TISSUE CULTURE STUDIES OF THE INHIBITION OF LEE INFLUENZA VIRUS BY ISOPROPYL BIGUANIDE

by

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TABLE OF CONTENTS

		Page
1.	Introduction	1
2.	Historical Review	3
3.	Experimental Methods	9
	Culture methods	9
	Equipment	9
	Nutrient fluid	10
	Tissue	11
	Virus	12
	Incubation	13
	Titrations	13
	Inhibition Studies	14
	Determination of inhibitory concentrations	14
	Toxicity to tissues	15
	Attempts to show reversal of inhibition	16
4.	Experimental Results	18
	Culture methods	18
	Virus	18
	Incubation	19
	Titrations	21
	Inhibition Studies	23
	Determination of inhibitory concentrations	23

TABLE OF CONTENTS

(continued)

		Page
	Attempts to show reversal of inhibition	35
5.	Discussion	46
6.	Summary and Conclusions	49
7.	Bibliography	51

LIST OF TABLES

Table		Page
1	Effect of Concentration of Inoculum and Length of Incubation Period Upon Growth of Lee Influenza Virus in Tissue Culture	20
2	Effect of Shaking Upon Growth of Lee Influenza Virus in Tissue Culture	22
3	Growth of Lee Influenza Virus in Tissue Cultures in the Presence of DL-citrulline	25
4	Inhibition of Lee Influenza Virus in Tissue Cultures by L-canavanine	26
5	Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide Hydrochloride.	29
6	The Effect of Isopropyl Biguanide Upon the pH of Tissue Culture Fluids	30
7	Relationship of Dosage of Isopropyl Biguanide and Virus Inoculum to the Inhibition of Lee Influenza Virus in Tissue Cultures	31
8	Growth of Lee Virus in Chorioallantoic Membrane After Exposure of the Tissue to Isopropyl Biguanide	33
9	Attempted Reversal by L-Arginine of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide	36
10	Influence of Arginine on the Inhibition of Lee Influenza Virus in the Chick Embryo by Isopropyl Biguanide	38
11	Attempted Reversal by L-Lysine of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide	40
12	Attempted Reversal by Adenine of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide	41

LIST OF TABLES

continued

Table		Page
13	Attempted Reversal by Desoxyribonucleic Acid of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide	42
14	Attempted Reversal by p-Aminobenzoic Acid of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide	44
15	Attempted Reversal by Folic Acid of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide	45

TISSUE CULTURE STUDIES OF THE INHIBITION OF LEE INFLUENZA VIRUS BY ISOPROPYL BIGUANIDE

INTRODUCTION

It is of interest to investigate the effect of certain chemical compounds upon the growth of the animal viruses, both as a fundamental study and in the search for chemotherapeutic agents. The use of metabolic antagonists is particularly well suited to both types of study. Our knowledge of the metabolism of the bacterial cell has been greatly increased through investigations with antimetabolites, and it is not unreasonable to assume that these types of compounds can also reveal valuable information in regard to the nutrition and metabolism of viruses.

The need for chemotherapeutic agents against the virus diseases is at once apparent, and recent studies have indicated that a valuable approach to this problem lies in the use of metabolic antagonists, especially those interfering with the amino acid and nucleic acid metabolism of the host cell-virus system. The present study was undertaken to investigate, by means of the tissue culture technique, the inhibitory effect upon Lee influenza virus which has been found in this laboratory when chick embryos infected with this virus are treated with isopropyl biguanide hydrochloride. Two other compounds which have been found to influence growth of the virus in the chick embryo, DL-citrulline

and L-canavanine, were also briefly studied.

HISTORICAL REVIEW

The ability of influenza virus to reproduce and to produce hemagglutinins in tissue cultures was first demonstrated by Weller and Enders (21, pp.127-128). Using the Maitland type of suspended cell culture, they observed that egg-adapted influenza A virus grew rapidly, the hemagglutinin appearing early in the incubation period and continuing to rise until reaching a maximum on the third day. Cultures prepared by suspending minced chorioallantoic membrane in a balanced salt solution gave an increase in the hemagglutinin titer of 535 times in 72 hours. Similar cultures of minced brain and of minced amniotic membrane also gave significant but lesser increases.

This study was of fundamental importance because the demonstration of virus growth in a simple tissue culture provided a rapid and convenient method of determing the effect of drugs and other compounds upon the multiplication of the virus and, with various modifications, this method is now being widely used in such investigations.

Tissue culture studies with several different viruses have established that various structural analogues of natural metabolites, which have been shown to be antagonistic to the latter in one or more biological systems, are capable of inhibiting development of such viruses in the cultures. Several analogues of amino acids and of the purine components of nucleic acids have been found active in this respect.

Ackermann (2, pp.339-342) found that graded concentrations of DL-methoxinine produced a proportional inhibition of the growth of influenza A virus. The inhibition was reversed by methionine, increasing concentrations of the amino acid giving progressively greater reversal. Methoxinine did not appear to be destructive to the virus itself and inhibitory concentrations did not affect the endogenous respiration of the tissue. The observation that a combination of betaine plus homocysteine was able to produce only partial reversal of the inhibition by methoxinine led Ackermann to conclude that the mode of action of this antimetabolite was interference with some function of methionine, rather than with its synthesis.

The inhibition of poliomyelitis virus in tissue cultures by another methionine analogue, DL-ethionine, (3, pp. 368-369) again demonstrated the effectiveness of this type of antimetabolite. The inhibitory action of ethionine was not due to a direct destructive action on either the virus or the tissue and appeared to be specifically directed against its analogue, methionine. Although only partial reversal of the inhibition of poliomyelitis virus was obtained by methionine, the action of ethionine was apparently similar to that of methoxinine in the case of influenza virus, and was considered to be due to interference with some phase of methionine metabolism involved in biosynthesis of the virus.

Thompson and Wilkin (19, p.435) investigated the action of β-2-thienylalanine on vaccinia virus in suspended cell tissue culture and found marked suppression of virus multiplication by this compound. The addition of both β-2-thienylalanine and its analogue phenylalanine to the cultures permitted growth of the virus, indicating that phenylalanine in some way participated in the proliferation of the virus.

Inhibition of influenza A virus by ϥ-amino acid derivatives in which reversal could not be demonstrated was that caused by several ϥ-aminosulfonic acids (1, pp.364-365). Of particular interest was the inhibition caused by ϥ-aminophenylmethanesulfonic acid. This compound is structurally similar to phenylalanine and tyrosine but all attempts to reverse the inhibition caused by the sulfonic acid by means of these two amino acids were unsuccessful. It was suggested that the action of the sulfonic acids was upon a cellular enzyme which had only a limited specificity for its substrate, that is, upon a single enzyme which normally had several substrates. Thus a complete reversal by any single substrate would not be expected.

Several diamidines and acridines have been observed to inhibit the growth of influenza A and B and mumps viruses in tissue cultures. Eaton, et al. (5, pp.323-325) found that pentamidine and stilbamidine inhibited mumps virus in concentrations of 0.5-5.0 μg per ml, while slightly higher

concentrations were required to suppress growth of the influenza viruses. The minimal inhibitory concentration of the diamidine necessary for inhibition of the virus increased with the initial concentration of virus present and with the time after inoculation of the virus at which the drug was added.

Eaton, Cheever and Levenson (6, pp.465-473) observed that the viruses of feline pneumonitis, meningopneumonitis, mumps, and influenza B were markedly or completely inhibited in the chick embryo by nitroacridine, while both the nitro and chloroacridine were effective against mumps and influenza A and B in tissue cultures.

