INHIBITION OF THE DEVELOPMENT OF LEE INFLUENZA VIRUS BY CANAVANINE AND ISOPROPYL BIGUANIDE

by

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INTRODUCTION

An investigation applying structural analogues of known metabolites to a host-virus system in an effort to inhibit the development of the viral agent has a two fold purpose. First, by inhibiting the infection of the host by the virus without causing any undue effects on the host, it may be possible to uncover some practical chemotherapeutic agent. Secondly, based on the fact that virus multiplication occurs only after some unknown modification of the host cell by the virus, wherein the host cell is forced to produce virus material instead of cell material, we may be able to obtain some information as to the basic nature of this change.

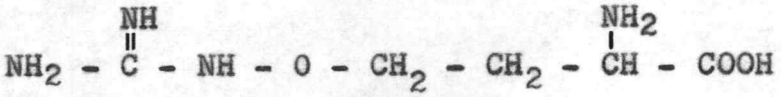
Any compound showing anti-viral activity may interfere with any one of the three phases of virus infection. The compound may prevent the initial adsorption of the virus particle to the cell. This may be done by directly inactivating the free virus, by altering the constituents of the virus particle essential for its reaction or combination with the host cell, or by destroying, altering or combining with receptor areas on the host cell. An active inhibiting compound may interfere with the pathways of synthesis of

the cell which are modified to produce virus material or it may be incorporated into the newly formed virus particles, and once incorporated, result in an abnormal virus unable to further multiply and cause infection of new cells. The final phase of the infection cycle in which the chemical antagonist may interfere is the release of newly formed virus particles from the cell. The compound may, by some unknown reaction, prevent this liberation of virus and, by this means, inhibit further infection.

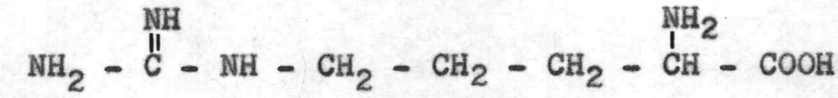
The probability of finding a compound active as an anti-viral agent and being able to pinpoint the exact reaction interfered with by this compound is indeed slight. However, by carefully observing for differences which might be found to occur between host-virus systems with and without treatment by these anti-viral compounds we might gain some information relating to the nature of virus multiplication. This study is an investigation of the inhibition of the Lee strain of influenza B virus induced by L-canavanine and by isopropyl biguanide hydrochloride, particularly in the embryonated chicken egg.

HISTORICAL REVIEW

Canavanine is a naturally occurring L-amino acid isolated from the jack bean, Canavalia ensiformis, where it makes up 2.5 per cent of the dry weight of the bean. Canavanine is a structural analogue of arginine whose relationship can be seen in the following formulae:



Canavanine



Arginine

It is seen that canavanine differs in structure from arginine by the substitution of an oxygen atom for one CH_2 group in the number five position of the carbon chain.

A number of investigators have shown canavanine to have antimicrobial activity. Horowitz and Srb (21, pp. 371-376) have studied the effect of canavanine upon three wild strains of Neurospora and found one strongly sensitive to inhibition by canavanine, one weakly sensitive, and one resistant. By addition of arginine to canavanine inhibited cultures they obtained reversal of the inhibition and evidence for a competitive antagonism between the two compounds. In the case of a number of typical

strains of yeasts of the genera Saccharomyces, Torula and Kloeckera, which were inhibited by canavanine, reversal of this inhibition was also demonstrated when arginine was available (27, p.1035). Canavanine sensitive Torulopsis utilis overcame the inhibition and indicated a competitive mechanism when arginine, lysine, or homoarginine were added to the culture medium (51, pp.209-211). Arginine and homoarginine were equally effective in reversing the inhibition while lysine showed only one half of the growth restoring activity of these two compounds.

Inhibition by canavanine of a number of lactobacilli and of both arginine requiring and arginine synthesizing strains of Escherichia coli (50, pp.894-899) and Salmonella enteritidis (42, p.10, 116) has been shown to be reversed by arginine apparently in a non-competitive manner in those cases thoroughly studied.

Canavanine has been shown to be an inhibitor of the elongation of Avena coleoptile sections induced by indole acetic acid (2, pp.328-329). Concentrations of canavanine between 3 and 10 mg per liter brought about a 50 per cent inhibition of this elongation. Reversal was shown to be possible by addition of arginine, glutamic acid or lysine with activity decreasing in that order. Further investigations by Bonner into effects on respiration indicate that canavanine acts to inhibit the increase

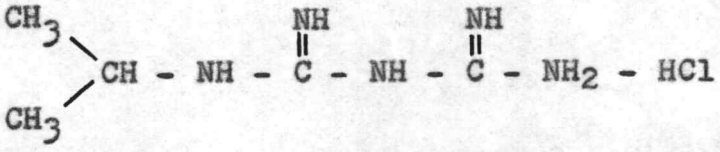
of respiration normally induced by indole acetic acid (3, pp.430-434). On the other hand, it has no effect on an increase of respiration resulting from the addition of adenylic acid. From this study he speculated that the increase in respiration caused by indole acetic acid might be mediated through some unknown arginine step. Canavanine, in high concentration, inhibited growth of the oat coleoptile and also reduced endogenous respiration by about 15 per cent.

Canavanine as an inhibitor of virus development has been noted in a report by Pearson, Lagerborg and Winzler in which they screened a number of amino acids and analogues of amino acids for activity against Theiler's GD VII strain of mouse encephalomyelitis virus (32, p.410). DL-Canavanine sulfate in a concentration of 1 mg per ml was reported to cause a 100 per cent reduction in the titer of the hemagglutinin produced by this virus in a tissue culture of one day old mouse brain.

The basic amino acids, arginine and lysine, have been shown to inhibit the development of the Lee strain of influenza B virus in tissue culture (7, pp.506-507). Concentrations of arginine and lysine necessary to effect this inhibition were of the order of two to five mg per ml. This is an interesting observation in view of the

fact that Knight has shown by chemical analysis of the influenza viruses that both the A and B strains possess good amounts of both of these amino acids (24, pp.125-126). The multiplication of mumps virus also was inhibited by arginine. Lysine added to tissue culture of one day old mouse brain resulted in a decrease in the hemagglutinin formed during infection with Theiler's GD VII strain of mouse encephalomyelitis virus (32, pp.410-411). Synthetic polypeptides, composed solely of lysine, were able to inhibit the multiplication of influenza, mumps, Newcastle disease and infectious bronchitis viruses in the chick embryo (14, pp.853-857; 15, pp.641-642; 16, pp.61-63).

Isopropyl biguanide, although a member of a group of compounds possessing the biguanide structure and showing a variety of biological activities, apparently has not been widely studied at this time. This compound bears the following structure:



Isopropyl biguanide hydrochloride

A closely related compound and one used clinically as an antimalarial is 1-(p-chloro-phenyl)-5-isopropyl biguanide hydrochloride, more commonly known as "Paludrine" or

chloroguanide (38, pp.96-97; 26, p.226). Chloroguanide has chemotherapeutic activity against the endoerythrocytic forms of Plasmodium falciparum and to a lesser degree against P. vivax. Chloroguanide has a fairly high degree of antifungal activity inhibiting growth in vitro of 45 strains of fungi tested at levels of 5 to 20 mg per cent (37, p.8996). Tuberculostatic activity has also been reported where in vitro experiments showed complete inhibition of Mycobacterium tuberculosis var.hominis at 100 γ per ml (39, pp.159-160). In experiments with Lactobacillus casei, inhibition by chloroguanide was overcome by addition of various purines and by pteroylglutamic acid (8, pp.107-108). In a wide variety of chemical compounds tested for activity against the development of Rickettsia typhi in the yolk sac of the embryonated chicken egg, chloroguanide was found to be completely ineffective at 0.8 mg per egg (34, p.443). However, the closely related structure 1-(p-chlorophenyl) biguanide hydrochloride significantly lengthened the time of survival of the infected eggs. These two compounds are similar in structure except that chloroguanide possesses the terminal isopropyl group. 1,1-Dimethyl biguanide was reported to relieve fever and headache in a number of clinical cases of influenza within a short period after administration (12, pp.289-292).

A number of substituted urea compounds have indicated some degree of anti-viral activity. Various cyclic aldyhyde semicarbazones inhibited the development of atypical pneumonia virus in the chick embryo and suppressed the formation of pulmonary lesions in the cotton rat (6, pp.423-424).

Several groups of investigators have found thiosemicarbazone derivatives to inhibit multiplication of vaccinia virus in both the chick embryo and in mice inoculated intracerebrally as shown by a significant delay in death and increase in the number of survivors (17, pp.306-311; 46, pp.229-233; 47, pp.11-12).

A wide variety of chemical compounds have been shown to be active in inhibiting virus development. Many of these inhibitors are analogues of known metabolites. Poliomyelitis virus has been inhibited in tissue culture by several amino acid analogues (4, pp.442-455). Active as inhibitors were cysteic acid, DL-ethionine, N-dichloro acetyl α-(p-nitrophenyl) glycine, and β-2 thienylalanine. β-2 Thienylalanine was also observed to be active against vaccinia virus in tissue culture (45, pp.435-436).

A variety of substituted purine and pyrimidine compounds have shown anti-viral activity. Benzimidazole has been shown to decrease the growth of vaccinia virus in

tissue culture (44, pp.530-532) and poliomyelitis virus in tissue culture (4, pp.442-445). 2,6-Diamino purine was able to inhibit development of Russian spring-summer encephalitis in tissue culture (11, p.151) as well as vaccinia virus in the same medium (44, pp.530-532). Both thiouracil (30, p.449) and 8-azaguanine (29, pp.278-280) were inhibitory to psittacosis virus developing in tissue culture.

The vitamin analogues, oxythiamine and desoxypyridoxine, have demonstrated antiviral activity when incorporated in tissue culture with either mumps virus or the PR-8 strain of influenza virus (5, pp.498-499). Oxythiamine has also been shown to offer some protection for mice infected with Lansing poliomyelitis virus (22, pp.455-458). Several folic acid analogues have inhibited psittacosis virus in both the embryonated chicken egg and in tissue culture (28, pp.272-274).

A polysaccharide from Klebsiella pneumoniae has been shown to antagonize infection of the chick embryo by mumps virus (13, pp.99-100) and apple pectin and various other complex carbohydrates inhibited hemagglutination of chicken red blood cells by influenza virus (16, pp.61-63). Apple pectin inhibited infection of the chick embryo when PR-8 influenza virus was inoculated.

Chemical compounds active as inhibitors of virus development are quite specific for a definite virus and host system. The majority of compounds showing antiviral activity are active only in tissue culture. The number of compounds inhibiting virus development in the embryonated chicken egg is apparently much lower and the number of compounds active against virus infections in the intact animal is but a very small percentage of those showing activity in other systems. Furthermore, definite differences as to metabolic requirements between various viruses exist. For example, 2,6 diamino purine was active in tissue culture against Russian spring-summer encephalitis (11, pp.151-152), 8-azaguanine inhibited psittacosis virus in tissue culture (29, pp.278-280) and β-2-thienylalanine showed antiviral activity against both poliomyelitis virus (4, pp.443-446) and vaccinia virus (45, pp. 435-436) in tissue culture in both cases. However, none of these compounds were able to inhibit the multiplication of either mumps or the PR-8 strain of influenza virus when these were grown in tissue culture (5, p.499).

