°C. The rate of development of the bacteria was followed by plating a measured amount from each tube onto nutrient agar plates at fifteen-minute intervals. (1) In concentrations of 5.0 units per milliliter the bacteria developed normally; (2) at 10.0 units per milliliter some inhibition of bacterial growth was detected; (3) at 15.0 units per milliliter and higher bactericidal action was indicated by a loss in viable bacteria.

In other experiments it was observed that when suspension of inactivated phage were allowed to stand at 37°C for three to four hours, it became difficult to estimate the number of residual phage particles that remained active. It was assumed, therefore, that since there was an over-all increase in the active phage count following vigorous shaking of the suspension, that the residual active phage particles were not distributed in a random manner but were agglutinated by the antibiotic.

With all these phages the rate of inactivation increased with an increase in temperature and was greater in phosphate buffer than in nutrient broth, the rate of inactivation being studied over a sixty-minute period.

In view of the differences in sensitivity of the three phages to the antibiotic, different concentrations of antibiotic were used with each of the phages for the medium-temperature-inactivation studies. T_1--.1 units per milliliter, T_3--5.0 units per milliliter, and T_7--15.0 units per milliliter of the antibiotic. Each phage was incubated at 2°, 23°, and 37°C in phosphate buffer and at 37°C in nutrient broth.

In continuation of the work at the Botanical Garden with the genus Streptomyces, Strelitz, et al. (26) have isolated a yellow crystalline antibiotic substance with high antiphage activity. The strain producing the substance is presently unidentifiable; nevertheless, the name chrysomycin has been assigned to it. Chrysomycin has a wide bacterial spectrum as well as activity against many phage systems. In vivo toxicity studies revealed that twenty mice tolerated two milligrams of chrysomycin suspended in peanut oil, intraperitoneally. However, when five milligram amounts were given by the same route, paralysis of the hind legs became evident after the first twenty-four hours and loss of appetite persisted for several days. No further untoward reactions appeared, and the mice seemed normal at the end of two weeks. This tolerance could prove useful in testing the antiviral activity in mice susceptible to other viruses for which the mouse has proved a successful experimental animal. Table 2 shows the antiphage spectrum of chrysomycin.

Table 2. Antiphage spectrum of chrysomycin assayed by paper disc method (concentration .2 mg/ml)

Phages	Number	Positive results
Staphylophages	9	9
Streptophages	4	1
Entercocci phages	5	3
B. subtilis phage	1	1
B. cereus phage	1	1
E. coli phages	19	7
B. megaterium phages	4	0
Paratyphoid phages	4	0
S. typhosa phages	1	0
Dysentery phages	3	0
Pseudomonas phages	2	7
Cholera phages	11	1
Streptomyces griseus phages	1	1

Summary of Review of Literature

At the present time there is no antibiotic available for commercial purchase which will completely suppress virus growth. Diseases such as influenza and poliomyelitis, rabies, and Newcastle disease all remain to challenge the experimental virologist and as an enigma to the clinician.

Nevertheless, in recent years there has been a breakthrough in the field of therapy. There are four main approaches to the problem that have been undertaken by virus workers: (1) Investigation of the broad-spectrum antibiotics already in wide use against bacterial infections; (2) the production of narrow-spectrum antibiotics experimentally effective against specific viruses; (3) studies in which polysaccharides or muco-protein substances extracted from cultures of proliferating bacteria; and (4) the use of bacteriophage and the possible relation of sensitivity demonstrated by phage to that of the animal viruses.

Experimental results obtained from the susceptible laboratory species were generally based on death of the animal, macroscopic lesions in the various organs, cell pathology (tissue culture), or plaque formation.

On this subject one cannot help but be impressed by one of the main obstacles confronting the research: the effect of experimental design on the results one obtains in the laboratory--the individual behavior to which biological phenomena are prone.

In reviewing the literature there are many methods of evaluating antibiotics in laboratory experimentation. The procedures most commonly employed were herein reviewed.

MATERIALS AND METHODS

Regardless of the type of virus strain employed, laboratory tests for antiviral activity fall into two main groups--screening and evaluating. The screening test used in the present study was not elaborate in scope. It employed few chick embryos and was designed as a relatively crude check for possible activity. The sensitivity of the virus to the antibiotic was set moderately high and all indications of possible inhibitory activity, no matter how slight, were rechecked. Embryonated hen eggs were used exclusively throughout the testing, employing Newcastle disease virus strains that killed the embryo within a rather narrow time range. All inoculations were made when embryos were ten days old, and four eggs were used for each dilution of virus in the titration procedure.