Both the diamidines and the acridines appeared to exert their influence by an interference with the host cell metabolism. The inhibitory concentration of pentamidine produced an inhibition of respiration of the host tissue and it was suggested that the diamidine possibly diverted energy from the synthetic processes of the host cell, thereby interfering with synthesis of the virus. Inhibitory concentrations of the acridines did not affect tissue respiration but were quite similar to the concentrations inhibiting multiplication of the tissue fibroblasts, suggesting that the acridines also acted by interfering with some synthetic process of the host cell.

Noting that many microorganisms are inhibited by analogues and derivatives of the nucleic acid components,

Thompson and co-workers (18, pp.530-532) investigated the effect of substituted purines and pyrimidines on vaccinia virus. Using suspended cell tissue cultures, they demonstrated the inhibition of the virus by 2,6-diaminopurine, benzimidazole, and several halogenated purines. Three triazolopyrimidines and 2-aminopurine were inactive. Reversal experiments revealed that the inhibition of the virus by benzimidazole was not reversed by adenine, guanine or yeast nucleic acid. However, both yeast nucleic acid and adenine counteracted the inhibition by 2,6-diaminopurine. One of the limitations of tissue cultures is shown by the finding that neither 2,6-diaminopurine nor benzimidazole protected mice against a mouse-adapted strain of the vaccinia virus.

Growth of Russian spring summer encephalitis virus in tissue culture has also been shown to be inhibited by 2,6-diaminopurine (9, p.151). A concentration of the purine of 10^{-3} molar suppressed virus growth, even when added to the culture as long as 24 hours after inoculation of the virus. As was found with vaccinia virus, the inhibition of the encephalitis virus by 2,6-diaminopurine was reversed by adenine but not by guanine. Partial reversal by adenine was obtained when the reversing agent was added 24 hours after the 2,6-diaminopurine.

Biguanide compounds have exhibited inhibitory action against diversified organisms. Acridyl biguanides (16, pp. 293-294) were shown to inhibit the growth of gram positive

and gram negative microorganisms, including staphylococci, hemolytic streptococci, Escherichia coli and Salmonella typhosa. A biguanide compound of particular interest is 1-p-chlorophenyl-5-isopropyl biguanide. This compound, commonly known as paludrine or chlorguanide, is an active antimalarial (4, pp.724-725) and has also shown fungistatic and tuberculostatic (15, pp.159-160) activity.

Isopropyl biguanide has been investigated for its effect on influenza B virus in the chick embryo (12, p.44) and was found to give marked inhibition of virus growth when administered in a dose of 10 mg per egg. The compound produced partial inhibition when given as late as 24 hours after inoculation of the virus. The inhibitory action of isopropyl biguanide did not appear to be due entirely to a direct effect on the virus. A concentration of 2 mg isopropyl biguanide per ml did not show an in vitro effect on the hemagglutinating activity of the virus when held at 37°C. for 24 hours. No effect on infectivity could be detected within 24 hours at 10°C., nor within 2 hours at 37°C. In 24 hours at the latter temperature, however, infectivity decreased to a significantly lower level in the presence of the compound than was observed in its absence.

EXPERIMENTAL METHODS

Culture Methods

Influenza virus has two properties which make it an especially useful agent for chemotherapeutic studies. It is easily cultivated in a simple tissue culture and the presence of the virus is easily detected by its property of agglutinating red blood cells. Although the virus is easily cultivated *in vitro* some difficulty was experienced in finding satisfactory methods of preparing the tissue and the virus inoculum. However, the procedures finally developed, which are essentially those of Tamm (17, pp.560-561), have given satisfactory results and high titer cultures were obtained.

Equipment. All glassware coming into contact with the tissue or the nutrient glucosol was cleaned in dichromatesulfuric acid cleaning solution, followed by rinsing fifteen times in running tap water. The glassware was further rinsed three times in distilled water and once in glass distilled water. The glassware was sterilized by dry heat at 160°C. for one hour. All cotton plugs and bakelite caps were sterilized separately by autoclaving and transferred aseptically to the tubes.

New rubber stoppers were boiled for about 20 minutes in approximately 0.5 per cent sodium hydroxide solution, rinsed in tap water, then boiled in dilute hydrochloric

acid, (a 1:150 dilution of concentrated HCl). The stoppers were then rinsed thoroughly in tap water and three times in distilled water. Used rubber stoppers and bakelite caps were rinsed for 30 minutes in hot running tap water and three times in distilled water. Rubber stoppers were sterilized by autoclaving at 15 pounds pressure for 20 minutes.

Metal instruments were washed with hot water and Tide detergent and rinsed thoroughly in hot running tap water, followed by distilled water. Instruments were sterilized by dry heat at 160°C. for one hour.

Nutrient Fluid. The nutrient fluid was prepared as two solutions having the following compositions:

Glucosol solution.	NaCl	8.0 grams
	$CaCl_2$	0.2 grams
	$MgCl_2.6H_2O$	0.5 grams
	Glucose	1.0 grams
	Glass distilled H_2O	to 1000 ml.
Buffer solution. pH 7.28	Na_2HPO_4 (anhydrous)	7.105 grams
	KH_2PO_4	2.269 grams
	Phenol red	0.02 grams
	Glass distilled H_2O	to 1000 ml.

The two solutions were autoclaved separately at 15 pounds pressure for 20 minutes in Pyrex bottles fitted with bakelite screw caps and combined in equal volumes immediately before use. Penicillin and streptomycin were added to the nutrient glucosol (combined glucosol and buffer solutions) to give final concentrations of 10 units per ml and 40 μg per ml respectively.

The antibiotics were prepared as stock solutions and

frozen at -20°C. in serological tubes in 1 ml amounts. The concentration of the penicillin solution was 1.499 mg per ml and the concentration of the streptomycin solution was 10.0 mg per ml. For use, the solutions were thawed and 0.1 ml of each antibiotic added to 25 ml of nutrient glucosol. Each tube of antibiotic solution was opened only once and unused portions were discarded.

Tissue. Chorioallantoic membranes from 10 or 11-day old chick embryos were obtained by first disinfecting the egg shells with tincture of iodine and then aseptically removing the shell and shell membrane from over the air sac by means of sterile forceps. The embryo was tipped into a sterile petri dish and the chorioallantoic tissue was grasped with sterile forceps and cut free from other tissues with sterile scissors. The membranes were washed by suspending in nutrient glucosol in centrifuge tubes, centrifuging at 1365 r.p.m. for 2 minutes, removing the fluid and repeating this process twice more. The membranes were then placed in sterile petri dishes and cut into pieces approximately 2 x 2 cm in size by means of sterile cataract knives. These tissue fragments varied in weight from 75-120 mg. The fragments were pooled and added to the culture tubes at random. One piece of tissue was added to each culture tube, which contained 2 ml of nutrient glucosol. Culture tubes were 25 x 100 ml Pyrex tubes and were closed with number 4 rubber stoppers.

Virus. The Lee strain of influenza B virus was used for all experiments. This strain was obtained from Dr. F. L. Horsfall of the Rockefeller Institute and had been through a large number of passages in the chick embryo. It was maintained in chick embryos throughout this study and the allantoic fluid from infected embryos was used as the source of the virus inoculum for the tissue cultures.

The stock virus was prepared by diluting infected allantoic fluid 1:1000 in veal infusion broth of pH 7.1-7.4 and inoculating 0.1 ml into the allantoic sac of 10 or 11-day old chick embryos. The eggs were incubated at 35°C. for 40 hours. After chilling for one hour at -20°C. the fluids were harvested and the hemagglutinin titer of each fluid was determined. Fluids with the highest titers, 1:320 or above, were pooled and stored at 4°C. until used.

For inoculation of the tissue cultures, the virus was used within 24 hours after harvesting. The inoculum for these cultures was prepared by diluting infected allantoic fluids in nutrient glucosol and adding 0.1 ml of the dilution to each culture tube. Preliminary studies revealed that satisfactory titers could be obtained with a virus dilution of 1:10 and this dilution was used as the inoculum in most experiments. It was not possible to use a standardized lyophilized or frozen virus inoculum and therefore the actual infectivity titer of the inoculum varied from experiment to experiment even though the same dilution was used.