A problem of this type, wherein we attempt to inhibit the development of the virus by interfering with some essential step required for its synthesis, suffers from the lack of information available on what pathways are

essential for this synthesis. Perhaps in time, when more investigations have been carried out and more instances of virus inhibition are known, we will then be able to visualize more clearly the pathways and systems involved in virus multiplication and be able to selectively inhibit one or more of these systems and minimize virus infection.

EXPERIMENTAL METHODS

Virus. The Lee strain of the type B influenza virus was obtained from the American Type Culture Collection and used in the majority of experiments involving the chick embryo. The history of this virus, when received, indicated a series of 8 ferret passages, 310 mouse passages and 3 egg passages.

For experiments involving tissue culture techniques another strain of Lee influenza B virus was used which was especially adapted to the chick embryo. This strain was made available through the courtesy of Dr. F. L. Horsfall of the Rockefeller Institute.

Mumps virus, originally from the American Type Culture Collection, was received from Cutter Laboratories. This virus strain had been transferred through 2 monkey passages, 12 amniotic sac passages in the chick embryo and 43 allantoic sac passages in the latter host.

The PR-8 strain of influenza A virus was obtained from the American Type Culture Collection and had a history of 198 ferret passages, 691 mouse passages and 3 egg passages when received.

A stock supply of each virus was maintained by lyophilization of allantoic fluid virus. Allantoic fluids from eggs infected 40 to 48 hours previously with

influenza virus or 5 days previously with mumps virus were harvested aseptically and centrifuged to remove cells and aggregates present in the fluid. The supernatant fluid was then mixed with an equal volume of sterile skim milk and dispensed in 0.1 ml volumes in sterile cotton plugged ampoules. The virus-skim milk mixture was quick frozen in an alcohol-dry ice mixture and placed in a desiccator chilled in dry ice to below -10°C. Water vapor was removed by phosphorous pentoxide held in a tray inside the desiccator. The latter was evacuated to a pressure of 100 to 200 microns of mercury and held at -20°C for a period of four days. The final phase of drying took place with the desiccator at room temperature for the fifth day. After this period of lyophilization, the ampoules were removed from the desiccator and sealed off individually at a pressure of 100 to 200 microns of mercury and stored at -20°C. The virus titer was determined periodically by infectivity titration and the 50 per cent infective dose for the chick embryo was calculated. For use, three ampoules were aseptically opened and the contents reconstituted with one ml sterile distilled water per ampoule to give a 1:20 dilution of the original allantoic fluid virus. The contents of the three ampoules were pooled and further

dilution was prepared in a buffered saline solution[1] at pH 7.2 to give a dose of 50 ID_{50} in 0.1 ml as determined by infectivity titration. Lyophilized stocks were used for a period of approximately three months before it was necessary to prepare a new stock.

In cases where fresh allantoic fluid virus was desired, lyophilized virus was reconstituted and diluted to give a dose of 50 ID_{50} in 0.1 ml and injected into five to ten eggs. After the desired incubation period, the allantoic fluid was harvested, pooled and an infectivity titration run on the pooled virus fluid. The pooled virus fluid was held at 4°C until the result of the infectivity titration was available, at which time the fluid was then diluted to give the proper dosage in a 0.1 ml volume. Occasionally, infectivity titrations were run again on the fluid after the 2 to 3 day period of storage at 4°C. In no case was the infectivity titer

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[1]Buffered salineof pH 7.2 was prepared by the following formula:

Na_2HPO_4 (anhydrous)	7.105 grams
KH_2PO_4	2.269 grams
NaCl	8.5 grams

Distilled water to one liter.

The saline solution was dispensed in 30 to 50 ml volumes in Pyrex bottles with screw caps and sterilized at 121°C for 20 minutes.

of the refrigerated virus found to differ from the initial titer beyond the experimental error of the titration procedure.

Eggs. Chicken eggs of various breeds were obtained from the Oregon State College poultry farm, Russell's Hatchery and Hansen's Leghorn Farm. White Leghorn eggs were used in the early portion of this investigation, while eggs from New Hampshire Reds were used chiefly in the latter part. Previous to infection, eggs were incubated at 38°C for 9 to 11 days for influenza virus and 8 to 9 days for mumps virus. Following infection, the eggs were incubated at 35°C.

Titrations. Hemagglutinin titers were determined by the pattern method of Salk (35, pp.88-92). Chicken red blood cells from four or five chickens were collected fresh each week by adding the blood to an equal volume of Alsever's solution.[2] The red blood cells were washed

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[2]Alsever's solution which was prepared had the following composition:

Dextrose	20.5	grams
NaCl	4.2	grams
Sodium citrate	8.0	grams
Citric acid	0.55	grams
Distilled water	1000	ml

The solution was distributed in 150 to 200 ml volumes in wide mouthed bottles and autoclaved at 15 pounds pressure for 15 minutes. After cooling, all bottles were refrigerated until used.

with physiological saline by centrifuging three times at 1500 r.p.m. for seven minutes and a final packing at 1500 r.p.m. for ten minutes. A ten per cent suspension by volume was prepared and stored at 4°C. For hemagglutinin titrations on allantoic fluids, a one per cent suspension was prepared immediately before use. A 0.5 per cent suspension was used for hemagglutinin titrations on tissue culture fluids.

Infected fluids were diluted serially by two fold steps from two initial dilutions of 1:10 and 1:15 to give a progression of dilutions as 1:10, 1:15, 1:20, 1:30, 1:40, 1:60, etc. Whenever practical, a separate pipet was used for each dilution. Mixing was accomplished by alternately withdrawing and blowing back into the tube a one ml volume eight times. The ninth time the one ml volume was transfered to the next tube in the dilution series and the pipet discarded. A fresh pipet was taken and the mixing and transfer continued. When the number of pipets was limited, fresh pipets were used as far as possible in the dilution series with the final pipet being used through the remaining dilutions in the series. The final volume in each tube was 1.0 ml. After dilution, an equal volume of a one per cent suspension of chicken red blood cells was added to each tube and the tubes were shaken. This resulted in a final volume of 2.0 ml and an

increase of the initial dilution by a factor of two.

After an incubation period of 45 minutes at room temperature, the titration was read with the highest dilution showing complete agglutination of the red blood cells being considered as the endpoint.

As hemagglutination was used as the principle measure of virus growth or inhibition in the investigation, the reproducibility of the results of hemagglutinin titrations was examined. This was done by two different methods. In one case a dried stock of mumps virus was titrated at various time intervals over a period of four months and the resulting titers compared. The hemagglutinin is quite stable and should not vary to any degree in the lyophilized virus preparation. This information is contained in Table 1. In eleven observations the maximum difference noted in hemagglutinin titers is a three fold difference. When represented as the logarithm of the hemagglutinin titer, it is evident that a logarithm difference as great as 0.4771 occurred once in eleven trials presumably due to chance factors. It was not considered necessary to pursue further this investigation concerning the significance of differences occurring between hemagglutinin titrations run on different days. The number of observations indicated is too small to allow for any statistical analysis but they do serve to indicate the degree of

Table 1

Reproducibility of Hemagglutinin Titration as Determined by Titrations on Reconstituted Dried Stock Mumps Virus Over a Four Month Time Interval

Titration Number	Date of titration	Hemagglutinin titer	Logarithm of hemagglutinin titer
1	4-10-51	160	2.2041
2	4-24-52	160	2.2041
3	5-15-52	240	2.3802
4	5-23-52	240	2.3802
5	5-29-52	160	2.2041
6	6-10-52	160	2.2041
7	6-13-52	160	2.2041
8	6-18-52	160	2.2041
9	6-28-52	80	1.9031
10	7-18-52	160	2.2041
11	8-8-52	240	2.3802

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.

2. Lyophilized stock mumps virus was reconstituted with 1.0 ml sterile distilled water to give the initial 1:20 dilution. Reconstituted virus from four to five ampoules were pooled for each titration.

variation in titration values obtained on titrating the same sample on different days. It is understood that a logarithm difference of 0.4771 $\log_{10}$ units may occur one time in eleven trials but it can not be said that any difference exceeding this value is necessarily a significant difference between day to day titrations in any experiment. Obviously, values obtained on different days would have to show considerably greater differences to be judged significant.

The second method of investigation of the reproducibility of the hemagglutinin titration was a series of duplicate titrations on different infected allantoic fluids. In Table 2, duplicate titrations on 21 samples of allantoic fluid virus are tabulated. Differences in the titration endpoint are noted in 7 of the 21 titrations and occur as only a one tube difference. By statistical analysis the standard error of the difference between two estimates on a given sample is 0.0860 logarithmic units (25, pp.199-207). Any difference of hemagglutinin titer resulting between treated and untreated control fluids which exceeds 0.1789 logarithmic units would appear to result through chance in only about five per cent of the cases. Such a difference, then, can be understood to be experimentally significant and is due to known factors controlled in the experiment. With respect to

Table 2

Reproducibility of Hemagglutinin Titrations as Determined by Duplicate Titrations on Twenty-one Samples of Mumps Virus Infected Allantoic Fluids

Allantoic fluid Number	Reciprocal of dilution (1)	(2)	Logarithm of reciprocal (1)	(2)	Logarithm differences
1	120	120	2.0792	2.0792	0
2	120	160	2.0792	2.2041	0.1249
3	60	60	1.7782	1.7782	0
4	30	30	1.4771	1.4771	0
5	30	30	1.4771	1.4771	0
6	80	60	1.9031	1.7782	0.1249
7	120	80	2.0792	1.9031	0.1761
8	80	80	1.9031	1.9031	0
9	80	80	1.9031	1.9031	0
10	120	120	2.0792	2.0792	0
11	240	160	2.3802	2.2041	0.1761
12	160	160	2.2041	2.2041	0
13	160	160	2.2041	2.2041	0
14	120	120	2.0792	2.0792	0
15	160	160	2.2041	2.2041	0
16	240	320	2.3802	2.5051	0.1249
17	320	320	2.5051	2.5051	0
18	80	120	1.9031	2.0792	0.1761
19	60	60	1.7782	1.7782	0
20	160	160	2.2041	2.2041	0
21	120	160	2.0792	2.2041	0.1249

Sum of squares of differences = 0.1554

Number of pairs = 21; division = 21 x 2 = 42

Mean square = $\frac{0.1554}{42} = 0.0037$

Standard deviation = $\pm\sqrt{0.0037} = \pm 0.06083$

Students "t" for degrees of freedom = 21, and P=95% is a value of 2.08.

Therefore, if the standard deviation represented simply accidental errors, the true value in an analysis would be unlikely to be above or below the value given by a single estimate by more than 2.08 x 0.06083 = 0.1265 of a logarithmic unit.