Virus Stocks

Two strains of Newcastle disease virus were obtained from the Department of Bacteriology Laboratory, Kansas State College. These two highly virulent strains, VN-26 (Manhattan) and VN-36 (Texas GB), were used to serve as a rigid test for protection of antibiotic substances. The virus stocks were harvested from the allantoic fluid of chick embryos that had succumbed to infection by these strains. Approximately fifty milliliters of allantoic fluid of each strain were obtained in this manner and each stock was titered. To prevent contamination of the virus stocks, penicillin-streptomycin mixture was added so that stocks would

contain 200 units of penicillin and 1000 micrograms of streptomycin per milliliter. The stocks were aseptically ampoulized. in 1.0 cc amounts and preserved at freezing temperatures. In this way loss of titer by constant freezing and thawing was avoided inasmuch as a new ampoule with a known virus titer was used for each experiment. Also, possible contamination of the stock was reduced by this measure. Periodically, samples were removed from the ampoule used in a particular study and inoculated into broth as a test for sterility.

Antibiotic Materials Tested

With the exception of aureomycin, terramycin and Pseudomonas viscosa the cultures of all organisms and their products were obtained from the Department of Bacteriology Laboratory, Kansas State College. The former two were commercial preparations and the latter was obtained from Dr. Vincent Groupe of the Department of Microbiology, Rutgers University, New Brunswick, New Jersey. Each of the cultures of Klebsiella, Alkaligenes, Pseudomonas, and Xanthomonas was examined for purity.

Having obtained and identified the necessary organisms, the next step was to grow large numbers of organisms until a thick capsular growth became evident. Since a large quantity suitable for a series of experiments was needed, a modification of the Kochi method for the culture of Pseudomonas viscosa (Groupe, et al., 14) was employed. In preliminary experiments with Pseudomonas viscosa, Alkaligenes viscosus, and Klebsiella pneumoniae

the composition of the medium in which the antibiotic substance was produced was as follows:

Tryptone	5 gm.
Beef Extract	5 gm.
Asparagin	1 gm.
KH_2PO_4	5 gm.
$MgSO_4 \cdot 7H_2O$	0.25 gm.
Glycerol	20 cc.
Distilled water	1000 cc.

The pH of the medium was adjusted to 7.0 before sterilization in the autoclave. The medium was cooled and poured into Blake bottles. The medium was then seeded with the culture of the organism under study, and the bottles placed in a horizontal position at suitable temperatures of incubation. Within seven to ten days a thick capsular growth was evident and the medium had a pH of 8.5. The broth was clarified by centrifugation, adjusted to pH 6 and sterilized by heating to 121°C for twenty minutes. Embryo inoculation followed.

This medium was substituted by Bacto-Nutrient Broth medium in subsequent experiments with the remainder of the organisms since capsular growth was just as profuse. Nutrient broth has been recommended for use in testing the sensitivity of microorganisms to antibiotics (29). The composition of Bacto-Nutrient Broth was Bacto-Beef Extract, three grams, and Bacto-Peptone, five grams per liter of medium.

Preparation of Inocula

Virus. Nutrient broth was used as the diluent in all experiments. Exactly 0.5 milliliter of virus, suspended in allantoic fluid, was added to the first tube in a ten-tube series (VN-26) or a seven-tube series (VN-36), each tube containing 4.5 milliliters of broth. The virus was further diluted serially to give final dilutions of 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}.

Terramycin and Aureomycin. Quantities of 500 milligrams of each of these antibiotics were obtained in sterile dispensing bottles. To this amount ten milliliters of distilled water was added and 0.1 milliliter was inoculated into chick embryos so that each embryo received 5.0 milligrams of antibiotic.

Uncrystallized compounds. Thirty milliliters of growing bacterial cultures were autoclaved for fifteen minutes at fifteen pounds pressure. The suspension was then centrifuged. Following centrifugation, the cells were resuspended in the supernatant in a ratio of 0.5 milliliter of cells in 7.5 milliliters of supernatant. This was done in order that bacterial cell suspensions would be standardized throughout all of the experiments. The suspension was transferred to sterile test tubes and steamed at 100°C for fifteen minutes to insure sterility.

Titration by Embryo Infectivity

Inoculation Procedure. Inoculation via the allantoic cavity in fertile hen's eggs has been most frequently employed in the

evaluation of antibiotics in virus diseases (16). The procedure used for inoculation of the virus and the substance tested was the same, the inoculum being standardized at 0.1 milliliter antibiotic per 0.1 milliliter of virus. Except, as in the case of time experiments, it was never the purpose of the experiment to delay inoculation of the substance. All embryos that were inoculated with virus were immediately reinoculated with the antibiotic substance to be tested. Additional inoculations were carried out to ascertain the level of toxicity of the antibiotic substance. The dosage tolerance was surveyed in a range of 0.1, 0.5, 0.2, 0.25 to 1.0 milliliter of antibiotic substance. At the 0.1 milliliter level none of the substances proved toxic.