Incubation. The cultures were incubated at 35°C. on a reciprocating shaker with a stroke length of 8 cm and a frequency of 90 strokes per minute.

Titrations. Hemagglutinin titers were determined by a modification of Salk's method (14, pp.87-98). The red blood cells were prepared by collecting the blood from 5-6 chickens in Alsever's solution. The cells were washed three times by centrifuging in physiological saline solution at 1500 r.p.m. for 7 minutes. Following the third washing the cells were packed in graduated centrifuge tubes by centrifuging for 10 minutes and a 10 per cent suspension by volume prepared in physiological saline. The cells were stored at 4°C. and were kept no longer than 7 days. A 0.5 per cent suspension was prepared immediately before use.

Serial two fold dilutions of infected fluids were made in physiological saline solution in serological tubes. The volume in each tube was 1.0 ml. An equal volume of a 0.5 per cent suspension of washed chicken red blood cells was added to each tube and the tubes thoroughly shaken.

Readings were made after 45 minutes at room temperature. The highest dilution showing complete agglutination was considered to be the endpoint and the titers were expressed as the reciprocal of this dilution. The hemagglutinin titer of the tissue cultures was determined after 48 hours incubation, as preliminary studies had shown that the titers did not increase after this time.

Infectivity titers were determined by preparing serial ten fold dilutions of virus in veal infusion broth of pH 7.1 to 7.4 and inoculating 0.1 ml of each dilution into groups of 10 or 11-day old chick embryos. At least 6 embryos were used for each dilution. After incubation for 48 hours and chilling, the fluid were harvested and tested for the presence of hemagglutinins at a final dilution of 1:2. If hemagglutination occurred the presence of infection was indicated. Eggs showing no hemagglutinin were considered to be uninfected. The 50 per cent embryo infectivity end point was calculated by the method of Reed and Muench (13, pp.493-497). The EID_{50} obtained from this data is the dose of virus causing infection in 50 per cent of the embryos.

Inhibition Studies

Determination of Inhibitory Concentrations. To determine the concentration range over which a compound might affect the virus in tissue cultures, the range of 5.0 to 0.008 mg compound per ml nutrient glucosol was arbitrarily chosen, intermediate concentrations differing by a factor of approximately two. The compounds were prepared by dissolving the final desired concentration of the compound directly into the nutrient glucosol and adding the resulting solution after sterilization to the culture tubes as the suspending medium. The pH of such solutions was adjusted with sodium hydroxide or hydrochloric acid if it had been altered from the desired range of 7.2-7.3 by addition of the compound.

All solutions containing supplements were sterilized by filtering through Pyrex sintered glass filters of UF porosity.

Tissue cultures containing the various concentrations of the compound, together with control cultures, were inoculated with virus and incubated on the shaker at 35°C. for 48 hours. At this time the virus concentration in each culture was measured by hemagglutinin titration. The amount of virus present in the cultures just before incubation was usually determined by titrating one culture immediately after inoculation. This provided a basis for demonstrating multiplication of the virus in the cultures.

It was desirable in the case of isopropyl biguanide to determine the relation between the effective concentration of the compound and the amount of virus present. This was done by varying the dilution of a virus inoculum of known infectivity while using a constant concentration of isopropyl biguanide.

Toxicity to tissue. Since viruses are known to propagate only in the presence of living cells, the effect of inhibitory compounds could be due to the killing of the host cells, rather than to any influence on the virus. It was therefore necessary to determine whether the concentrations of isopropyl biguanide employed in the experiments caused irreversible damage to the tissue. There are several methods which may be used for this type of investigation but the following procedure appeared to be the most satisfactory for

the present study.

The tissue was prepared and added to culture tubes containing the desired concentration of isopropyl biguanide, as previously described. Virus was added to these tubes and to the control tubes. Control tubes were also included which were not inoculated. After incubation for 40 hours the fluids from all the culture tubes were removed as completely as possible by 5 ml pipets and replaced by nutrient glucosol containing no drug. All tubes, including the control tubes not previously inoculated, were reinoculated with virus and the cultures again incubated for 40-48 hours. The hemagglutinin titer of the fluids was determined at the end of each incubation period.

If the tissue had not been irreversibly damaged by contact with the compound, then growth of the virus should occur during the second period of incubation, when the compound had been removed. Fulton and Armitage (10, p.225) have reported that deterioration of the tissue begins to occur at 60 hours, even when not infected by virus. The control tubes not inoculated until the second incubation period would therefore show the extent of the lowering of the virus titer as a result of changes occurring in the tissue during the prolonged incubation period.

Attempts to Show Reversal of Inhibition

The procedure used in the experiments attempting

reversal of inhibition was identical with that used in investigating the inhibitory effects of the compound, except that the proposed reversing agent was put into solution with the isopropyl biguanide. The cultures were incubated and the hemagglutinin titer determined as previously described.

In these experiments isopropyl biguanide was used in a concentration of 0.1 mg per ml in the culture field. Compounds being examined for possible reversing effect were used over a concentration range of 5.0-0.008 mg per ml. Intermediate concentrations differed by a factor of 5.

EXPERIMENTAL RESULTS

Culture Methods

To obtain satisfactory growth of the virus in tissue cultures it was necessary to determine the optimal dilution of the virus inoculum, the optimal length of the incubation period and to compare results with both shaken and stationary cultures.

Virus. To determine the concentration of the inoculum which would give the most satisfactory growth of the virus in the tissue cultures, several experiments were carried out in which the growth given by different virus dilutions was compared.

A composite of two such experiments is presented in Table 1. Reference to this table shows that, as would be expected, the final virus titer was directly related to the initial concentration of the virus added to the culture. The undiluted virus inoculum gave the greatest growth in the cultures, the geometric mean of the hemagglutinin titer of these cultures being 317.2 on the second day. A 1:10 dilution of the inoculum also gave good growth of the virus, resulting in a mean titer of 139, while a 1:100 dilution of the inoculum gave very poor growth of the virus in the tissue cultures. The initial titer of the cultures receiving the undiluted virus inoculum was 10, and the initial titer of the lower virus dilutions was 0. The initial titer was

determined by titrating one culture immediately after inoculation. Thus the virus titer in cultures receiving the undiluted virus increased about 32 fold on the average in 44 hours.

The initial titers of the cultures in all subsequent experiments fell between 20 and 0 when the inoculum was 0.1 ml of a 1:10 dilution of allantoic fluid virus. In most cases the titer at this point was 0.

On the basis of the data in Table 1 a virus inoculum consisting of 0.1 ml of a 1:10 dilution of fresh allantoic fluid virus was chosen as the routine inoculum. The growth obtained with this inoculum was large enough so that multiplication or interference with multiplication of the virus could be unquestionably demonstrated, and yet inhibition would be more easily shown with the relatively smaller amount of virus.

Incubation. Both shaken and stationary tissue cultures are used in various laboratories and the stationary type were used in preliminary experiments in the present investigation. However, very poor growth of the virus was obtained in these cultures and therefore a series of experiments was made to determine the effect upon virus growth of shaking during incubation.