Table 2, continued

The standard error of the difference between two estimates is:

$$\sqrt{(0.06083)^2 + (0.06083)^2}$$

$$= 0.06083\sqrt{2}$$

$$= 0.08601$$

$2.08 \times 0.08601 = 0.1789$ logarithmic units.

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1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.

2. Eight day old chicken embryos were inoculated with 100 EID_{50} mumps virus into the allantoic sac and incubated for four days at 35°C.

differences in dilution that might be considered significant, it can be seen in Table 2 that differences of one tube may correspond to dilution differences of 1.33 or 1.5 which correspond to logarithmic differences of 0.1249 and 0.1761 respectively. Both figures are below the 0.1789 $\log_{10}$ difference required for significance. The next dilution step beyond the 1.33 or the 1.5 difference is a dilution of two, corresponding to a logarithm difference of 0.3010. This value is significant and we can conclude that such a two fold difference occurring between two titrations performed on the same day under the same experimental conditions is a significant difference and may occur by chance in less than one time in twenty.

The results obtained through analysis of Table 2 seem to differ from those obtained in Table 1. However, this can be easily reconciled by the fact that the observations recorded in Table 1 were each obtained on different days over a four month interval. Each pair of results tabulated in Table 2 were collected on a single day. It is easily visualized that differences occurring from day to day are greater than those noted on any single day. Factors contributing to these day by day differences that may effect the hemagglutinin endpoint are variations in temperature, in sensitivity of the chicken red cells, and variations in chickens.

Infectivity titrations were run by preparing serial ten fold dilutions of virus infected fluids in buffered saline solution of pH 7.2, and inoculating 0.1 ml of each dilution into the allantoic sacs of six or more 9 to 11 day old embryos. After 40 to 48 hours incubation, 0.5 ml of allantoic fluid of each egg was removed and added to 0.5 ml of saline, followed by the addition of 1.0 ml of a one per cent suspension of chicken red blood cells. After a 45 minute incubation period, presence or absence of virus infection in each egg was determined by presence or absence of hemagglutination. The 50 per cent embryo infectivity endpoint was calculated by the method of Reed and Muench (33, pp.494-497).

Investigations into the reproducibility of infectivity titrations by Knight (23, pp.488-490) indicate that if five embryos were used per group with ten fold dilution steps, the standard deviation of a single titration was equal to 0.225 $\log_{10}$ units. By further statistical analysis it was seen that in using this method of titration a difference between titrations exceeding 0.62 $\log_{10}$ units was significant at the 95 per cent level of significance. If the number of eggs per dilution was increased to ten, the standard deviation was equal to 0.132 and a difference of 0.37 $\log_{10}$ units or greater is significant at the same significance level.

Preparation of compounds. Isopropyl biguanide was obtained from the American Cyanamid Company as a white crystalline compound readily soluble in water. The concentration most commonly used in these studies was 50 mg per ml. Heating briefly in a boiling water bath effected a clear colorless solution of approximately pH 6.2. Upon injection of 10 mg in a 0.2 volume into the allantoic sac of a 9 to 11 day old chick embryo, no lowering of the pH of the allantoic fluid could be detected. On the basis of this observation, no adjustment of the pH of the solution was deemed necessary. All solutions of isopropyl biguanide were sterilized by filtration through a sintered glass filter of ultra fine porosity.

The canavanine used in this investigation was isolated from jack bean meal by Victor E. Smith of the Oregon State College Department of Chemistry. In the earlier experiments in this study the canavanine was prepared as L-canavanine sulfate. However, about midway in this investigation the canavanine was made available as the free base. The method of isolation was that of Horowitz and Fling (20).

Canavanine, as either the sulfate or free base, is a slightly yellow crystalline compound very soluble in water. Canavanine sulfate at a concentration of 100 or 200 mg per ml in distilled water is a solution of a golden

yellow hue with a pH of approximately 4.0. The free base of canavanine at this concentration also forms a golden yellow solution with a pH of approximately 8.3 In neither case was the adjustment of the pH to neutrality attempted. The sulfate preparation injected in a 20 mg quantity in a 0.1 or 0.2 ml volume into the allantoic sac of the 9 to 11 day old chick embryo caused a decrease in the pH of the allantoic fluid of between 0.3 and 0.6 of one pH unit. The pH of the solution of the free base at 8.3 is within the pH range of the normal allantoic fluid so that no effect on the pH of the fluid was noted.

All solutions of canavanine were sterilized by filtration through an ultra fine sintered glass filter.

Inhibition studies. Previous to any investigation of inhibitory activity against virus development in the chick embryo it was necessary to determine the maximum tolerated dose of the test compound for the chick embryo. This was done by preparing a concentrated solution of the compound, usually limited by solubility or physical characteristics of the solution, in distilled water and injecting decreasing doses of this compound into groups of four to five 9 to 11 day old chick embryos. The eggs were then candled daily for four days to observe for death of the embryos. The dose used as the maximum tolerated dose was that concentration noted to cause the death of

but 25 per cent or less of the embryos in a group.

Upon completion of this test of compound toxicity for the chick embryo, the maximum tolerated dose was injected into the allantoic sac of ten or more chick embryos. A similar group of control eggs either received no injection, or the same volume of sterile distilled water injected into the allantoic sac. After a one hour period all eggs were inoculated into the allantoic sac with 50 ID_{50} of Lee influenza virus and then sealed with a sterile vaseline-paraffin mixture. All eggs were then incubated at 35°C for 40 to 48 hours.

At the end of the 40 to 48 hour incubation period, the eggs were removed from the incubator and candled to observe for deaths of any eggs. Any dead eggs were then discarded and the surviving eggs chilled for one hour at -20°C. After the one hour chilling period, the eggs were opened aseptically and the allantoic fluids removed individually by means of a sterile needle and syringe. Sterility tests were made by inoculating 0.1 ml of the fluid from each egg into thioglycollate broth and the results read at seven days. Hemagglutinin titrations were run on each individual fluid as well as on a pool of equal volumes from all eggs from the same group.

Following the observation of inhibition of virus development by the compound at its maximum tolerated dose,

various modifications were made in the basic experimental plan to determine the extent of the inhibition and the possible mechanism of this inhibition. These modifications involved variation in the dosage of the compound injected and increases in the dose of virus inoculated. Variations in the time of injection of the compound were made, injecting the compound one hour before, and two hours and twenty-four hours after the injection of the virus. Any possible direct effect of the compound upon the virus hemagglutinin and infectivity was investigated *in vitro*. Attempts to reverse the inhibitory activity of this compound were carried out with related metabolites. Respiration studies were carried out to investigate any effect of the compound upon respiration of the chorioallantoic membrane tissue. Possible inhibitory activity of this compound against the development of other viruses was also investigated. Besides investigations of inhibition *in ovo*, activity against virus development in tissue cultures and in mice was also studied.

Tissue cultures. The tissue culture techniques used were similar to those of Tamm, Folkers and Horsfall (43, pp.559-561), wherein the virus was found to grow easily *in vitro* with a satisfactory titer. All equipment used in the tissue culture was cleaned in dichromate sulfuric acid cleaning solution or boiled in Labtone solution,

followed by careful rinsing ten times in running tap water, five times in distilled water and two times in deionized water. All glassware was sterilized at 160°C for one hour while rubber stoppers and bakelite caps were sterilized by autoclaving.

The nutrient fluid for tissue culture was prepared in two solutions having the following composition:

Glucosol solution.	NaCl	8.0	grams
	$CaCl_2 \cdot 2H_2O$	0.2	grams
	$MgCl_2 \cdot 6H_2O$	0.5	grams
	Glucose	1.0	grams
	Deionized water to	1000	ml
Buffer solution.	Na_2HPO_4 (anhydrous)	7.105	grams
	KH_2PO_4	2.269	grams
	Phenol red	0.02	grams
	Deionized water to	1000	ml

Both solutions were sterilized in Pyrex bottles with bakelite screw caps by autoclaving at 15 pounds pressure for 20 minutes. The two solutions were mixed in equal volumes immediately before use at which time penicillin and streptomycin were added to give a final concentration of 10 units per ml and 40 micrograms per ml, respectively. The final pH of this mixture was 7.28.

The chorioallantoic membrane tissue was removed aseptically from 9 to 11 day old chick embryos by cleaning the surface of the egg with tincture of iodine, breaking away the shell with sterile forceps and pouring the egg contents into sterile petri dishes. The chorioallantoic

membrane was removed with fresh forceps and washed by centrifuging three times at 1500 r.p.m. for two minutes with fresh changes of nutrient glucosol. After the tissue was washed it was cut into 2 cm x 2 cm squares using sterile Bard Parker scalpel blades and distributed at random into sterile Pyrex culture tubes (25 x 100 mm), fitted with rubber stoppers and containing 2 ml nutrient glucosol, with or without the addition of the compound to be tested. The compound to be studied was dissolved in nutrient glucosol solution at a concentration of ten to twenty times the highest concentration to be tested and sterilized by filtration through a sintered glass filter. Dilutions containing the compound at the proper concentration were prepared in sterile nutrient glucosol from the concentrated solution. The virus inoculum consisted of 0.1 ml of a 1:100 dilution of allantoic fluid virus maintained in the frozen state at -60°C or the same dilution of freshly harvested allantoic fluid virus previously titrated for presence of a high hemagglutinin titer.

The tissue culture tubes were incubated at 35°C on a reciprocating shaker having a stroke length of 8 cm and a frequency of 90 strokes per minute. After an incubation period of 40 to 48 hours the culture fluids were tested for sterility by inoculation of 0.1 ml from each

tube into thioglycollate broth. The amount of virus present in each tube was determined by hemagglutinin titration.

In experiments designed to measure the amount of virus present in the tissue, and therefore not released into the tissue culture fluid, the membranes of each group receiving similar treatment were pooled, rinsed three times in sterile distilled water and ground in sterile mortar and pestle with sterile Pyrex glass to a 1:20 dilution, weight per volume, in sterile buffered saline. After centrifuging, the virus content of the supernatant was determined by hemagglutinin and infectivity titrations.

Respiration Studies. In an effort to determine whether either canavanine or isopropyl biguanide has any direct effect on the respiration of the tissue, several studies were carried out using Warburg manometry with uninfected chorioallantoic membranes. No significant difference in the respiration of normal membranes and membranes infected with influenza virus in vitro was noted in a study undertaken by Ackermann (1, pp.422-427).

Chorioallantoic membrane tissue of 15 to 20 ten day old embryos was removed aseptically and washed three times in sterile nutrient solution by centrifuging at 1500 r.p.m. for three to five minutes. After blotting on sterile filter paper to remove excess water, pieces

approximating 200 to 250 mg were weighed aseptically and dispensed in sterile Warburg flasks.