Aside from the toxicity controls, virus controls were inoculated at each dilution. Consequently, for every four embryos inoculated with virus plus test substance, there were four embryos inoculated with virus alone from the same dilution. This measure served as a check on virus titer as well as the chemotherapeutic value of the substance tested.

Titration by Hemagglutination

Hemagglutination Test. The agglutinating antigen for hemagglutination tests was obtained by aseptically bleeding a chicken that was supplied by the Department of Bacteriology, Kansas State College. A 0.5 per cent suspension of red blood cells was used in all hemagglutination tests: 1:10 dilutions in the first tube of a ten-tube series were made. The final dilutions being recorded were 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280,

1:2560, and 1:5120. An eleventh tube served as control. The hemagglutination titer of the virus stock was obtained before any other tests were run. Then the allantoic fluid was harvested from two embryos at each dilution in which deaths had occurred and used as virus source in the hemagglutination tests. If the titer of the virus was reduced, the antibiotic substance was judged to have been operative in its reduction of infection.

The Beta-Hemagglutination-Inhibition Test. This test was employed using the antibiotic substances tested. Virus stock was diluted so that ten hemagglutinating units per tube were obtained. This method is used for the identification of unknown antiserum in which the antiserum instead of the virus is diluted. For the purposes of this study the unknown serum was substituted by the antibiotic substance. Any inhibition in the most concentrated dilutions indicated its effectiveness. The dilutions were the same as reported for the hemagglutination tests.

Use of LD_{50} in Evaluating Antibiotic Substances

It is essential that some definite means of evaluating the test substance be employed. This was done in terms of LD_{50} comparisons made with the controls and the treated embryos. By definition the LD_{50}, as calculated by the Reed and Muench Assay (22), is the dilution at which fifty per cent of the test animals survive and fifty per cent are killed. Results depend on: (1) The positive response which is the manifestation of

successful infection in that a proper amount of virus material reaches a susceptible host cell and infects the cell; (2) the propagation of infection to other cells; and (3) the titer of the virus--number of infectious units under the specific conditions of the test. An example of the calculated LD_{50} is given below. Column "C" refers to embryos dying during the first twenty-four hours of non-specific causes. Column "G" is obtained by adding the number of dead in Column "E" from the bottom, the number alive in Column "F" from the top and dividing the total number dead in Column "F" by the total number dead and alive in the particular dilution. This number should be multiplied by 100 to obtain per cent mortality.

Table 3. Calculation of the fifty per cent endpoint according to the method of Reed and Muench

Column A	Column B	Column C	Column D	Column E	Column F		Column G
Dilution:	No.	Traumatics : 1st day :	2nd day D	3rd day D :	Total A	D :	Mortality (%)
10^1	3	(1)	2	-	0	8	100
10^2	3		3	-	0	6	100
10^3	3		3	-	0	3	100
10^4	3		-	-	3	0	0
10^5	3		-	-	6	0	0

Proportional distance = $\frac{50\% - \text{last dilution above}}{\text{Last dilution above} - \text{first dilution below}}$

Table 3 (concl.).

P. D. = $\frac{100 - 50}{100 - 0}$ = .5

P. D. = $10^{3.5}$ per 0.1 ml

LD_{50} = log .5 = 3.17 x log 10 (dilution factor) = 31,700 virus particles per ml, or 3.17×10^4

LD_{50} = log of 3.17 = 5.0

LD_{50} = 10^5 or 5 virus particles per ml are present at 10^3 dilution

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Screening Procedure of Antibiotic Substances for Activity

Having described the general methods used in screening and evaluating the test substance, a description of the experiments follows.

Fertile eggs were marked as they were candled to note the position of the embryo and thereby to facilitate inoculation of the allantoic membrane. Dilutions of each virus strain were made and 0.1 milliliter of each dilution was inoculated allantoically into the embryonated eggs with a sterile syringe. The openings were then sealed with paraffin and the eggs incubated at 37°C for seventy-two hours. These eggs were candled twice daily. The infectivity titers of the viruses were established in this way, and rechecked using this same procedure whenever embryo infectivity titrations were run.

As embryo deaths occurred, the eggs were placed in the refrigerator so that the allantoic fluids could be harvested. The fluids were obtained by pipette and placed into sterile bottles. The hemagglutination titer of the virus stock had to be obtained

so that a comparison could be made with the titer of fluids harvested from treated embryos. Simultaneously, another series of tubes was set up for hemagglutination titration using the fluids harvested from controls as the virus source. The hemagglutination titers were found to be the same.