The data in Table 2 will show that incubation under shake conditions was definitely favorable to growth of the virus. With an undiluted virus inoculum, the cultures

TABLE 1

Effect of Concentration of Inoculum and Length of Incubation Period Upon Growth of Lee Influenza Virus in Tissue Culture

Culture Number	Virus inoculum dilution[3]	Virus hemagglutinin[1] present in tissue cultures incubated[2] for periods of	
		44 hours	68 hours
1	Undiluted	480	240
2		480	240
3		320	240
4		240	240
5		240	160
6		240	
	Geometric mean	317.2	221.3
1	1:10	160	160
2		160	160
3		160	120
4		120	80
5		120	80
6		120	60
	Geometric mean	139.0	102.6
1	1:100	20	
2		10	
3		10	
4		10	
5		10	
6		10	
7		2-10	
8		2-10	
	Geometric mean	8.9	

1. All figures in the table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:20 were not made. Cultures entirely negative at 1:20 were assigned a value of 0. Those showing 3+ agglutination at 1:20 were assigned a value of 10. Those showing very slight agglutination at 1:20 were assigned a range value of 2-10.

2. Virus titrations were made after incubation at 35°C. on a reciprocating shaker.

3. The virus inoculum was 0.1 ml of various dilutions of allantoic fluid virus freshly harvested from chick embryos.

incubated on the shaker were 2-5 times higher in virus titer than identical cultures which were not shaken. The effect of shaking was even more striking with the cultures inoculated with a 1:10 virus dilution. In experiment 1 the mean titer of the cultures which were shaken was at least 25 fold greater than the titer of the stationary cultures, while in experiment 2 the mean titer of the shaken cultures was almost 200 fold greater than that of the stationary cultures. For this reason incubation of the cultures on the shaker was employed throughout this study.

It should be pointed out that the titers of stationary cultures did increase somewhat if incubated for a sufficiently long period of time, 96 hours or longer. There was therefore a time advantage in using shaken cultures, which reached a high titer by 48 hours.

Titrations. It was desirable to establish the point during incubation at which the maximum hemagglutinin titers were reached. A number of experiments were carried out in which the titers of the cultures were determined at 44 and 68 hours. A typical experiment is shown in Table 1.

Examination of the data in Table 1 reveals that the hemagglutinin titer of the cultures did not increase after the second day of incubation, the titers appearing to be even slightly lower on the third day. These results are in accord with those reported in the literature and on the basis of these determinations the length of incubation chosen for the present experiments was 44-48 hours. The final

TABLE 2

Effect of Shaking Upon Growth of
Lee Influenza Virus in Tissue Culture

Culture Number	Virus dilution[3]	Virus hemagglutinin[1] present in tissue cultures incubated[2] under the following conditions: Cultures shaken during incubation	Cultures stationary during incubation	
Experiment 1				
1	Undiluted	60	10	
2		40	10	
		49	10	Geometric mean
1	1:10	30	0	
2		20	0	
		24.5	0	Geometric mean
Experiment 2				
1	Undiluted	160	80	
2		120	60	
		138.5	69.3	Geometric mean
1	1:10	240	0	
2		160	0	
		195.9	0	Geometric mean
Experiment 3				
1	Undiluted	120	40	
2		60	30	
		84.9	34.6	Geometric mean
1	1:10	30	2-10	
2		20	2-10	
		23.4	4.5	Geometric mean

1. All figures in the table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:20 were not made. Cultures entirely negative at 1:20 were assigned a value of 0. Those showing 3+ agglutination at 1:20 were assigned a value of 10. Those showing very slight agglutination at 1:20 were assigned a range value of 2-10.

2. Virus titrations were made after 44-48 hours incubation at 35°C. on a reciprocating shaker.

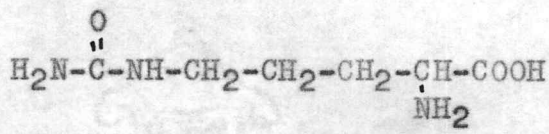
3. The virus inoculum was 0.1 ml of various dilutions of allantoic fluid virus freshly harvested from chick embryos.

hemagglutinin titration was made at the end of this time.

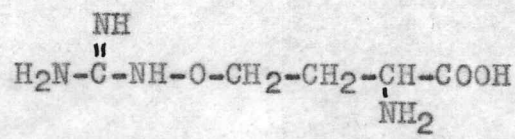
Inhibition Studies

Three compounds, DL-citrulline, L-canavanine, and isopropyl biguanide hydrochloride, were chosen for preliminary investigation. The latter two were selected because they have been found to produce marked inhibition of the Lee influenza virus in the intact chick embryo in studies carried out in this laboratory. DL-citrulline has been found slightly stimulatory to the virus under these conditions.

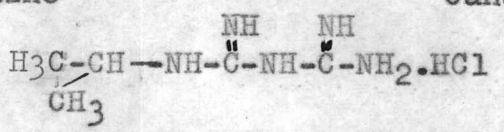
These compounds have the following structural formulas:



Citrulline



Canavanine



Isopropyl biguanide hydrochloride

Determination of inhibitory concentrations. When satisfactory conditions for growth of the Lee influenza virus in tissue cultures had been worked out, experiments were then set up to study the effects of certain compounds previously found to influence the development of the Lee virus in the chick embryo. Two of these were studied only very briefly. These were DL-citrulline and L-canavanine.

Although DL-citrulline has shown a slightly stimulatory effect upon this virus in the chick embryo it did not

appear to have any influence, either stimulatory or inhibitory, upon the virus in tissue cultures. Cultures containing citrulline in a concentration range of 10-2 mg per ml did not exhibit increased growth of the virus, as shown in Table 3. This data also reveals that inhibition of the virus did not take place either, as the final hemagglutinin titer was about the same in all citrulline containing cultures as in the control. Thus it would appear that the slight stimulation of the virus produced by citrulline in the chick embryo depends on some factor present in the intact embryo itself and not found in the tissue cultures.

L-canavanine was found to be quite inhibitory for Lee influenza virus in tissue cultures. Table 4 shows that various degrees of inhibition of the virus occurred over a concentration range of 4-0.02 mg per ml. Complete or nearly complete suppression of virus growth was observed with canavanine in concentrations of 4-0.2 mg per ml. Partial inhibition was found with 0.05 and 0.02 mg per ml, the mean titers in these cultures being about half of the control titers. These differences were found to be statistically significant when analyzed by Fischer's "t" test (8, pp.114-174). A canavanine concentration of 0.01 mg per ml had no effect upon the virus growth; the final titers of cultures containing this concentration were almost identical with the control titers.

It is of interest to note that a marked degree of

TABLE 3

Growth of Lee Influenza Virus in Tissue Cultures in the Presence of DL-citrulline

Culture Number	Virus hemagglutinin present in tissue cultures containing the following concentration of DL-citrulline				
	10.0 mg per ml	5.0 mg per ml	2.0 mg per ml	Controls, no citrulline	
1	40	40	40	60	
2	30	30	40	40	
3	30	20	40	40	
4	30	20	30	40	
5	20	20	30	20	
6	20	20	20	20	
7	20	10	20	20	
8	20	2-10	20	20	
9	10	2-10	2-10	2-10	
	22.9	15.0	23.3	24.1	Geometric mean

1. All figures in the table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:20 were not made. Cultures entirely negative at 1:20 were assigned a value of 0. Those showing 3+ agglutination at 1:20 were assigned a value of 10. Those showing very slight agglutination at 1:20 were assigned a range value of 2-10.

2. Virus titrations were made after 44-48 hours incubation at 35°C. on a reciprocating shaker.

3. The virus inoculum was 0.1 ml of a 1:10 dilution of allantoic fluid virus freshly harvested from chick embryos.

TABLE 4

Inhibition of Lee Influenza Virus in Tissue Cultures by L-canavanine

Culture Number	Virus Hemagglutinin Present in Tissue Cultures Containing the Following Concentration of L-canavanine			
Experiment 1	4 mg per ml	2 mg per ml	1 mg per ml	Control, no canavanine
1	0	2-10	0	160
2	0	0	0	160
3	0	0	0	120
4	0	0	0	120
5	0	0	0	120
6	0	0	0	
Geometric mean	0		0	125.9
Experiment 2	0.5 mg per ml	0.2 mg per ml	0.1 mg per ml	Control, no canavanine
1	0	0	120	320
2	0	0	40	240
3	0	0	20	240
4	0	0	10	240
5	0	0	0	160
6	0	0	0	160
Geometric mean	0	0	9.9	220
Experiment 3	0.05 mg per ml	0.02 mg per ml	0.01 mg per ml	Control, no canavanine
1	160	120	240	240
2	120	120	160	240
3	120	120	160	160
4	80	80	160	160
5	60	80	160	120
6	40	80	160	120
Geometric mean	87.3	97.7	171.2	166.4

1. All figures in the table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:20 were not made. Cultures entirely negative at 1:20 were assigned a value of 0. Those showing 3+ agglutination at 1:20 were assigned a value of 10. Those showing very slight agglutination at 1:20 were assigned a range value of 2-10.