Respiration studies were carried out at different times using two methods. In one case, a CO_2-free atmosphere was maintained by the presence of 0.2 ml of 20 per cent potassium hydroxide in the center well. The nutrient fluid used in this system was the nutrient glucosol solution of pH 7.2 of the same formula as used in the tissue culture experiments. The other system used involved the maintenance of a partial pressure of CO_2 in the flask by the method of Pardee (31, pp.1085-1088). An atmosphere of 3 per cent CO_2 was obtained by using a mixture of diethanolamine, 6N hydrochloric acid and potassium bicarbonate in the center well. A nutrient medium buffered at pH 8.33 containing bicarbonate and described by Ackermann (1, p.422) was present in the main compartment of the Warburg flask. Penicillin and streptomycin were included at the concentrations employed in tissue cultures to inhibit development of any bacterial contaminants. The compound to be investigated, either L-canavanine as the free base or isopropyl biguanide hydrochloride, was contained at the desired concentration in the 3.0 ml volume of nutrient fluid in each Warburg flask. This was obtained by appropriate dilution of a filter sterilized solution of known concentration. The total fluid volume was 3.2 ml per flask. Each preparation was run in duplicate or triplicate

and incubation was carried out at a temperature of 37°C. Respiration, as oxygen uptake, was followed by application of the conventional methods described by Umbreit, Burris and Stauffer (49, pp.1-37). Observations were generally carried out for an eight to ten hour period. In two experiments, respiration was followed over an extended period. Observations were made during the 20 to 26 hour incubation period in one and during the 44 to 50 hour period in the other. At the end of each experiment, the flasks were removed from the water bath and tested for sterility by inoculation of 0.2 ml of the fluid from each flask into thioglycollate browth. Tissues from each flask were removed, rinsed in distilled water, dried individually in weighing bottles at 110°C for 24 hours and then weighed. Oxygen uptake was calculated for each flask in terms of microliters of oxygen per milligram of dry weight of tissue.

EXPERIMENTAL RESULTS

Inhibition Studies Involving L-Canavanine

Investigations in the chick embryo. Inhibition of the development of Lee influenza virus was first observed in an experiment wherein one group of ten day old embryonated chicken eggs received an intra-allantoic injection of L-canavanine and a second group of eggs received no canavanine treatment. After one hour all eggs received 50 EID_{50} of Lee virus by the same route. Following a 44 to 48 hour incubation period at 35°C, all eggs were candled and the survivors were chilled one hour at -20°C. At the end of this period the fluid contained within the allantoic sac was removed from each egg and each fluid was individually titrated for hemagglutinin content. The results of such an experiment are indicated in Table 3. The figures in this table represent the reciprocals of the highest dilutions of allantoic fluids causing complete agglutination of chicken red blood cells. The lowest dilution of allantoic fluid in this experiment was a 1:4 dilution and, therefore, a zero being recorded as the hemagglutinin titer indicates that no agglutination of chicken erythrocytes occurred at this dilution. Equal volumes of allantoic fluids from each egg in each group were pooled. The hemagglutinin titer was determined for these pooled fluids

Table 3

Inhibition of Lee Influenza Virus Development in the Chick Embryo by L-Canavanine Sulfate at 20 mg per Egg

Egg Number	Hemagglutinin in untreated control eggs	Hemagglutinin in eggs treated with 20 mg canavanine
1	480	80
2	320	30
3	240	20
4	120	4
5	120	0
6	80	0
7	80	0
8	40	0
9	0	0
10	0	0
11	- -	0
Hemagglutinin titers of pooled fluids	160	10
Geometric mean	59.4	4.7

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluids causing complete hemagglutination of chicken red blood cells.

2. Treated eggs received 20 mg canavanine injected into the allantoic sac. One hour later all eggs received 50 EID_{50} of virus by the same route and were incubated at 35°C.

3. Virus titrations were carried out 44-48 hours after inoculation of the eggs with 50 EID_{50} of Lee influenza virus.

from both the treated and the untreated groups in an effort to obtain an experimental average value, representative of each group. The geometric mean for each group is also indicated. The geometric mean is the Nth root of the product of N numbers and is used to equalize ratios or percentages of numbers in a series. As the lowest dilution titrated was the 1:4 dilution, and not even partial agglutination occurred at this point, it is justifiable to assume that the hemagglutinin endpoint for those fluids with a titer recorded as zero, is at the 1:2 dilution or lower and this value was used in the calculation of the geometric mean. Any error involved in this procedure would tend to decrease the significance of an experimental result rather than increasing it.

It can be seen from the results obtained that treatment of the group of eggs with L-canavanine before infection with the virus reduced the amount of hemagglutinin produced. Hemagglutination by influenza virus is known to be caused by the virus itself; thus it appears that the development of the virus is hindered or retarded. However, it must be noted that though hemagglutinating activity is low or even not apparent in the treated eggs, we can not conclude that no virus was formed. It has been shown that in order to obtain visible hemagglutination,

the virus concentration as measured by infectivity titer, must be in the range of 10^6 to 10^7 EID_{50} per ml (9, pp. 173-176). Results such as those described in Table 3 in which a 13 to 16 fold difference in hemagglutinin titer was observed would suggest a difference in infectivity titer of approximately one logarithmic unit; that is, if canavanine produces a corresponding decrease in infectivity as well as in hemagglutinin.

In Table 4 are recorded results of two experiments run similarly which indicate a reduction of the infectivity titer of the virus contained in allantoic fluids of eggs treated with canavanine. Allantoic fluids were removed aseptically from a group of canavanine treated eggs and an untreated control group, each having received 50 EID_{50} Lee influenza virus. Equal volumes of fluids from each group were pooled and the infectivity titer determined by preparation of serial ten fold dilutions of each pool and injection of each dilution into six or more chick embryos. After 44 to 48 hours incubation at 35°C, the presence or absence of infection in each egg of each group was determined by testing for the presence or absence of hemagglutinin in the allantoic fluid.

In experiment 1, treatment with 20 mg of canavanine resulted in a reduction of the infectivity titer of

Table 4

Effect of Canavanine upon the Development of Lee Influenza Virus as Determined by Infectivity Titration

Experiment Number	Virus Dose	Canavanine dose mg per embryo	Hemagglutinin titer of pooled fluids	EID_{50} per ml of pooled fluids
1	50 EID_{50}	0	- -	$10^{7.19}$
	50 EID_{50}	20	- -	$10^{6.09}$
2	50 EID_{50}	0	256	$10^{9.12}$
	50 EID_{50}	20	0	$10^{5.80}$

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluids causing complete hemagglutination of chicken red blood cells.

2. Treated eggs received 20 mg canavanine injected into the allantoic sac. One hour later all eggs received 50 EID_{50} of virus by the same route and were incubated at 35°C.

3. Virus titrations were carried out 44-48 hours after inoculation of the eggs with 50 EID_{50} Lee influenza virus.

4. Infectivity titers were determined by injection of 0.1 ml of serial ten fold dilutions of pooled allantoic fluids into groups of six or more eggs per dilution. After 44 hours incubation at 35°C presence or absence of infection was determined by testing for hemagglutination at a 1:4 dilution.

slightly more than one logarithmic unit as compared with a similar group of untreated control eggs. In experiment 2, a marked difference in hemagglutinin titer is noted. At the same time, a corresponding decrease in infectivity of greater than three logarithmic units occurs. Canavanine at 20 mg per egg appears to cause a significant reduction in virus development as indicated by both lower hemagglutinin production and a lower number of infective particles.

It was found that as the dose of canavanine was decreased, the degree of inhibition also decreased. Results of inhibition studies using doses of canavanine ranging from 40 mg per egg to 2 mg per egg are tabulated in Table 5. This is a composite of several experiments and the geometric mean is calculated on the individual titers of all eggs tabulated. The lowest dilution titrated in these experiments was the 1:20 dilution. In the calculation of the geometric mean, those fluids giving no agglutination or a partial agglutination at the 1:20 dilution were arbitrarily given an endpoint value of 1:10.

As the dose of canavanine was decreased, the per cent of eggs containing greater amounts of virus hemagglutinin increased, as did the geometric mean of the hemagglutinin titer. By the use of the "t" test for comparison of means, it was found that the effect of 2 mg

Table 5

Relation of Dosage of Canavanine to the Degree of Inhibition of Lee Influenza Virus in the Chick Embryo

Dosage of canavanine per egg	Total number of eggs treated	Per cent of eggs showing titers of (10 or below)	(20-80)	(120-320)	(480 or above)	Geometric mean of all fluids
40 mg	10	90	10	0	0	11.4
20 mg	23	61	17	22	0	23.1
10 mg	45	27	27	40	6	53.8
5 mg	33	36	33	21	10	57.8
2 mg	20	25	30	35	10	67.9
Control	51	10	15	53	22	155.2

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluids causing complete hemagglutination of chicken red blood cells.

2. Treated eggs received their indicated dose of canavanine injected into the allantoic sac. One hour later all eggs received 50 EID_{50} of virus by the same route and were incubated at 35°C.

3. Virus titrations were carried out 44 to 48 hours after inoculation of the eggs with 50 EID_{50} Lee influenza virus.

4. As the lowest dilution titrated was the 1:20 dilution, all eggs recorded in the column "10 or below" were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

Table 5, continued

5. Comparison of the mean values for the hemagglutinin titers of the control and 2 mg groups by means of the "t" test (25, pp.151-174) using logarithms of the individual titers, gives a "t" value of 2.37. For 69 degrees of freedom this indicates that the difference is highly significant at the .05 probability level and is unlikely to be due to chance.

dose of canavanine was still significant[3] (25, pp.147-170; 10, pp.114-128). The 20 mg dose is the one used in the majority of experiments reported in this study and causes a definite inhibition with a minimum of toxicity.

The next variable to be studied was the effect of the dosage of virus in the inoculum upon the inhibition by canavanine. It was found that as the dose of virus was increased, the inhibitory effect of canavanine decreased. The results of such a study are tabulated in Table 6. Inhibition is evident with all lower virus doses and is still striking in the group receiving 4500 EID_{50} of Lee influenza virus. When lyophilized virus

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[3] Using the logarithms of the individual titers of the 51 eggs in the control group and 20 eggs in the group receiving 2 mg of canavanine, mean logarithmic values of 2.1910 and 1.8320 were obtained for the control and treated groups respectively. The difference in mean values was equal to 0.3590 $\log_{10}$ units.

Sum of the squares of the deviations from the mean for the control group = 14.77800.

Sum of the squares of the deviation from the mean for the treated group = 7.99607.

Combined sum of squares = 22.77407.

Degrees of freedom = (51-1) + (20-1) = 69.

Variance = $\frac{22.77407}{69} = 0.330058$

Estimated standard deviation = $\pm\sqrt{0.330058} = \pm 0.5744$

Table 6

Influence of Virus Dosage on Inhibition
of Lee Influenza Virus by Canavanine in the Chick Embryo

EID_{50} of virus inoculated	Canavanine Dose mg per egg	Number of eggs with titers of (10 or below)	(20-80)	(120-320)	(480 or above)	Hemagglutin titers in pooled fluids from all eggs	Geometric mean
8.4	0	2	5	3	0	60	54.0
8.4	20	9	1	0	0	0	10.7
84	0	2	4	4	0	120	62.1
84	20	8	1	0	0	0	10.8
800	0	0	4	6	0	120	82.9
800	20	4	5	1	0	30	24.2
4500	0	0	0	2	8	640	567.4
4500	20	6	1	0	1	60	18.6
13,000	0	3	0	4	3	320	102.0
13,000	20	1	2	7	0	120	98.0
130,000	0	1	0	6	2	320	166.4
130,000	20	2	1	6	0	160	72.7

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluids causing complete hemagglutination of chicken red cells.