In order to establish the dosage levels of aureomycin, terramycin, substances produced by Alkaligenes viscosus, Pseudomonas viscosa, Klebsiella pneumoniae, and the effect of broth, twenty embryos were inoculated with 0.1, 0.2, 0.25, 0.5, and 1.0 milliliter amounts of the substances. Four embryos were inoculated per quantity of substance.

To another set of eggs, treated as mentioned above with virus, was added 0.1 milliliter of the test substance, this dosage tolerance having been established.

The allantoic fluids of the treated embryos were harvested and refrigerated in the usual manner as was the fluid from the controls and a comparison of hemagglutination titers was made. The allantoic fluids from controls in embryo infectivity experiments were used as controls in hemagglutination tests. In further assay of hemagglutination activity of the antibiotic substance, the beta-hemmagglutination-inhibition test was employed. A ten-tube series was set up and instead of diluting the virus as in the hemagglutination titration, the antibiotic substance was diluted to find whether, in less concentrated amounts it had the ability to inhibit hemagglutination. Using the protocol of Cunningham for the beta-hemagglutination test, the antibiotic substance was substituted for antiserum.

The LD_{50} for the embryo infectivity in control and treated embryos was calculated and the whole series of tests and procedures was repeated.

Experiments in which Xanthomonas pruni-11 was used have been reported separately because, according to the original plan, the substance showing the greatest amount of significant antiviral activity would be subjected to further studies. The general screening for Xanthomonas species followed the plan used for the other antibiotic substances, i. e. infectivity of embryos and hemagglutination activity.

Identification and Characterization of Active Component of Xanthomonas pruni-11

In the experiments with Xanthomonas pruni-11 four sterile 150 milliliter Erlenmeyer flasks containing twenty-five milliliters nutrient broth were seeded with one loopful of stock culture Xanthomonas pruni-11. This culture was constantly agitated from forty-eight to ninety-six hours and the cells and supernatant were separated by centrifugation at 3000 rpm or by filtration. Subsequently, chick embryos were inoculated with the cellular suspension of Xanthomonas pruni-11 or with the cells or supernatant alone. Four embryos per dilution served as controls. Other embryos were inoculated for the establishment of the toxicity level.

In order to identify and characterize the active component originating from Xanthomonas pruni-11, this broth culture received several treatments as described in Table 8 of the results.

RESULTS

It is the purpose of the results to show that products of microorganisms were obtained that showed virustatic activity. The results have been presented in tabular form on the pages that follow. Tables 4 and 5 show the organisms from which the substance tested was obtained. In addition, they include the calculated LD_{50} for each of the substances used in the initial screening procedure. Table 4 reports tests of antibiotic against Newcastle virus strain VN-26 (Manhattan); Table 5, Newcastle strain VN-36 (Texas GB). The results are tabulated so that the LD_{50} of each virus strain may be compared with the LD_{50} obtained after treatment with the antibiotic substance.

It can be seen from these tables that terramycin and the inhibitory substance from Xanthomonas pruni-11 gave the lowest LD_{50}, repeatedly, with both strains of Newcastle disease virus. Zero per cent mortality was not recorded for terramycin in the calculation of LD_{50} until those dilutions were reached at which the controls showed fifty per cent survival, i. e. 10^8 dilution of virus, when infected with the VN-26 (Manhattan) strain of Newcastle disease virus. However, against the same virus strain, Xanthomonas pruni-11 gave protection to four out of four embryos at the 10^7 dilution; all four of the controls died at this dilution.

Table 6 includes the LD_{50} for additional screening of Xanthomonas species. This was done as a verification of the results of tables 4 and 5. Table 6 shows that Xanthomonas

pruni-11 gave consistently good results against both strains of Newcastle disease virus. However, in the initial screening Xanthomonas papvericola-5 gave a lower LD_{50} than did pruni-11. The results obtained in the initial screening using Xanthomonas papvericola-5 were not reproducible. The consistency of the results from Xanthomonas pruni-11 lead to its selection from this group of species for more precise studies.

Table 7 presents hemagglutination titers of Newcastle virus after treatment with the antibiotic substances. In addition, a standard hemagglutination-inhibition test of strain VM-26 is presented in the last column of Table 7. The test is usually run with antiserum, but in this case dilutions of the antibiotic substance were substituted for dilutions of antiserum. Antiviral activity is represented by a drop in titer in cases where the compounds were mixed with virus (columns 1 and 2), but in the last column, antiviral activity is represented by an increase in titer. The results recorded for Xanthomonas pruni-11 were obtained before the cells and the supernatant were separated. Subsequent titrations, using the concentrated filtrate, resulted in complete inhibition of hemagglutination and a beta-hemagglutination-inhibition titer of 1/6400.