2. Virus titrations were made after 44-48 hours incubation at 35°C. on a reciprocating shaker.

3. The virus inoculum was 0.1 ml of a 1:10 dilution of allantoic fluid virus freshly harvested from chick embryos.

Table 4, continued

When the mean value for the hemagglutinin in the group of cultures containing .05 mg of canavanine per ml in Experiment 3 is compared with the control group using Fischer's "t" test, (8, pp.114-174), and using logs of individual titers, a "t" value of 2.63 is obtained. For 10 degrees of freedom this corresponds to a P value between .02 and .05, indicating the observed difference is very unlikely to be due to chance.

In a similar manner the mean value for the group containing .02 mg of canavanine per ml is shown to be significantly lower than the control group.

inhibition of the virus was obtained in these cultures with a canavanine concentration of 0.1 mg per ml. This is in contrast to earlier work with this compound employing the intact chick embryo, where a concentration roughly 10 fold greater, or about 1.0 mg per ml, was required in the allantoic fluid to produce a comparable degree of inhibition.[1] It thus appears that under the conditions existing in tissue cultures the growth of the virus is more sensitive to this antagonist than under the conditions found in the allantoic cavity of the chick embryo.

The major part of the work described in this thesis was devoted to a study of the effect of isopropyl biguanide hydrochloride upon the development of the Lee virus in tissue cultures. This compound had previously been found inhibitory to the virus in the intact chick embryo. It was felt that a study of this activity in tissue cultures, where it might be magnified in degree, might serve to throw further light on the mechanism of the inhibition.

In the first part of the study of this compound in tissue culture it was necessary to determine if the compound was inhibitory to the virus under these conditions and if so, what minimum concentration would be effective. The results of experiments bearing on this point are shown in

[1] Soike, Kenneth F. Unpublished research on the inhibition by canavanine of Lee influenza virus in the chick embryo. Oregon state college, Dept. of bacteriology, 1952.

Table 5. It will be noted that isopropyl biguanide was definitely inhibitory for the influenza B virus over a concentration range of 2-0.1 mg per ml. Inhibition appears less complete at the lowest concentration. Although the data in Table 5 do not show the minimum inhibitory concentration, below which inhibition does not occur, other results indicated that this concentration was probably near 0.1 mg per ml. Depending upon the particular stock virus used, slight virus growth has been shown to occur in some cases at this concentration.

The inhibition by isopropyl biguanide was not due to an adverse effect upon the pH of the culture fluids. The data in Table 6 show that the pH of cultures incubated in the presence of 0.2 mg isopropyl biguanide was only about 0.1 pH unit lower than control cultures.

The second point to be investigated was the relationship between the minimum inhibitory concentration of isopropyl biguanide and the size of the virus inoculum. A summary of experiments concerned with this relationship appear in Table 7. It can be seen that the concentration of isopropyl biguanide required for inhibition increased with an increase in the virus inoculum. A virus inoculum giving a concentration of $10^{6.1917}$ EID_{50} per ml of culture fluid was only partially inhibited by 0.1 and 0.2 mg isopropyl biguanide per ml but almost completely inhibited by 0.5 mg per ml. Virus in a concentration of $10^{5.1917}$ EID_{50} per ml was almost

TABLE 5

Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide Hydrochloride

Culture Number	Virus hemagglutinin present in tissue cultures containing the following concentration of isopropyl biguanide hydrochloride					
	2.0 mg per ml	1.0 mg per ml	0.5 mg per ml	0.2 mg per ml	0.1 mg per ml	Controls no IBG[4]
1	0	0	10	2-10	30	80
2	0	0	0	2-10	10	60
3	0	0	0	0	10	60
4	0	0	0	0	0	40
5	0	0	0	0	0	40
6	0	0	0	0	0	30
7			0	0	0	20
8			0	0	0	20
9			0	0	0	10

1. All figures in the table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:20 were not made. Cultures entirely negative at 1:20 were assigned a value of 0. Those showing 3+ agglutination at 1:20 were assigned a value of 10. Those showing very slight agglutination at 1:20 were assigned a range value of 2-10.

2. Virus titrations were made after 44-48 hours incubation at 35°C. on a reciprocating shaker.

3. The virus inoculum was 0.1 ml of a 1:10 dilution of allantoic fluid virus freshly harvested from chick embryos.

4. IBG is isopropyl biguanide hydrochloride.

TABLE 6

The Effect of Isopropyl Biguanide Upon the pH of Tissue Culture Fluids

Cultures containing:	pH of fluids from cultures before and after incubation at 35°C. for 45 hours:	
0.2 mg IBG[2] per ml	Initial pH	Final pH
Culture number		
1	7.28	6.99
2	7.28	6.98
3	7.28	6.94
4	7.28	6.92
5	7.28	6.92
6	7.28	6.92
Control cultures, containing no IBG		
Culture number		
1	7.27	7.10
2	7.27	7.01
3	7.27	7.0
4	7.27	7.0
5	7.27	7.0
6	7.27	7.0

1. The virus inoculum was 0.1 ml of a 1:10 dilution of allantoic fluid virus freshly harvested from chick embryos.

2. IBG is isopropyl biguanide.

TABLE 7

Relationship of Dosage of Isopropyl Biguanide and Virus Inoculum to the Inhibition of Lee Influenza Virus in Tissue Cultures

Culture Number	Concentration IBG[4] per ml	Virus hemagglutinin present in tissue cultures containing the following concentration of isopropyl biguanide with the indicated virus inoculum: $10^{6}.1917$ EID_{50} virus per ml	$10^{5}.1917$ EID_{50} virus per ml	$10^{4}.1917$ EID_{50} virus per ml
1	0.1 mg	80	20	0
2		80	2-10	0
3		80	0	0
4		40	0	0
5		30	0	0
6		30	0	0
7	Control,	640	480	40
8	no IBG	640	160	20
1	0.2 mg	80	10	0
2		30	2-10	0
3		30	2-10	0
4		30	0	0
5		20	0	0
6		10	0	0
7	Control,	480	320	60
8	no IBG	320	160	60
1	0.5 mg	20	0	0
2		10	0	0
3		2-10	0	0
4		2-10	0	0
5		2-10	0	0
6		0	0	0
7	Control,	960	320	60
8	no IBG	480	240	10

1. All figures in the table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:20 were not made. Cultures entirely negative at 1:20 were assigned a value of 0. Those showing 3+ agglutination at 1:20 were assigned a value of 10. Those showing very slight agglutination at 1:20 were assigned a range value of 2-10.
2. Virus titrations were made after 44-48 hours incubation at 35°C. on a reciprocating shaker.
3. The virus inoculum was a 1:10 dilution of a frozen stock of allantoic fluid virus held at -20°C. for no longer than 3 days.
4. IBG is isopropyl biguanide.

completely inhibited by the two lower concentrations of isopropyl biguanide and was completely inhibited by 0.5 mg per ml. Growth of the next lower concentration of virus, $10^{4.1917}$ EID_{50} per ml was completely prevented by all concentrations of isopropyl biguanide. It should be pointed out that here also a concentration of 0.1 mg of this compound per ml is able to produce significant inhibition of the virus in tissue culture. In the case of chick embryo infections, it was found earlier that about ten times this concentration was needed to give a similar degree of inhibition.