2. Treated eggs received 20 mg canavanine injected into the allantoic sac. One hour later all eggs received 50 EID_{50} of virus by the same route and were incubated at $35^{o}C$.

3. Virus titrations were carried out 44-48 hours after inoculation of the eggs with 50 EID_{50} Lee influenza virus.

Table 6 (continued)

4. Doses of 4,500, 13,000 and 130,000 EID_{50} were obtained by use of freshly harvested and titrated allantoic fluid virus. Doses of 8.4, 84 and 800 EID_{50} were prepared from lyophilized virus titrated on the day of the experiment.

5. As the lowest dilution titrated was the 1:20 dilution, all eggs recorded in the column "10 or below" were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

6. The 50 per cent egg infective dose in each case was determined by infectivity titrations run on the same day of the experiment using the same virus preparation used in the experiment.

was used, the dose administered was calculated from infectivity titrations on the preparation of virus performed on the same day as the experiment. Doses of 4500 EID_{50} and greater were obtained by the use of freshly harvested allantoic fluid virus. When freshly harvested virus was employed, the virus titer was determined in each case by infectivity titration on the day of harvest while the virus was held at 4°C. When the infectivity titer was known, the refrigerated virus was then diluted to that dilution calculated to contain the desired dose in 0.1 ml. An infectivity titration was run again on this preparation following inoculation of the experimental group. In this manner we expect to know, within limits of the experimental error of the infectivity titration, the dose of virus administered in each experiment.

It may be seen that as the dose of virus was increased to 13,000 EID_{50} and above, the inhibition of virus

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[3](continued)

Standard error of difference between means =

$$\pm\sqrt{\frac{(0.5744)^2}{51} + \frac{(0.5744)^2}{20}} = \pm\ 0.15153$$

$$\text{"t"} = \frac{\text{difference in means}}{\text{standard error of difference}} = \frac{0.3590}{0.15153} = 2.37$$

For 69 degrees of freedom this value of "t" corresponds to a P value of between 0.025 and 0.01 and indicates that the observed difference is very unlikely to occur by chance.

development produced by 20 mg canavanine was too slight to be significant with this small number of eggs. It was also noted that as the virus dose was increased to very high levels, the infection was partially inhibited by the virus itself. An exceedingly high number of infecting particles decreased the amount of hemagglutinating virus produced. This is known as auto-interference and has been commonly encountered with influenza viruses. It tends to confuse and make impractical inhibition studies involving doses exceeding 100,000 EID_{50} per embryo.

When canavanine was injected into the yolk sac of the chick embryo instead of the allantoic sac, and then followed one hour later by an intraallantoic inoculation of 50 EID_{50} Lee influenza virus, the virus developing in the allantoic sac was also inhibited. Results of such an experiment are given in Table 7. It appears as though the compound was rapidly absorbed from the yolk sac and distributed to the cells of the allantoic membrane where multiplication of the virus normally takes place. This result suggested that the inhibitory action of canavanine is not due to a direct effect of the compound upon the virus while both are present together in the allantoic sac.

A study was next made of the relationship of the time of injection of canavanine with respect to the virus inoculation and the effect upon virus inhibition. Results

Table 7

Inhibition of Lee Influenza Virus in the Chick Embryo Resulting from the Yolk Sac Injection of 20 mg Canavanine

Egg Number	Hemagglutinin in untreated control eggs	Hemagglutinin in eggs treated with 20 mg canavanine
1	960	640
2	640	120
3	640	80
4	480	80
5	480	80
6	480	40
7	480	0
8	480	0
9	320	0
10	320	0
Hemagglutinin titers of pooled fluids	640	80
Geometric mean	502.3	41.6

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.

2. Treated eggs received 20 mg canavanine injected into the yolk sac. One hour later all eggs received 50 EID_{50} of virus by the same route and were incubated at 35°C.

3. Virus titrations were carried out 44-48 hours after inoculation of the eggs with 50 EID_{50} Lee influenza virus.

4. As the lowest dilution titrated was the 1:20 dilution, all eggs recorded as zero showed no agglutination at this dilution and were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

of this study are shown in Table 8. When the compound was administered one hour before the virus inoculum, the inhibition was most striking; however, if canavanine followed the virus dose by two hours, the inhibition was still very definite. This is indicated by the greater number of eggs showing low titers in the treated groups as well as in differences noted on comparison of their geometric mean titers with that of the untreated control group. When canavanine was administered by intraallantoic injection 24 hours after the virus inoculation, the degree of inhibition was considerably less and by statistical analysis it did not appear to be significant.

Results obtained from this experiment could be interpreted in at least two ways. Either there may be a direct interference by canavanine with some path of synthesis involved in the early stage of virus development, or perhaps canavanine had a direct inactivating effect upon the virus inoculated into the allantoic sac. Although virus absorption occurs fairly rapidly, with Lee influenza virus a time interval of about five hours elapses before the maximum amount of virus introduced is absorbed (18, pp.2-4). During this period of pre-adsorption, canavanine might have reacted directly with the virus, interfering with the properties necessary for infection. Even though this was not indicated to be the

Table 8

Influence of Time Interval between Infection and Injection of Canavanine on Inhibition of Lee Influenza Virus

Canavanine 20 mg per egg	Number of eggs showing titers of (10 or below)	(20-80)	(120-320)	(480 or above)	Geometric mean of individual hemagglutinin titers
1 hour before virus	14	3	2	1	20.4
2 hours after virus	10	8	2	0	25.3
24 hours after virus	4	6	7	3	84.8
Control, no canavanine	2	4	11	3	119.8

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluids causing complete hemagglutination of chicken red blood cells.

2. All eggs received 50 EID_{50} Lee influenza virus inoculated into the allantoic sac and were incubated at 35°C for 44 hours after virus inoculation.

3. As the lowest dilution titrated was the 1:20 dilution, all eggs recorded in the column "10 or below" were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

4. Comparison of the mean values of the hemagglutinin titers of the control group and the group receiving canavanine 24 hours after the virus injection by means of the "t" test (25, pp.151-174) using logarithms of the individual titers, gives a "t" value of 1.04. For 38 degrees of freedom the difference is not significant at the usually accepted .05 probability level.

case in experiments involving the yolk sac injection of canavanine, it was desirable to obtain further information by a direct in vitro experiment wherein the virus was incubated with canavanine at a concentration approximately equal to that present in the allantoic fluid. Such experiments were carried out in the following manner.

Investigations in vitro. Infected allantoic fluid from embryos inoculated 44 hours earlier with 50 EID_{50} Lee influenza virus was harvested, centrifuged to remove aggregates and coarse particles and then dispensed in two equal volumes. To one volume was added sterile canavanine solution to give a concentration of canavanine of 4 mg per ml of allantoic fluid. This was assumed to be the maximum concentration of canavanine present in the allantoic sac of a ten day old chick embryo following injection of 20 mg intra-allantoically. To the second volume of allantoic fluid virus was added a similar volume of sterile distilled water. Each volume was then divided into two tubes, with one tube of each preparation to be incubated at 35°C, and the other at 10°C for a period of 24 hours. Infectivity titrations were run before incubation on the control fluid and after incubation on each of the four samples. Results of an experiment of this type are recorded as experiment 1 in Table 9. In no case was

Table 9

Investigation of an In Vitro Effect of Canavanine upon Lee Influenza Virus

Experiment	Preparation	Incubation time in hours	Incubation temperature	Hemagglutinin titer	ID_{50} per ml	pH of fluid after incubation
I	virus only	0	before incubation	640	$10^{9.30}$	8.32
	virus only	24	10°C	480	$10^{9.40}$	8.67
	virus+canavanine 4 mg per ml	24	10°C	480	$10^{8.76}$	8.52
	virus only	24	35°C	640	$10^{2.48}$	8.86
	virus+canavanine 4 mg per ml	24	35°C	640	$10^{5.50}$	8.54
II	virus+H_2O diluent	0	before incubation	--	$10^{9.00}$	8.36
	virus+H_2O diluent	24	35°C	640	$10^{4.46}$	8.66
	virus+buffer pH 8.00	24	35°C	480	$10^{5.43}$	8.40
	virus+canavanine 4 mg per ml	24	35°C	480	$10^{6.79}$	8.45

Table 9, continued

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.

2. Infectivity titers were determined by injection of 0.1 ml of serial ten fold dilutions of virus fluids into groups of six or more eggs per dilution. After 44 hours incubation at 35°C presence or absence of infection was determined by hemagglutination at a 1:4 dilution.

the hemagglutinin affected, whether canavanine was included or absent and whether the temperature of incubation was 10°C or 35°C for a 24 hour period. The amount of infective virus was not decreased by incubation in the absence of canavanine at 10°C and although a slight decrease was noted when canavanine was present at 10°C, this difference did not appear in two subsequent experiments. At an incubation temperature of 35°C for 24 hours, the infectivity of the virus was decreased considerably in both cases. However, this loss of infectivity was much greater in the absence of canavanine than in its presence, indicating that canavanine does not have a direct inactivating effect on the virus, but rather may exert some sort of protective effect. The final pH of untreated allantoic fluids after incubation in all cases showed a tendency to be slightly more alkaline in reaction than the similar fluids which contained canavanine. This was probably due to a dissociation or decomposition of some of the urates present in the fluid. Canavanine seemed to more or less buffer the allantoic fluid. This same resistance to pH change was shown when arginine was present in the allantoic fluid. In order to throw more light on the mechanism of the protective effect of canavanine, it was then desirable to attempt to buffer the allantoic fluid with a phosphate-glycine buffer of pH 8.0

to see whether the buffered fluid also possessed this same effect. A concentrated buffer of M/2 glycine in M/2 NaCl was sterilized by autoclaving and mixed in equal volume with M/3 Na_2HPO_4 immediately before use and then added to infected allantoic fluid in the proportion of one part buffer to nine parts of fluid. A similar control mixture was prepared in which nine parts of infected allantoic fluid were diluted with one part of sterile distilled water. A third preparation of the infected fluid was made up with the same degree of dilution to contain canavanine at a concentration of 4 mg per ml. All preparations were incubated at 35°C for 24 hours and then assayed for virus content. The results appear in Table 9. Again, no significant variation in hemagglutinin was noted between the three groups. The pH of the control fluid receiving distilled water again was slightly more alkaline than the buffered fluid or the canavanine fluid. Infectivity titrations showed that less loss of infectivity occurred in the buffered fluid than in the unbuffered control fluids, but that the fluid containing canavanine still contained a significantly larger amount of infective virus.

It appears as though canavanine does not have any deleterious effect upon the virus infectivity but rather exerts some sort of protective effect. This protection

may in part be a pH effect, but this does not seem to be the only explanation.