Although Klebsiella pneumoniae and Xanthomonas papvericola-5 showed the most hemagglutination-inhibition activity, the LD_{50} of embryo infectivity was not correspondingly impressive.

Table 4. LD_{50} of embryo infectivity after antibiotic treatment, using strain VN-26 (Manhattan) of Newcastle virus.

Source of compound	LD_{50} strain VN-26	Rating of compound
Nutrient broth (control)	$10^{8.5}$	
Aureomycin (Lederle commercial)	$10^{8.3}$	Poor
Terramycin (Pfizer commercial)	$10^{5.8}$	Good
Alkaligenes viscosa	$10^{6.2}$	Good
Klebsiella pneumoniae	$10^{6.5}$	Fair
Pseudomonas viscosa-122	$10^{6.4}$	Good
Xanthomonas campestris-3	$10^{9.0}$	Negative
Xanthomonas campestris-119	$10^{8.7}$	Negative
Xanthomonas hyacinthi-8	$10^{7.3}$	Fair
Xanthomonas juglandis-107	$10^{7.3}$	Fair
Xanthomonas papavericola-5	$10^{5.1}$	Excellent
Xanthomonas pelargonii-126	$10^{8.6}$	Negative
Xanthomonas pelargonii-139	$10^{9.3}$	Negative
Xanthomonas phaseoli-20	10^{7}	Fair
Xanthomonas pruni-11	10^{6}	Good
Xanthomonas (unidentified species) -9 U	10^{6}	Good

≤ 8.5	Negative
8.4-7.5	Poor
7.4-6.5	Fair
6.4-5.5	Good
≥ 5.4	Excellent

Table 5. LD_{50} of embryo infectivity after antibiotic treatment, using strain VN-36 (Texas GB) of Newcastle virus

Source of compound	LD_{50} strain VN-36	Rating of compound
Nutrient broth (control)	$10^{5.2}$	
Aureomycin (Lederle commercial)	$10^{6.2}$	Negative
Terramycin (Pfizer commercial)	$10^{3.2}$	Good
Alkaligenes viscosa	$10^{4.3}$	Poor
Klebsiella pneumoniae	$10^{3.8}$	Fair
Pseudomonas viscosa-122	10^{6}	Negative
Xanthomonas campestris-3	$10^{3.5}$	Fair
Xanthomonas campestris-119	$10^{3.5}$	Fair
Xanthomonas hyacinthi-8	$10^{5.4}$	Negative
Xanthomonas juglandis-107	$10^{5.2}$	Negative
Xanthomonas papavericola-5	$10^{2.23}$	Good
Xanthomonas pelargonii-126	10^{3}	Good
Xanthomonas pelargonii-139	10^{4}	Poor
Xanthomonas phaseoli-20	$10^{3.7}$	Fair
Xanthomonas pruni-11	10^{3}	Good
Xanthomonas (unidentified species) -9 U	10^{5}	Negative

$\leq 10^{4.5}$ Negative
4.4- 4.0 Poor
3.9- 3.5 Fair
≥ 3.4 Good

Table 6. Screening of inhibitory substances produced by species of Xanthomonas. The bases for ratings are the same as those used in tables 4 and 5.

Species	LD_{50} VN-26	Rating of compound	LD_{50} VN-36	Rating of compound
Controls (virus alone)	$10^{8.5}$		10^{5}	
Xanthomonas campestris-3	$10^{9.0}$	Negative	$10^{3.5}$	Fair
Xanthomonas campestris-119	$10^{8.6}$	Negative	$10^{3.5}$	Fair
Xanthomonas hyacinthi-8	$10^{7.3}$	Negative	$10^{4.5}$	Negative
Xanthomonas juglandis-107	$10^{7.3}$	Negative	10^{5}	Negative
Xanthomonas papavericola-5	$10^{5.6}$	Good	10^{3}	Good
Xanthomonas pelargonii-126	$10^{8.6}$	Negative	10^{3}	Good
Xanthomonas pelargonii-139	$10^{9.3}$	Negative	10^{4}	Poor
Xanthomonas phaseoli-20	10^{7}	Fair	$10^{3.7}$	Fair
Xanthomonas pruni-11	10^{6}	Good	10^{3}	Good
Xanthomonas (unidentified species) -9 U	10^{9}	Negative	10^{5}	Negative

Table 7. Hemagglutination titer of Newcastle disease virus after treatment with compound. The first two columns represent hemagglutination titers after the virus was mixed with compound. The last column represents standard beta HI results wherein dilutions of the compound were substituted for antiserum.