Toxicity to tissues. Obviously many chemical agents could prevent the growth of a virus in a tissue culture system by causing irreversible damage to the tissue and making it incapable of supporting virus multiplication. Although the results of the chick embryo work seemed to indicate that this was not the mechanism it was desirable to obtain additional evidence directly from tissue cultures. The method employed has been described in detail earlier.

The results of experiments of this type with isopropyl biguanide appear in Table 8. It will be noted that the data suggest some slight degree of toxicity of isopropyl biguanide for allantoic membranes in tissue cultures at a concentration of 0.2 mg per ml. After contact with this concentration of the compound for 40 hours the tissue appeared to yield slightly less virus when reincubated in the absence

TABLE 8

Growth of Lee Virus in Chorioallantoic Membrane After Exposure of the Tissue to Isopropyl Biguanide

Culture Number	Hours of Incubation	Virus hemagglutinin present in tissue cultures containing the following concentration of isopropyl biguanide hydrochloride			
		0.2 mg IBG per ml	0.1 mg IBG per ml	Control plus virus	Control; uninoculated
1	40[1]	2-10	30	160	0
2		2-10	20	120	0
3		0	20	120	0
4		0	0	120	0
5		0	0	80	0
6		0	0	80	0
		Virus hemagglutinin developing after removal of fluids, replacement with nutrient glucosol, reinoculation with virus, and reincubation			
1	88[2]	40	40	40	40
2		16	30	30	30
3		8	30	30	20
4		8	20	30	20
5		8	20	30	20
6			2	30	8

1. All culture fluids replaced with nutrient fluid containing no isopropyl biguanide after 40 hours incubation.

   All tubes, including control tubes not inoculated during first incubation period, were reinoculated with a 1:10 virus dilution.

2. All culture tubes were reincubated for 48 hours, the final titer therefore was determined after a total of 88 hours incubation.

of the drug than was found in control cultures under similar conditions. However, the difference is not great enough to be statistically valid with the small number of cultures.

The tissue did not appear to be irreversibly damaged by an exposure to 0.1 mg isopropyl biguanide. Tissue which had been incubated with this amount of the compound for 40 hours supported virus growth equally as well as control cultures when the test cultures had been reincubated in the absence of isopropyl biguanide. If the tissue had been damaged by the inhibitor the final titer of the test cultures after the reincubation would not have been as high as the control cultures.

Examination of the titers of the control cultures inoculated with virus at the end of the first and second incubation period shows that the tissue was less favorable for virus growth after a prolonged incubation period. The titers after the second incubation period were markedly lower than after the first incubation, but were still sufficiently high to demonstrate that growth of virus had occurred during the second period. This evidence seems to indicate that inhibition of growth of the virus by isopropyl biguanide at a concentration of 0.1 mg per ml is not due to irreversible damage to the tissue. Even at 0.2 mg per ml no significant evidence for such damage was obtained.

Attempts to Show Reversal of Inhibition

The mode of action of isopropyl biguanide would be more clearly understood if the inhibition could be reversed by one or more known metabolites. Because paludrine, a closely related compound, has been implicated in the nucleic acid metabolism of other organisms, especially at the level of purine and pyrimidine synthesis, these types of compounds offered a logical starting place for experiments attempting reversal of the inhibition of virus growth by isopropyl biguanide. Accordingly folic acid, p-aminobenzoic acid, adenine, and desoxyribonucleic acid were investigated as possible reversing agents.

Because of some structural similarity between isopropyl biguanide and arginine, this amino acid was also included in these tests as was another basic amino acid lysine, which is known to be an antagonist of arginine in some biological systems.

Results. The results from experiments attempting reversal of the effect of isopropyl biguanide on influenza virus in tissue cultures by L-arginine are presented in Table 9. It will be noted that the data from experiment 1 suggested a very slight reversing action of arginine at a concentration of 0.04 mg per ml. Higher concentrations did not appear to prevent the inhibition by isopropyl biguanide. In attempts to establish a more definite reversal, concen-

TABLE 9

Attempted Reversal by L-Arginine of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide

Culture Number	Virus hemagglutinin present in tissue cultures containing 0.1 mg IBG[4] per ml and the indicated concentration of L-arginine monohydrochloride					
Experiment 1	5.0 mg per ml	1.0 mg per ml	0.2 mg per ml	0.04 mg per ml	Controls, no arginine, 0.1 mg IBG per ml	Controls, virus only
1	0	0	20	30	20	120
2	0	0	10	30	10	120
3	0	0	2-10	30	10	120
4	0	0	2-10	20	10	80
5	0	0	0	20	2-10	80
6	0	0	0	2-10	0	80
Geometric mean	0	0	4.0	19.5	6.7	97.9
Experiment 2	0.08 mg per ml	0.04 mg per ml	0.02 mg per ml	0.008 mg per ml	Controls, no arginine, 0.1 mg IBG per ml	Controls, virus only
1	20	30	20	40	8	240
2	8	20	20	20	4	160
3	4	0	16	0	4	160
4	4	0	2	0	2	120
5	0	0	2	0	2	80
6	0	0	0	0	0	80
Geometric mean	3.7	2.90	5.4	3.1	2.9	129.5
Experiment 3	0.04 mg per ml	Controls, no arginine, 0.1 mg IBG per ml	Controls, virus only			
1	40	2-10	240			
2	30	2-10	160			
3	20	2-10	160			
4	20	0	120			
5	10	0	80			
6	0	0	80			
Geometric mean	13.2	2.1	129.5			

1. All figures in the table represent reciprocals of

Table 9, continued

highest dilutions of tissue culture fluids giving complete hemagglutination. In experiment 2, dilutions below 1:4 were not made. Cultures giving 3+ agglutination at 1:4 were assigned a value of 2. Those showing very slight agglutination at 1:4 were assigned a value of 0. In experiments 1 and 3, dilutions below 1:20 were not made, and values were assigned as previously described.

2. Virus titrations were made after 40-48 hours incubation at 35°C. on a reciprocating shaker.

3. The virus inoculum was 0.1 ml of a 1:10 dilution of allantoic fluid virus either frozen at -20°C. for not more than 4 days or freshly harvested from chick embryos.

4. IBG is isopropyl biguanide.

5. If the data of experiments 1 and 3 comparing cultures containing only IBG at 0.1 mg per ml with cultures containing 0.04 mg of arginine per ml in addition to the IBG, are combined and analyzed by Fischer's "t" test (8, pp.114-174) a "t" value of 3.29 is obtained. For 22 degrees of freedom this corresponds to a P value less than 0.01, indicating that it is highly unlikely the observed difference could be due to chance.

trations just above and just below 0.04 mg were investigated but in this experiment (experiment 2), not even a slight reversal by any concentration of arginine was obtained. However, a slight reversing effect by 0.04 mg arginine per ml was again noted in experiment 3. In this experiment the titers of the cultures containing arginine in addition to isopropyl biguanide appeared to be somewhat higher than those of the cultures containing only isopropyl biguanide, although the arginine titers were obviously much lower than the titers of the virus control cultures.

When the data of experiments 1 and 3 were combined and tested statistically it was found that the differences in virus titers in the cultures containing 0.1 mg of isopropyl biguanide per ml and arginine in a concentration of 0.04 mg per ml were significantly higher than the controls containing only isopropyl biguanide. In experiment 2 some unrecognized difference apparently existed which interfered with the results as observed in the other experiments.

To obtain additional information on this point a similar experiment was carried out in intact chick embryos. The results are shown in Table 10. Here again the same trend was noted; in the presence of the proper ratio of arginine to isopropyl biguanide, the inhibitory effect of the latter compound for the virus seem to be partially reversed.