Investigations on adsorption. Although canavanine does not destroy the infectivity of the virus when both are present together in the allantoic fluid, it might still interrupt the first phase of virus multiplication by preventing adsorption of the virus to the susceptible cell, perhaps by altering, inactivating or combining with its specific receptor area on the cell. In Table 10 an experiment investigating any possible effect of canavanine upon the adsorption of the virus is recorded. Freshly harvested allantoic fluid virus was subjected to a low speed centrifugation to remove any aggregates and adsorbed virus and then two dilutions of 1:2 and 1:5 were prepared in saline. Each dilution was divided into two equal volumes. To one tube of each dilution was added a small volume of canavanine solution to give a final concentration of 4 mg per ml. To the second tube of each dilution was added similar small volumes of sterile distilled water. Each preparation was then further divided into two tubes. Chorioallantoic membrane tissue which had been washed three times in sterile saline was added at one gram per milliliter to one preparation containing canavanine and one without canavanine in both the set of tubes containing the 1:2 dilution of virus and in the set containing the

Table 10

Investigation of the Effect of Canavanine Upon the Adsorption of Lee Influenza Virus to Chorioallantoic Membrane

Allantoic fluid virus dilution	Canavanine concentration	Incubation time	Tissue present	Hemagglutinin titer
1:2	0	Before incubation	0	160
1:2	0	30 min.	0	160
1:2	4 mg per ml	30 min.	0	240
1:2	0	30 min.	1 gm per ml	20
1:2	4 mg per ml	30 min.	1 gm per ml	20
1:5	0	Before incubation	0	80
1:5	0	30 min.	0	60
1:5	4 mg per ml	30 min.	0	80
1:5	0	30 min.	1 gm per ml	<20
1:5	4 mg per ml	30 min.	1 gm per ml	<20

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluids causing complete hemagglutination of chicken red blood cells.

2. The tissue used was freshly harvested chorioallantoic membrane tissue which had been washed three times in sterile saline and weighed using aseptic conditions.

3. Incubation was carried out at 35°C in a water bath with occasional agitation.

1:5 dilution. After an initial titration of hemagglutinin before incubation, all tubes were incubated at 35°C for 30 minutes. At the end of the 30 minute incubation period the tubes containing tissue were centrifuged and hemagglutinin titrations were conducted on all fluids. The degree of adsorption was then measured by the decrease in the hemagglutinin in the fluids.

In the tubes containing the virus at 1:2 and chorioallantoic membrane tissue at one gram per milliliter, the hemagglutinin titer decreased to about one-eighth of that present in the tubes incubated without tissue, indicating an adsorption of approximately 85 per cent of the hemagglutinating virus present. No difference occurred between tubes containing canavanine and those without canavanine. In the tubes containing the virus at 1:5 the degree of adsorption was at least as great. It can be concluded from this experiment that canavanine has no effect upon the adsorption of the virus to the cells of the chorioallantoic membrane *in vitro*, at least within the limits of sensitivity of the test employed. It seems reasonable to assume that there is no effect in the allantoic sac of the intact chick embryo.

Investigations of reversal of virus inhibition in tissue culture. In view of other results obtained with canavanine inhibition of a large variety of microorganisms,

and its reversal by arginine, it seemed likely that the inhibition of Lee influenza virus was due to competition with arginine in some system required for virus synthesis (21, pp.371-376; 50, pp.894-899; 51, pp.209-211). The most sensitive method for studying this possibility would be the use of virus inhibition studies in tissue culture. Canavanine has been shown in this laboratory to markedly inhibit the development of Lee influenza virus in tissue culture in concentrations as low as 0.2 mg per ml of culture fluid (48, pp.23-27). Inhibition, though definite, at 0.1 mg per ml was not complete.

For these experiments, a strain of Lee influenza virus was used that was well adapted to the chick embryo and would multiply in tissue culture to produce a fairly high hemagglutinin titer. It was the Rockefeller Institute strain described in the "materials and methods" secion. The tissue was aseptically removed from 9 to 11 day old embryos, washed in nutrient glucosol solution, cut into 2 cm x 2 cm pieces and dispensed in rubber stoppered Pyrex tubes. Concentrated solutions of canavanine and arginine were prepared in nutrient glucosol solution and sterilized individually by filtration. Dilutions and mixtures of canavanine and arginine were prepared and dispensed in 2.0 ml volumes in six culture tubes per dilution. After inoculation with 0.1 ml of a 1:100 dilution

of Lee influenza virus, all tubes were incubated at 35°C and continuously aerated by shaking. Hemagglutinin titrations were carried out on each tube after 44 hours incubation.

The results reported in Table 11 indicate that canavanine inhibition of the virus is definitely overcome by arginine. However, it is not possible with the data available to state whether the inhibition is of a competitive nature. At 0.1 mg canavanine per ml of culture fluid there is only a partial inhibition of virus development and it appears as though equal quantities of arginine will reverse this degree of inhibition as shown by the tubes containing both canavanine and arginine at 0.1 mg per ml. Higher doses of arginine of 0.35 and 0.70 mg per ml also cause essentially a complete reversal.

The ratio of arginine to canavanine to effect the greatest degree of reversal of canavanine inhibition at the 0.2 mg per ml level seems to be better than 7:1 as indicated in that group receiving arginine at a concentration of 1.4 mg per ml. As the concentration of arginine in the tissue cultures was increased to beyond 2.0 mg per ml to reverse still larger doses of canavanine, the arginine itself seemed to have an inhibitory effect upon virus development (7, pp.506-507). This effect is apparent in experiment 3 when the arginine dose was increased

Table 11

Reversal of Canavanine Induced Inhibition of Lee Influenza Virus by L-Arginine HCl

Culture Number	Control, Virus Only	Virus hemagglutinin in tissue culture containing indicated concentration of L-arginine HCl plus L-canavanine at 0.1 mg per ml: 0.0 mg per ml	0.1 mg per ml	0.35 mg per ml	0.7 mg per ml
Experiment 1					
1	160	80	240	160	240
2	160	60	160	80	160
3	120	40	160	80	160
4	80	40	160	80	160
5	60	10	160	40	160
6	40	10	80	40	80
Geometric mean	91.6	30.3	152.5	71.3	152.5

Culture Number	Control, Virus Only	Virus hemagglutinin in tissue culture containing indicated concentration of L-arginine HCl plus L-canavanine at 0.2 mg per ml: 0.0 mg per ml	0.2 mg per ml	0.7 mg per ml	1.4 mg per ml
Experiment 2					
1	120	0	80	120	120
2	120	0	20	60	60
3	80	0	20	40	40
4	80	0	20	40	30
5	80	0	0	30	30
6	60	0	0	0	20
Geometric mean	91.4	10.0	20.0	38.9	41.6

Culture Number	Control, Virus Only	Virus hemagglutinin in tissue culture containing indicated concentration of L-arginine HCl plus L-canavanine at 0.3 mg per ml: 0.0 mg per ml	1.4 mg per ml	2.1 mg per ml	4.2 mg per ml
Experiment 3					
1	160	0	160	240	60
2	160	0	160	160	60
3	120	0	120	80	60
4	120	0	80	60	40
5	80	0	60	30	30
6	80	0	40	10	10
Geometric mean	115.3	10.0	91.5	61.7	37.8

Table 11 (continued)

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.

2. The virus inoculum consisted of 0.1 ml of a 1:100 dilution of freshly harvested Lee influenza virus, approximately equal to 10^5 EID_{50} per culture. This inoculum produced no detectable hemagglutinin titer in a culture tube containing 2 ml nutrient glucosol without tissue when titrated before incubation.

3. As the lowest dilution titrated was the 1:20 dilution, a zero being recorded as the hemagglutinin titer indicates that no agglutination occurred at the 1:20 dilution. In calculating the geometric mean, the endpoint of the zero titration value was assumed to be 10 or below and the value of 10 was used in the calculation.

to 4.2 mg per ml and the canavanine concentration was 0.3 mg per ml. It may also be indicated with arginine at the 2.1 mg per ml level when the extent of the reversal effect was less than that resulting from 1.4 mg of arginine per ml.

A similar condition was found to exist in attempts to reverse this canavanine antagonism in the chick embryo. The inhibition induced by canavanine is quite striking at 20 mg per embryo, but is considerably less apparent at 10 mg per embryo. If the canavanine dose is increased to 40 mg we exceed the maximum tolerated dose as determined by toxicity tests and the majority of the embryos do not survive the injection. To obtain a satisfactory arginine to canavanine ratio to effect reversal of inhibition by 20 mg of canavanine, the arginine dose would probably have to exceed 100 mg per embryo, at which concentration arginine is toxic. In two experiments wherein arginine was administered at 75 mg and 80 mg per embryo and canavanine at 20 mg per embryo, no reversal of inhibition was apparent.

Lysine has been reported by some investigators to reverse canavanine inhibition of Neurospora, yeast and _Avena_ coleoptiles (21, pp.373-376; 2, pp.328-329; 27, 1035). In some reports of bacterial inhibition, lysine was without effect (50, pp.896-899). Lysine was investigated briefly in tissue culture at 0.25, 1.0 and 4.0 mg

per ml in an attempt to reverse the inhibition of Lee influenza virus by 0.2 mg per ml of canavanine. At these concentrations, lysine showed no antagonistic effect on the canavanine inhibition.

Glutamic acid is reported to be a precursor of arginine synthesis in several microorganisms and was reported by Bonner to reverse the inhibitory effect of canavanine for Avena coleoptiles (2, pp.328-329; 19, pp.40-41). In tissue cultures of canavanine inhibited Lee influenza virus, glutamic acid at 0.2, 1.0 and 5.0 mg per ml had no effect on this inhibition.

Investigations of the effect on respiration. In a further attempt to determine the mechanism by which canavanine effects this inhibition of virus development, respiration studies were carried out employing the Warburg respirometer. Ackermann has shown that inhibition of respiration of the cells of the chorioallantoic membrane results in a marked decrease in virus production (1, pp. 422-427). It was desirable to determine whether canavanine at the concentrations shown to be inhibitory for virus multiplication in tissue culture would decrease the rate of oxygen uptake of chorioallantoic membrane tissue in the presence of glucose. Chorioallantoic membrane tissues from ten day old embryonated chicken eggs were removed aseptically and washed three times in the buffered

nutrient solution. Membranes were weighed on a torsion balance after first removing free water on sterile blottin paper. Membranes approximating 250 mg wet weight of tissue were distributed in sterile Warburg flasks containing either nutrient glucosol solution of pH 7.2 for use in the CO_2-free atmosphere, or the bicarbonate nutrient solution of pH 8.33 for use with the CO_2 buffer system of Pardee (31, pp.1085-1088). Canavanine, where desired, was incorporated into the solution at a concentration of 0.2 mg per ml. Penicillin and streptomycin were added to suppress growth of any accidental bacterial contaminants. A solution of 20 per cent KOH was contained in the center well when a CO_2-free atmosphere was to be maintained. The diethanolamine mixture was used in studies employing a partial pressure of CO_2. Oxygen uptake was followed manometrically according to the standard methods of Umbreit, Barris and Stauffer (49, pp.1-37). Readings were made at half hour intervals for periods extending from six to ten hours. The thermostat was maintained at 37°C.