Source of compound	Compound plus VN-26	Compound plus VN-36	HI titer with compound VN-26
Nutrient broth (control)	640	320	10
Aureomycin	40	10	400
Terramycin	0	0	1600
Alkaligenes viscosus	80	0	10
Klebsiella pneumoniae	0	0	6400
Pseudomonas viscosa	80	0	800
Xanthomonas campestris-3	80	80	800
Xanthomonas campestris-119	320	40	400
Xanthomonas hyacinthi-8	320	80	3200
Xanthomonas juglandis-107	320	80	3200
Xanthomonas papavericola-5	0	0	6400
Xanthomonas pelargonii-126	40	40	200
Xanthomonas pelargonii-139	320	320	0
Xanthomonas phaseoli-20	80	40	1600
Xanthomonas pruni-11	40	40	3200
Xanthomonas (unidentified species) -9 U	40	20	600

In the first two columns a drop in titer indicates antiviral activity. In the last column antiviral activity is represented by an increase in titer.

Table 8 shows the LD_{50} obtained by treating Xanthomonas pruni-11 in different ways as compared with the LD_{50} of the controls. It can be seen that the cells of Xanthomonas pruni-11, when inoculated without the supernatant, gave no protection against Newcastle disease virus strain VN-26 (Manhattan). The LD_{50} for the controls was consistent as shown by comparison with the LD_{50} of treated embryos. Ultraviolet light was used as a substitute for heat-killing the organisms in the event that heat might have destroyed antibiotic activity present in the cells. Whereas the ultraviolet treated cells did not effect embryo infectivity, the ultraviolet treated supernatant gave a higher degree of protection than did the filtered or autoclaved supernatant.

It was thought that concentration of the supernatant might result in an increased protection for chick embryos inoculated with Newcastle disease virus. The broth constituents dialyzed through the cellophane membrane leaving the active component inside. The LD_{50} of embryo infectivity in the interim between dialysis and concentration is recorded in Table 8. Also, the LD_{50} after two-thirds of the water was removed from the supernatant is recorded. The partially purified supernatant afforded increased protection to chick embryos when dialyzed and concentrated. This is evident from the lowered LD_{50}: $10^{5.2}$ as compared with $10^{6.0}$ when the crude culture of cells and supernatant was inoculated.

Table 8. LD_{50} obtained by various treatments of Xanthomonas pruni, using strain VN-26 (Manhattan)

Inoculum	LD_{50}
Virus alone (controls)	$10^{8.5}$
Autoclaved cells and supernatant	10^{6}
Ultraviolet inactivated cells	Toxic
Antibiotic inactivated cells	Toxic
Autoclaved cells (suspended in saline)	Toxic
Autoclaved supernatant	$10^{7.6}$
Seitz filtered supernatant	$10^{6.6}$
Ultraviolet treated supernatant	10^{6}
Dialyzed supernatant (before concentration)	$10^{5.2}$
Dialyzed supernatant (after concentration 2/3 evaporated)	$10^{5.2}$

The designation "toxic" refers to embryos that did not survive even at dilutions in which controls survived. The antibiotics used for inactivation of cells were penicillin and streptomycin.

Table 9 includes data from a time experiment using autoclaved Xanthomonas pruni-11 cells, suspended in the supernatant, as the inoculum. The purpose of the experiment was to see how long after infection the antibiotic substance could be administered and still show protective activity.

Table 10. shows a comparison of total deaths of the two most inhibitory substances used to treat chick embryos infected with Newcastle disease virus. Terramycin and Xanthomonas pruni-11 were compared with the controls.

Table 10 shows a comparison of the cumulative deaths of embryos treated with terramycin, Xanthomonas pruni-11 inhibitory substance and the controls. These data were subjected to statistical analysis and it was concluded that the presence of Xanthomonas pruni-11 reduced the rate of deaths as dilution rate increased to a significantly higher degree than for terramycin. For example, at the 10^5 dilution, it would be expected by statistical analysis that the cumulative deaths for terramycin would be 10.3 embryos while for Xanthomonas pruni-11 it would be only 5.2, approximately half. However, a more critical analysis, using a larger number of embryos per dilution, obviously would have greater significance.

Table 9. Time experiment using autoclaved cells and supernatant *Xanthomonas pruni*-11 strain VN-26 (Manhattan)

Inoculum	Time interval between virus and antibiotic inoculation	LD_{50}
Virus alone (controls)		$10^{8.5}$
Cellular suspension	0	10^{6}
Cellular suspension	40 minutes	10^{7}
Cellular suspension	80 minutes	10^{7}
Cellular suspension	120 minutes	10^{8}
Cellular suspension	180 minutes	$10^{8.5}$

Table 10. Comparison of cumulative deaths for dilution for statistical analysis. Virus treated with terramycin and with Xanthomonas pruni are compared with control (untreated) virus. A means alive. D means dead.