The foregoing evidence cannot be considered conclusive, but definitely suggests that arginine in the proper

TABLE 10

Influence of Arginine on the Inhibition of Lee Influenza Virus in the Chick Embryo by Isopropyl Biguanide

Egg No.	Hemagglutinin Titers of Fluids from Eggs Receiving 50 EID_{50} of Virus + 5 mg Isopropyl Biguanide	Hemagglutinin Titers of Fluids from Eggs Receiving 50 EID_{50} of Virus + 5 mg Isopropyl Biguanide + 2 mg L-arginine monohydrochloride
1	320	320
2	120	240
3	60	160
4	60	160
5	60	120
6	30	120
7	10	80
8	2-10	80
9	0	60
10	0	0
	Geometric mean 20.2	80.1

1. Figures in table represent reciprocals of highest fluid dilutions giving complete hemagglutination.

2. One group of eggs received an injection of 5 mg of isopropyl biguanide in the allantoic sac. The second group received an injection containing a mixture of 5 mg of isopropyl biguanide with 2 mg of L-arginine monohydrochloride. After 1 hour all eggs received 50 EID_{50} of Lee influenza virus. Fluids from all eggs were titrated individually for virus content after 44 hours incubation at 35°C.

   Comparison of these mean values for the hemagglutinin in the two groups by means of the "t" test (8, pp. 114-174) using the logs of the individual titers gives a "t" value of 1.702, which for 18 degrees of freedom, corresponds to a P value of about 0.1. Hence the difference is not significant at the usually accepted .05 probability level.

concentration can interfere to a slight extent with the inhibition of Lee influenza virus by isopropyl biguanide.

Lysine did not cause even a slight reversal of the inhibition of the virus by 0.1 mg isopropyl biguanide. Reference to Table 11 will show that the hemagglutinin titers of cultures containing a concentration range of 10-0.08 mg lysine per ml were almost identical with those containing only isopropyl biguanide.

Neither adenine nor desoxyribonucleic acid were able to reverse the inhibition by isopropyl biguanide. This data is presented in Tables 12 and 13. Neither of these compounds over a concentration range of 5-0.04 mg per ml increased the titers of cultures inhibited by 0.1 mg isopropyl biguanide. It might be noted in Table 13 that a relatively good virus growth occurred in the control cultures containing only 0.1 mg isopropyl biguanide, while complete inhibition of the virus occurred in similar controls in the experiment represented in Table 12. This well illustrates the point mentioned previously in regard to the variation in response shown by different virus stocks. Although the virus inoculum was a 1:10 dilution in both experiments, apparently the inoculum used in the DNA studies contained a larger amount of active virus.

In one experiment p-aminobenzoic acid appeared to give results suggesting slight reversal of the inhibition by isopropyl biguanide but these results could not be duplicated

TABLE 11

Attempted Reversal by L-Lysine of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide

Culture Number	Virus hemagglutinin present in tissue cultures containing 0.1 mg IBG per ml and the indicated concentrations of L-lysine monohydrochloride					
	10.0 mg per ml	2.0 mg per ml	0.4 mg per ml	0.08 mg per ml	Control, no lysine 0.1 mg IBG per ml	Control, virus only
1	0	0	0	0	2-10	240
2	0	0	0	0	2-10	160
3	0	0	0	0	0	160
4	0	0	0	0	0	120
5	0	0	0	0	0	120
6	0	0	0	0	0	80

1. All figures in the table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:20 were not made. Cultures entirely negative at 1:20 were assigned a value of 0. Those showing 3+ agglutination at 1:20 were assigned a value of 10. Those showing very slight agglutination at 1:20 were assigned a range value of 2-10.

2. Virus titrations were made after 44-48 hours incubation at 35°C. on a reciprocating shaker.

3. The virus inoculum was 0.1 ml of a 1:10 dilution of a frozen stock of allantoic fluid virus held at -20°C. for no longer than 3 days.

4. IBG is isopropyl biguanide.

TABLE 12

Attempted Reversal by Adenine of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide

Culture Number	Virus hemagglutinin present in tissue cultures containing 0.1 mg IBG[4] per ml and the indicated concentration of adenine sulfate					
	5.0 mg per ml	1.0 mg per ml	0.2 mg per ml	0.04 mg per ml	Control. No adenine, 0.1 mg IBG per ml	Control. Virus only
1	0	0	0	0	0	80
2	0	0	0	0	0	60
3	0	0	0	0	0	30
4	0	0	0	0	0	30
5	0	0	0	0	0	30
6	0	0	0	0	0	2-10

1. All figures in the table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:20 were not made. Cultures entirely negative at 1:20 were assigned a value of 0. Those showing 3+ agglutination at 1:20 were assigned a value of 10. Those showing very slight agglutination at 1:20 were assigned a range value of 2-10.

2. Virus titrations were made after 44-48 hours incubation at 35°C. on a reciprocating shaker.

3. The virus inoculum was a 1:10 dilution of a frozen stock of allantoic fluid virus held at -20°C. for no longer than 4 days.

4. IBG is isopropyl biguanide.

TABLE 13

Attempted Reversal by Desoxyribonucleic Acid of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide

Culture Number	Virus hemagglutinin present in tissue cultures containing 0.1 mg IBG[3] per ml and the indicated concentration of sodium desoxyribonucleate					
	5 mg per ml	1 mg per ml	0.2 mg per ml	0.04 mg per ml	Control, no DNA. 0.1 mg IBG per ml	Control, virus only
1	40	40	60	60	40	160
2	30	8	20	60	40	160
3	20	8	16	20	30	160
4	8	8	16	8	20	160
5	8	4	8	8	8	80
6	4	4	4	8	8	80
Geometric mean	13.5	8.3	14.6	18.2	19.8	126.9

1. All figures in table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination.

2. Virus titrations were made after 44-48 hours incubation at 35°C. on a reciprocating shaker.

3. The virus inoculum was 0.1 ml of a 1:10 dilution of allantoic fluid virus freshly harvested from chick embryos.

4. IBG is isopropyl biguanide.

in later experiments. This is shown in Table 14, experiment 1, with p-aminobenzoic acid at a concentration of 5 mg per ml. However, when this experiment was repeated not even a suggestion of reversal was obtained, and the titers of cultures containing p-aminobenzoic acid plus isopropyl biguanide were quite similar to those containing only isopropyl biguanide.

The data in Table 15 show that folic acid did not give a definite reversal of the inhibition by isopropyl biguanide. Again, a suggestion of reversal was obtained in one experiment but when this experiment was repeated no reversal appeared to take place. In experiment 2, folic acid in a concentration of 0.2-0.04 mg per ml appeared to prevent the inhibition by isopropyl biguanide to a slight extent, the titers of these two groups of cultures being somewhat higher than those of the isopropyl biguanide controls. However, in experiment 3 the titers of all cultures containing isopropyl biguanide were almost identical, whether or not folic acid was also present. An additional experiment also failed to show any effect of the folic acid.

TABLE 14

Attempted Reversal by p-Aminobenzoic Acid of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide

Culture Number	Virus hemagglutinin present in tissue cultures containing 0.1 mg IBG per ml and the indicated concentration of p-Aminobenzoic acid					
	0.04 mg per ml	0.2 mg per ml	1.0 mg per ml	5.0 mg per ml	Control, no PABA, 0.1 mg IBG per ml	Control, virus only
Experiment 1						
1	20	0	0	20	0	160
2	0	0	0	20	0	80
3	0	0	0	2-10	0	80
4	0	0	0	0	0	60
5	0	0	0	0	0	40
6	0	0	0	0	0	30
Experiment 2						
1				30	60	480
2				30	60	480
3				20	40	480
4				20	30	320
5				16	30	320
6				8	30	320
7				8	20	320
8				8	20	320
9				8	20	240
10				8	16	240
11				4	8	240
12					8	240
		Geometric mean		12.0	23.7	321.7

1. All figures in table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:10 were not made in Experiment 1. Cultures negative at 1:10 were assigned a value of 0.
2. Virus titrations were made after 40-48 hours incubation at 35°C. on a reciprocating shaker.
3. The virus inoculum was 0.1 mg of a 1:10 dilution of allantoic fluid virus either frozen at -20°C. for not more than 4 days or freshly harvested from chick embryos.
4. IBG is isopropyl biguanide.