At the completion of each experiment, the contents of each flask were tested for sterility by inoculation of 0.1 ml into thioglycollate broth. The membranes from each flask were removed and washed in distilled water and dried at 110°C for approximately 24 hours. The dry weight of tissue contained in each flask was determined and the

the oxygen uptake was calculated in terms of microliters per mg of dry weight of tissue.

In Table 12 three experiments are tabulated involving the effect of canavanine upon the respiration of chorioallantoic membrane tissues. The rate of oxygen uptake per milligram of dry weight of tissue per hour in a CO_2-free atmosphere in a nutrient glucosol solution is indicated at periods of two, four and six hours and, in two experiments, after eight hours of incubation. No significant inhibition of respiration was noted during this period of incubation. In examining the rates of oxygen uptake indicated for those tissues incubated in the presence of canavanine, it appeared that a gradual decrease in rate of oxygen uptake might be occurring. This suggested that longer periods of contact of the tissue with the canavanine solution might result in a significant depression of respiration by the compound. Furthermore, the period of maximum virus formation in the tissue is probably between 12 and 36 hours. Accordingly, in experiment three, indicated on Table 12, the period of incubation was extended to 18 hours during which time the flask was shaken continuously in the 37°C water bath with the manometer open to the air. After the 18 hour period the manometer was closed off and the rate of oxygen uptake again followed over a five hour period. It can be

Table 12

The effect of L-canavanine upon the Rate of Oxygen Uptake by Chorioallantoic Membrane Tissue in the Presence of Glucose

Period of incubation	Rate of oxygen uptake in microliters per milligram of dry weight of chorioallantoic membrane tissue per hour					
	Experiment 1		Experiment 2		Experiment 3	
	Control, no canavanine	Canavanine, 0.2 mg per ml	Control, no canavanine	Canavanine, 0.2 mg per ml	Control, no canavanine	Canavanine, 0.2 mg per ml
2 hours	6.8	6.4	7.1	6.9	6.2	7.1
4 hours	6.7	6.2	7.3	6.6	6.2	6.9
6 hours	6.7	6.1	7.0	6.2	6.1	6.5
8 hours	6.7	6.1	7.0	6.0	---	---
18 hours incubation plus						
1 hour					4.8	4.5
2 hours					4.7	4.8
3 hours	- - - - -		- - - - -		5.0	5.0
4 hours					5.0	5.0
5 hours					4.8	5.0

Table 12 (continued)

1. Respiration of chorioallantoic membrane tissue was measured in a CO_2 free atmosphere in nutrient glucosol solution buffered at pH 7.2. The formula of the nutrient glucosol solution is identical to that used in tissue culture studies.

2. Each flask contained a total fluid volume of 3.2 ml and approximately 250 mg wet weight of chorioallantoic membrane, which upon completion of the experiment was dried at 110°C for 20 hours and yielded roughly 10 to 12 mg dry weight of tissue.

3. Oxygen uptake is calculated on the basis of microliters per milligram of dry weight of membrane.

seen from these results that the rate of oxygen uptake in both cases is lower than the rate during the first six hours. However, after this 18 to 23 hour incubation period, canavanine seemed to cause no depression of the respiration of the chorioallantoic membrane tissue.

The effect of canavanine upon the rate of respiration of chorioallantoic membrane when both were incubated together for a longer period was investigated using diethanolamine in the center well and a partial pressure of CO_2, estimated to be 3 per cent, in the flask. Oxygen uptake was followed during the first seven hours incubation after which time the flasks were removed from the water bath, stoppered with a sterile rubber stopper and incubated at 35°C for the next 36 hours. The diethanolamine was carefully removed and replaced with fresh diethanolamine and again placed on the manometer and incubated with shaking in the 37°C water bath. After equilibration the rate of oxygen uptake was again followed. Oxygen uptake was calculated per milligram of dry weight of tissue. Results of this experiment are indicated in Table 13.

Canavanine appeared to cause a slight initial depression of respiration of the tissue in this experiment when compared with control flasks containing no canavanine. The depression is only of the order of eight to ten per

Table 13

The Effect of L-Canavanine upon the Rate of Oxygen Uptake by Chorioallantoic Membrane Tissue after an Extended Incubation

Period of incubation	Rate of oxygen uptake in microliters per milligram of dry weight of tissue per hour in a 3 per cent CO_2 atmosphere	
	Control, no canavanine	Canavanine at 0.2 mg per ml
2 hours	6.9	6.0
4 hours	7.3	6.4
6 hours	7.2	6.4
7 hours	7.1	6.5
43 hours incubation plus		
2 hours	2.7	2.7
4 hours	2.8	2.9
6 hours	2.9	3.2
7 hours	2.9	3.2

1. Each Warburg flask contained 0.6 ml diethanolamine mixture in the center well, 2.6 ml bicarbonate-nutrient solution of pH 8.33 and approximately 250 mg wet weight of chorioallantoic membrane in the main compartment.

2. Oxygen uptake was measured manometrically during incubation at 37°C with readings being made every half hour.

3. At the end of the first seven hours of incubation, the flasks were removed from the bath and their manometers, stoppered with sterile rubber stoppers and held at 35°C for the next 36 hours. At the end of this time, the diethanolamine was removed and replaced with fresh solution and respiration was followed for the next seven hours.

4. Rates of oxygen uptake are calculated in microliters per milligram of dry weight of tissue per hour.

cent. What is of more importance is that this effect does not appear to persist. During the 44 to 50 hour incubation period at which time the virus titer would reach a maximum, no difference in respiration can be observed between membranes in the presence of canavanine at 0.2 mg per ml or with no canavanine present. In both cases the rate fell to no more than half the initial rate. The combined data in Tables 12 and 13 seem to indicate that canavanine does not exert its effect upon virus development through inhibition of the respiration of the tissue.

The rates of respiration of chorioallantoic membrane tissue appear to be lower than respiration rates of bacterial suspensions. The rates of oxygen uptake indicated for the membranes of Table 12 and 13 are in agreement or slightly higher than data indicated by Ackermann (1, pp.423-425). Some difficulty in direct comparison between this data is noted due to differences in calculation of oxygen uptake. Ackermann's respiration experiments were carried out with membranes removed from 14 day old embryos and rates of oxygen uptake were calculated as microliters of oxygen per 200 mg of moist tissue per 30 minute period.

Investigations of the effect on virus content of the tissue. The final phase of virus development during which canavanine might exert an interfering effect is the

period of release of the virus from the cell. If canavanine did interfere with virus liberation, we would expect to find, upon breaking of the cell and subsequent release of the virus, comparable hemagglutinin titers between untreated infected control tissues and canavanine treated infected tissues. This question was investigated in one experiment in which canavanine was administered to one group of ten day old chick embryos in a 20 mg dose and a control group received no canavanine. After one hour, all eggs were inoculated with 50 ID_{50} Lee influenza virus by the allantoic route. Following 44 hours incubation the allantoic fluids were removed from each egg surviving the treatment and the chorioallantoic membranes of six eggs in each group were harvested. The hemagglutinin titer of allantoic fluid virus of the control group was much higher than the titer of the treated group, the difference being of the order of 200 fold.

The membranes from each egg were washed in three changes of saline and, after blotting, weighed. A ten per cent suspension of each membrane was prepared in a chilled Waring blender in chilled saline to avoid excess heating. Each membrane was subjected to an arbitrary but definite time of five minutes in the blender. Each tissue suspension was centrifuged at about 1500 r.p.m. for 7 minutes and hemagglutinin titrations were carried out on the

supernatant fluids. It was found that the inhibition of virus development by canavanine as noted by titration of the allantoic fluid was also evident by titration of the membranes. This data lent additional support to the view that canavanine interferes with some step in the biosynthesis of the virus in the tissue, so that insufficient virus is produced to be observed by the hemagglutinin titrations.

Investigations on Lee influenza virus infection in mice. It was of interest to determine whether canavanine could exert this same inhibitory effect upon Lee influenza virus infection in mice. Canavanine was passed through a 250 mesh screen to produce a small and fairly constant particle size. No sterilization of the canavanine was attempted due to its instability to heat. A known quantity was weighed out and a suspension made in sterile sesame oil containing two per cent of aluminum monostearate to give a final concentration of 300 mg of canavanine per ml. The virus used as the inoculum was maintained in mouse lung, coarsely minced and frozen. An infectivity titration in mice was performed on a portion of this mouse lung virus previous to initiation of this experiment.

Five groups of ten Swiss mice per group ranging in weight from 16 to 19 grams were selected. Two groups

were inoculated under etherization by nasal installation of three mouse ID_{50} of the virus in 0.05 ml volumes. Two more groups received an inoculation of 30 mouse ID_{50} intranasally in the same 0.05 ml volume. Over a ten day period, daily injections of 15 mg of canavanine, equivalent to approximately 850 mg per kilogram of body weight, were given intramuscularly to one group of mice at each of the two virus dose levels. One group of each dose level received no canavanine. The fifth group of mice received daily canavanine injections but was not inoculated with the virus. Deaths were recorded during the progress of the experiment as 4+. At the end of ten days of treatment, all remaining mice were sacrificed by over-etherization and the lungs of each mouse removed and graded as to the extent of the area of lung involvement. Lungs showing approximately one-fourth of the area consolidated were graded as +; lungs one-half consolidated were graded as 2+, and lungs three-fourths consolidated were graded as 3+. Results of this experiment are indicated in Table 14. At these dose levels of Lee influenza virus and L-canavanine, no significant differences can be noted among the infected groups. The majority of the mice in all groups succumbed to the infection whether treated with canavanine or not.

Table 14

Investigation of the Effect of L-Canavanine upon the Progress of Lee Influenza Infection in the Mouse

Mouse Number	Severity of infection resulting from the following treatments: No virus, canavanine	30 MID_{50} Control	30 MID_{50} Canavanine	3 MID_{50} Control	3 MID_{50} Canavanine
1	4+	4+	4+	4+	4+*
2	--	4+	4+	4+	4+
3	--	4+	4+	4+	4+
4	--	4+	4+	4+	4+
5	--	4+	4+	4+	4+
6	--	4+	4+	4+	4+
7	--	4+	4+	4+	4+
8	--	4+	4+	3+	3+
9	--	4+	4+	2+	3+
12	--	4+	4+	+	3+

*Died during inoculation with virus at beginning of the experiment.

1. Severity of infection is graded on a plan of 4+ indicating the death of the mouse and 3+, 2+ and + representing areas of three-fourths, one half and one-fourth of the lung area, respectively, showing consolidation on sacrifice of the mouse at ten days following infection.
2. MID_{50} = Dose causing death of 50 per cent of a group of 5 or more mice. Inoculation of the mice is made by intranasal instillation under light etherization of 0.05 ml of inoculum containing the desired dose.
3. Canavanine was administered intramuscularly in 15 mg doses of a sesame oil suspension daily over the period of ten days.