Dilution of virus (VM-26)	Controls				Terramycin				Xanthomonas pruni			
	Actual		Accumulated		Actual		Accumulated		Actual		Accumulated	
	A	D	A	D	A	D	A	D	A	D	A	D
10^1	0	4	0	32	0	4	0	20	0	4	0	19
10^2	0	4	0	28	3	1	3	16	0	4	0	15
10^3	0	4	0	24	3	2	5	15	0	4	0	11
10^4	0	4	0	20	2	2	7	13	0	4	0	7
10^5	0	4	0	16	1	3	8	11	3	1	3	3
10^6	0	4	0	12	2	2	10	8	3	1	6	2
10^7	0	4	0	8	1	3	11	6	3	1	9	1
10^8	2	2	2	4	2	2	13	3	4	0	13	0
10^9	2	2	4	2	3	11	16	1	4	0	17	0
10^{10}	4	0	8	0	3	0	20	0	4	0	21	0

The analysis of these data by the Kansas State College Statistical Laboratory indicated that Xanthomonas pruni-11 protected embryos to a significantly greater degree than terramycin.

DISCUSSION

Experimental results obtained were based on death of the embryo and upon suppression of hemagglutination ability by the virus. The effectiveness of the antibiotic in suppressing virus multiplication was rated as "good," "fair," "poor," or "negative."

Treatment with Broad-Spectrum Antibiotics

Aureomycin and terramycin did not show equal effectiveness when used against Newcastle disease infection. Terramycin was the more active of the two and gave a lower LD_{50} than did other crude extracts tested. Terramycin prolonged the life of embryos at a given dilution. This effect was reproducible but indeed short-lived; embryos succumbed by the sixth day of infection instead of the usual seventy-two-hour period. The LD_{50}, obtained when terramycin was used in embryo infectivity titrations, indicated that the strains of Newcastle disease virus under study were sensitive to the action of terramycin. However, the fact that some embryos died at almost every dilution of the virus (see Table 10) would lead to the interpretation that the effectiveness of terramycin should be minimized.

Effect of *Klebsiella pneumoniae* Polysaccharide

While inhibiting the virus of Newcastle disease, as evidenced by the LD_{50} obtained, the activity of this substance was

at times inconsistent. It was thought that this effect might have been due to the broth used; consequently, several runs were made using saline as the diluent. The same effect was obtained and the variance in protection was a factor in rejecting this substance for more intensive study.

Effect of Pseudomonas viscosa Inhibitory Substance

The negative results obtained by Groupe (14) with viscosin were verified in the embryo infectivity titration when the Kochi method was employed. However, when nutrient broth was substituted for the special medium used by Groupe, protection was obtained from the crude culture. It was possible that the virus was affected by a synthesized product in the medium which rendered it unsusceptible to the inhibitory effect of the antibiotic substance. Or, the organism Pseudomonas viscosa could have been inhibited in its production of a specific factor necessary for the inhibition of Newcastle disease virus. Further studies with this organism could prove valuable.

Effect of Alkaligenes viscosus Inhibitory Substance

This substance, also, was increased in its protective effect when cultured on nutrient broth instead of the special medium used by Groupe. It could be that dialysis of the culture before inoculation into chick embryos could rid the special medium of some toxic substance that inhibited the protection effect of the antibiotic substance.

Effect of Inhibitory Substance from Xanthomonas pruni-11

Xanthomonas pruni-11 was chosen because of its reproducible good results both in embryo infectivity titrations and hemagglutination titrations. As Table 8 indicated there was an increase in inhibition with increased purification of the substance. Dialysis against distilled water resulted in the antibiotic substance being left inside the membrane and the components of the broth diffusing out. The fact that the active substance is heat stable and possibly colloidal has led to the supposition that the active component might be a protein linked to a stable molecule. There exists also the possibility of its being a polysaccharide, but this seems less likely since the thick capsular growth was brought down by centrifugation along with the cells, and the filtrate in the Seitz system was free of the capsular material which was apparently left with the cells.

It appeared from the results of the experiment in which the cells of Xanthomonas pruni-11 were inoculated alone that they contained a substance toxic to chick embryos. This poisonous substance was not extracted from the cell by saline solution. Heating at 121°C did not destroy the deleterious effect of the cells on the chick embryos, nor did ultraviolet light destroy this toxicity. Cell inactivation by antibiotic mixture only resulted in the embryos treated in this manner dying before the controls, i. e. before seventy-two hours. Therefore, it was established that the protective component produced by Xanthomonas

pruni-11 was retained in the supernatant fluid when centrifuged, inside the dialyzing membrane when subjected to dialysis. Furthermore, it was heat stable and effectively concentrated, to allow the survival of all embryos at 10^5 dilution of virus, whereas at this same dilution, all embryos in the control group died.