TABLE 15

Attempted Reversal by Folic Acid of the Inhibition of Lee Influenza Virus in Tissue Cultures by Isopropyl Biguanide

Culture Number	Virus hemagglutinin present in tissue cultures containing 0.1 mg IBG per ml and the indicated concentration of crystalline folic acid			
Experiment 1	5 mg per ml	1 mg per ml	Control, no folic acid 0.1 mg IBG	Control, virus only
1	0	0	0	40
2	0	0	0	40
3	0	0	0	40
4	0	0	0	40
5	0	0	0	30
6	0	0	0	0
Experiment 2	0.2 mg per ml	0.04 mg per ml	Control, no folic acid 0.1 mg IBG	Control, virus only
1	40	30	8	160
2	30	30	4	120
3	30	20	4	120
4	20	8	2	120
5	8	8	2	120
6	4	2	0	80
Experiment 3	0.2 mg per ml	0.04 mg per ml	0.008 mg per ml	Control, no folic acid 0.1 mg IBG	Control, virus only
1	8	8	16	16	160
2	4	4	16	8	120
3	4	2	8	4	120
4	2	2	8	2	80
5	0	0	4	2	
6	0	0	0	0	

1. It was necessary to adjust the fluids in Experiment 1 to pH 8.0 to keep the folic acid in solution. Control cultures were also adjusted to pH 8.0.

2. All figures in the table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:20 were not made. Cultures entirely negative at 1:20 were assigned a value of 0. Those showing 3+ agglutination at 1:20 were assigned a value of 10. Those

Table 15, continued

showing very slight agglutination at 1:20 were assigned a range value of 2-10.

3. Virus titrations were made after 44-48 hours incubation at 35°C. on a reciprocating shaker.

4. The virus inoculum was 0.1 ml of a 1:10 dilution of allantoic fluid virus freshly harvested from chick embryos.

5. IBG is isopropyl biguanide.

DISCUSSION

The inhibitory action of isopropyl biguanide for the Lee influenza virus does not appear to be due to an irreversible toxic effect on the tissue. Without doubt this factor is of importance at the higher concentrations of the compound, above 0.2 mg per ml, but nearly complete inhibition of virus growth is obtained with concentrations which are not toxic.

In view of this fact, another explanation of the mode of action of this compound must be sought. The experiments attempting reversal by several known metabolites, of the inhibition by isopropyl biguanide should be of value in this respect.

The results of such experiments with L-arginine strongly suggest a slight reversal of the action of isopropyl biguanide but as the results were not conclusive any statement in regard to an antagonism between arginine and isopropyl biguanide would be quite speculative. The fact that completely negative results were obtained with lysine seems to suggest that the guanidino group, which is shared by arginine and isopropyl biguanide but not by lysine, is of significance.

Falco (7, p.107) observed the inhibition of the growth of Lactobacillus casei by paludrine (1-p-chlorophenyl-5-isopropyl biguanide) and by several pyrimidine

derivatives which were structurally similar to paludrine. The inhibition by paludrine was reversed by guanine, and to a slightly lesser extent by adenine, xanthine, and hypoxanthine. Thus a compound rather closely related to isopropyl biguanide appears to be in some way involved in the nucleic acid metabolism of L. casei. The results obtained in the present study do not indicate that the purine adenine, or even DNA itself, is capable of reversing the inhibition of influenza B virus by isopropyl biguanide. Reversal studies with guanine and possibly the pyrimidine bases should be helpful in shedding further light on the question of possible interference by isopropyl biguanide with the nucleic acid metabolism of the host cell-virus complex.

In addition to the reversal by purines of the inhibition caused by paludrine, Falco also found that folic acid was an effective reversing agent. This observation was also made by Greenberg (11, pp.275-276), who found that inhibition of the malaria parasite Plasmodium gallinaceum could be partially reversed by either folic acid or p-aminobenzoic acid. However, Thurston (20, p.438) found that inhibition of Plasmodium berghei by paludrine, although reversed by p-aminobenzoic acid, was not reversed by folic acid.

Because of the similarity in structure between paludrine and isopropyl biguanide, p-aminobenzoic acid and folic acid were investigated as possible reversing agents for the inhibition of Lee influenza virus by isopropyl biguanide.

However, the data do not indicate a reversing action by either of these two compounds, suggesting that the mode of action of isopropyl biguanide in inhibiting influenza virus is not the same as the action of paludrine in inhibiting the malaria parasites.

The data obtained in the present investigation do not seem to offer any valid explanation of the mode of action of isopropyl biguanide. It apparently does not act by causing irreversible damage to the host cells. Arginine in proper concentration may interfere slightly with its action. Previous work (12, p.44) has shown that it has some in vitro effect on the virus, causing more rapid loss of infectivity if exposure is long enough and the temperature is optimum (24 hours at 37°C.). No effect was observed within 2 hours at this temperature.

It still seems possible that isopropyl biguanide may act in some manner as an antimetabolite in virus biosynthesis. It is also possible that the in vitro effect noted above may play a part in the in vivo activity.

SUMMARY AND CONCLUSIONS

The optimal dilution of the inoculum of Lee influenza virus to be used in the tissue cultures was determined. It was found that the final virus titer was dependent upon the initial concentration of the virus in the inoculum. Concentrated inocula gave higher virus titers than more dilute inocula; 0.1 ml of a 1:10 dilution of fresh allantoic fluid virus gave a satisfactory inoculum for most experiments.

The effect on virus growth of incubating the cultures on a reciprocating shaker was investigated. Virus titers of the cultures shaken during incubation were noticeably higher than those of stationary cultures. The maximum titers of the shaken cultures were also reached more quickly than those of stationary cultures.

Titration of the cultures after 44-48 hours of incubation was found to be desirable, as the hemagglutinin titers of the cultures did not increase after this time.

Three compounds were studied for possible effects upon Lee influenza virus in tissue cultures. These compounds were DL-citrulline, L-canavanine, and isopropyl biguanide. DL-citrulline has no effect on the growth of the virus under these conditions. Both L-canavanine and isopropyl biguanide in concentrations of 0.1 mg per ml caused marked inhibition of the virus. Both of these compounds were much more active in tissue culture than they had previously been found to be in the chick embryo. In tissue

culture the minimum effective concentration in each case was only about one tenth of that required in the chick embryo.

Isopropyl biguanide was investigated more extensively. It was found that concentrations of this compound which were inhibitory to the virus did not appear to cause irreversible damage to the tissue, so that it would be unable to support virus growth. Hence this does not seem to be the mechanism by which the Lee virus is inhibited in tissue cultures.

Several metabolites were tested for ability to reverse the inhibition of the virus by isopropyl biguanide. L-arginine, L-lysine, adenine, desoxyribonucleic acid, p-aminobenzoic acid, and folic acid were investigated as possible reversing agents but none of these compounds showed any ability to reverse the inhibition of the virus, with the possible exception of arginine. In 2 of 3 tissue culture experiments and in one chick embryo experiment a slight reversing effect was noted. The concentration of arginine appeared to be critical.

Further studies are needed to clarify the mode of action of isopropyl biguanide in inhibiting the Lee influenza virus.

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