The data reported on cumulative deaths obtained for the two most protective substances, terramycin and Xanthomonas pruni-11 was analyzed statistically. It was implied by this analysis that the presence of Xanthomonas pruni-11 reduces the rate of deaths as dilution rate increases to a significantly different degree than for terramycin. There are, of course, some precautions to be used in extending these results at this point: (1) The numbers used per dilution are small and hence the time relationship might differ considerably; (2) the analysis is made on the cumulative deaths, which are not independent observations for the different dilution rates; and (3) the number of dilutions is small so that considerable variability might be encountered if the experiment is repeated in exactly the same manner. However, these difficulties do not reduce the significance of the relationships noted, but only leave open the question of reproducing the effects a number of times with a larger number of test embryos.

SUMMARY

The extraction from microorganisms of substances which inhibit the growth of other organisms has become a standard approach to therapy of disease. It was the purpose of this thesis to investigate the production of bacterial substances effective against Newcastle disease virus. The bacteria, Klebsiella pneumoniae, Alkaligenes viscosus, Pseudomonas viscosa, and Xanthomonas species, were cultured in nutrient broth. The cultures were partially purified by autoclaving at 121°C and the cellular suspension inoculated into ten-day-old chick embryos previously inoculated with Newcastle disease virus strains VN-26 (Manhattan) and VN-36 (Texas GB). Protection was measured by calculation of LD_{50} after treatment with the antibiotic substance as compared with the controls receiving virus alone. Measurement of additional inhibition of virus was made by hemagglutination titration. From the initial screening using this procedure on all the bacterial substances tested, one substance was selected that afforded the best reproducible results. Xanthomonas pruni-11 was selected and subjected to further study. After centrifugation the inoculation of Xanthomonas pruni-11 cells and supernatant was effected separately. The cells were inactivated by ultraviolet light, antibiotic mixture, and autoclave and resuspended in broth or saline. In each case, inoculation of cells was found to be toxic to embryos. However, the supernatant alone gave increased protection.

Following dialysis of the pruni-11 filtrate, concentration was effected by the use of a water aspirator. The hemagglutina-

tion-inhibition titer was increased to maximum inhibition with concentration. Embryos treated with this dehydrated filtrate survived for twenty days while the untreated controls failed to survive beyond seventy-two hours.

The conclusion is drawn from these experiments that a heat stable, non-dialyzable substance originating from Xanthomonas pruni-11 is capable of affording protection to chick embryos previously infected with Newcastle disease virus.

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THE EFFECT OF CERTAIN ANTIBIOTIC SUBSTANCES ON NEWCASTLE DISEASE VIRUS

by

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The chemotherapy of virus diseases meets with difficulties which are not present in the control of bacterial infections. Virus multiplication occurs only inside of cells and utilizes the enzymatic machinery of the cells. Therefore, any therapeutic agent that interferes with virus multiplication may also damage cellular activity. However, a review of literature indicates that certain chemical and biological materials may interrupt certain virus infections.

The purpose of the thesis was to investigate products of bacterial origin which might be effective against Newcastle disease virus.

The first phase of the research dealt with the screening of bacterial cultures which produced heavy capsules, since other investigators have found antiviral activity in such materials. The cultures screened included those readily available in the Department of Bacteriology, i. e. Klebsiella pneumoniae, Alkaligenes viscosus, Pseudomonas viscosa, and several species of Xanthomonas. After cultures had grown in nutrient broth, they were autoclaved and the cellular suspensions were inoculated into ten-day-old chick embryos previously infected with Newcastle disease virus. Protection was measured by comparison of LD_{50} with controls that received virus alone. In addition, measurements were made of the ability of the antibiotic substances to inhibit the hemagglutination activity of the virus.

The second phase of the research was to select the organism (Xanthomonas pruni-11) which produced the most effective antibiotic

substance and to identify and characterize, partially, the active component. Upon centrifugation, the supernatant was found to be more active than the sedimented cells and heavy capsular material. The active component of the supernatant was not dialyzable and was not destroyed by autoclaving. The activity could be increased by removal of excess water either by evaporation or by a vacuum-aspirator process.

The conclusion is drawn from these experiments that a heat stable, non-dialyzable substance originating from Xanthomonas pruni-11 is capable of affording protection to chick embryos previously infected with Newcastle disease virus.