Investigations on the inhibition of other viruses. It was of further interest to see if canavanine would influence the development of other viruses in the chicken embryo. Mumps virus and the PR-8 strain of influenza A virus were investigated in this respect. Due to the slower development of mumps virus in the chick embryo, canavanine was administered in two doses. An initial dose of 10 mg of canavanine in the allantoic sac preceded the virus inoculation of 100 EID_{50} by one hour. After three days incubation, a second injection of 10 mg of canavanine was given in an effort to maintain a higher canavanine level. Four days after the virus inoculation the allantoic fluids were harvested and titrated for hemagglutinin, as with influenza virus. In Table 15 no significant difference was seen to occur between the eggs infected with mumps virus, whether receiving canavanine or not, at the dose levels used in this experiment. It is possible that a difference in biosynthetic pathways exists between mumps virus and Lee influenza virus and that the reaction interfered with by canavanine is not essential for the multiplication of mumps virus. Other explanations are possible, however, as will be noted below.

The development of the PR-8 strain of influenza virus was also not inhibited by canavanine when administered in a single 20 mg dose to embryonated chicken eggs

Table 15

Investigation of the Effect of Canavanine upon the Hemagglutinin Titer of Mumps Virus in the Chick Embryo

Egg Number	Hemagglutinin in untreated control eggs	Hemagglutinin in eggs treated with two 10 mg canavanine injections
1	160	160
2	160	160
3	120	120
4	80	80
5	80	60
6	80	40
7	80	40
8	60	30
9	30	20
10	20	20
Hemagglutinin titers of pooled fluids	120	80
Geometric mean	73.3	58.2

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.

2. Treated eggs received two 10 mg doses of canavanine injected into the allantoic sac at one hour before virus inoculation and 30 hours after virus inoculation.

3. Virus titrations were carried out four days after inoculation of the eggs with 100 EID_{50} mumps virus.

receiving 50 EID_{50} of the virus. Results of a single experiment involving the attempted inhibition of PR-8 influenza virus by canavanine are reported in Table 16. In the chick embryo, a single injection of 20 mg of canavanine did not appear to influence the development of PR-8 influenza virus to any significant extent. However, when canavanine was subsequently tested in the nutrient medium of a tissue culture, the growth of PR-8 virus was markedly inhibited. This inhibition was noted at levels of 0.4 mg per ml and 0.2 mg per ml in cultures inoculated with $10^{5.38}$ EID_{50} of freshly harvested PR-8 influenza A virus per culture. In considering an explanation for this difference, the most striking variable is in the method of cultivation, with inhibition occurring in vitro but not in ovo. It appears that the Lee strain of influenza virus is more susceptible to canavanine and is, perhaps, inhibited by a lower concentration of canavanine than is the PR-8 strain. Perhaps in the chick embryo, the concentration of canavanine injected decreases at a relatively rapid rate through the normal routes of metabolism, enzymatic degradation and even the instability of the compound. This decrease in concentration may not occur to the same extent or at the same rate in tissue culture as it does in the intact embryo and, possibly, the inhibitory concentration of canavanine is more easily maintained. If this

Table 16

Investigation of the Effect of Canavanine upon the Hemagglutinin Titer of PR-8 Influenza Virus in the Chick Embryo

Egg Number	Hemagglutinin in untreated control eggs	Hemagglutinin in eggs treated with 20 mg canavanine
1	480	320
2	480	240
3	320	240
4	320	240
5	320	120
6	240	120
7	160	---
8	160	---
Hemagglutinin titers of pooled fluids	240	320
Geometric mean	305.1	199.8

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.

2. Treated eggs received 20 mg canavanine injected into the allantoic sac. One hour later all eggs received 50 EID_{50} fresh PR-8 influenza virus by the same route and were incubated at 35°C.

3. Virus titrations were carried out 44-48 hours after inoculation of the eggs with 50 EID_{50} PR-8 influenza virus.

is the case, and Lee influenza virus is more susceptible to canavanine, we might expect its inhibition in the chick embryo, whereas the more resistant PR-8 strain is not inhibited.

In the light of these results it is possible that the failure of canavanine to inhibit mumps virus in the chick embryo may also be due to similar factors, rather than some specific difference between the biosynthetic pathways of the two viruses. Tests with mumps virus in tissue culture have not been carried out.

Inhibition Studies Involving Isopropyl Biguanide Hydrochloride

Because canavanine was able to produce a definite inhibition of the development of the Lee strain of influenza virus, it was desirable to investigate whether other compounds possessing the guanidine group might also exert a similar effect upon virus development. A number of preparations, synthesized by the American Cyanamid Company as substituted guanylurea and biguanide compounds, were made available for this purpose. The following chemical compounds were tested for anti-viral activity: guanylurea phosphate, benzoyl guanylurea, cyclohexyl guanylurea, dodecyl guanylurea, isopropyl biguanide hydrochloride and phenyl biguanide hydrochloride. Of these, only isopropyl biguanide and benzoyl guanylurea appeared to inhibit virus development. As but a small amount of benzoyl guanylurea was available, only a limited number of experiments involving dose relationships of virus and compound were performed. Isopropyl biguanide exerted a more complete inhibition of virus development than did the benzoyl guanylurea compound, and for this reason it was of interest to investigate its anti-viral activity.

A sample of 1,1-dimethyl biguanide hydrochloride which was similar to isopropyl biguanide in structure

except for the substitution of two methyl groupings in place of the isopropyl portion of the molecule, also inhibited the development of Lee influenza virus in the chick embryo at the same dose level. The dosage necessary to cause complete inhibition of hemagglutinin in tissue culture was similar to the dose of isopropyl biguanide required to show the same activity. This compound is identical to the one reported by Garcia (12, pp.289-292) to be effective in suppressing the symptoms of clinical cases of influenza.

Chloroguanide, the antimalarial mentioned earlier to be 1-(p-chlorophenyl)-5-isopropyl biguanide hydrochloride, produced no inhibition of Lee influenza virus multiplication in the chick embryo.

A number of biguanides and guanidine derivatives which were made available as part of a study in organic synthesis by David Nyberg of the Department of Chemistry at Oregon State College also failed to show any evidence of anti-viral activity. The compounds investigated were ethoxydiguanide bisulfate, n-propoxy diguanide bisulfate, iso-propoxydiguanide bisulfate, ethoxyguanidine sulfate, carboxy-methoxyguanidine and 3-methyl butoxyguanidine nitrate.

In early experiments employing isopropyl biguanide this compound exhibited a marked inhibitory effect on the multiplication of Lee influenza virus in the chick embryo. This activity was easily demonstrated for about a nine month period. Toward the end of the investigation, great variability was noted among the treated groups. In the latest studies the degree of inhibition was much less complete, though still significant. Ratios of hemagglutinin titers of control to treated groups in later tests ranged from 2 to about 8, where in earlier investigations, hemagglutinin was almost completely absent in the allantoic fluid of chick embryos treated with the 10 mg dose of isopropyl biguanide, and the ratio of titers control to those of treated groups would be more in the neighborhood of 40 to 160.

In this report involving the inhibition of Lee influenza virus development by isopropyl biguanide, the data will be presented more or less chronologically and, when it is necessary to deviate from this plan it will be noted. The early portion of the investigation is reported first, during which time the extent of the inhibition was examined and some studies concerning the mechanism of the inhibition were carried out. At the point where the change in the degree of inhibition was noted, the factors that

might have accounted for this difference are discussed as well as some experiments that were aimed at uncovering the responsible factors. Finally, the effect of isopropyl biguanide in tissue culture is examined and the effect of the compound on Lee influenza virus in mice investigated.

Investigations in the chick embryo. The development of Lee influenza virus was found to be markedly inhibited by an intra-allantoic injection of 10 mg of isopropyl biguanide. In Table 17 is reported the results of a typical experiment wherein ten day old chick embryos were inoculated with 50 EID_{50} Lee influenza virus one hour after treating the eggs with isopropyl biguanide. The ratio of the hemagglutinin titer of the pooled fluids of the untreated control group to that of the treated group is indicated to be 16 to 1 or greater. In Table 17 the lowest dilution titrated was the 1:20 dilution and a zero titer indicates that no significant agglutination occurred at this dilution. If agglutination was present but was incomplete, it is assumed it would have been complete at 1:10 and a titer of 10 is indicated. For calculation of the geometric mean, all zero titers were assumed to be 10. When this calculation was made, a fourteen fold difference still was noted between the two groups of Table 17. The actual difference was probably greater.

Table 17

Inhibition of the Development of Lee Influenza Virus in the Chick Embryo by 10 mg Isopropyl Biguanide Hydrochloride

Egg Number	Hemagglutinin in untreated control eggs	Hemagglutinin in eggs treated with 10 mg IBG
1	320	10
2	320	10
3	320	0
4	240	0
5	240	0
6	240	0
7	160	0
8	80	0
9	40	0
10	0	0
Hemagglutinin titer of pooled fluids	160	0
Geometric mean	137.0	10

1. IBG is isopropyl biguanide hydrochloride.

2. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.

3. Treated eggs received 10 mg IBG injected into the allantoic sac. One hour later all eggs received 50 EID_{50} of virus by the same route and were incubated at 35°C.

4. Virus titrations were carried out 44-48 hours after inoculation of the eggs with 50 EID_{50} Lee influenza virus.

5. As the lowest dilution titrated was a 1:20 dilution, all eggs recorded as zero showed no agglutination at this dilution and were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

6. A titer of 10 indicates the presence of some hemagglutinin at the 1:20 dilution but the endpoint was below that dilution. The value of 10 was arbitrarily assigned to these fluids.

With such a noticeable effect on the virus as measured by hemagglutinin, it was next of interest to observe whether a similar reduction might be found in the amount of infective virus formed. In Table 18, three experiments are reported. The first two of these experiments were performed during the period when marked inhibition of hemagglutinin was noted. Experiment three was carried out at a later date and the degree of inhibition of hemagglutination was considerably less. The infectivity titers in this latter experiment were at levels that might be predicted with hemagglutinin titers of 96 and 12 and the difference of one logarithmic unit in infectivity titer between the two groups would be expected. The difference in infectivity titers obtained in experiment three was not statistically significant when 5 to 6 eggs per dilution were used. In experiment one a significant difference was indicated between the infectivity titers, but this difference would probably not greatly exceed one logarithmic unit. From the data available it appears that isopropyl biguanide at 10 mg per egg caused a reduction of infectivity of the Lee virus of the order of one logarithmic unit, indicating a roughly ten fold reduction in the concentration of infective virus in the allantoic fluids.

Table 18

Effect of Isopropyl Biguanide upon the Development of Lee Influenza Virus as Determined by Infectivity Titration

Experiment Number	Virus dose	IBG dose in mg per embryo	Hemagglutinin titer of pooled fluids	EID_{50} of virus per ml of pooled fluids
1	50 EID_{50}	0	640	$>10^{9}$
	50 EID_{50}	10	10	$10^{8.22}$
2	50 EID_{50}	0	320	$10^{8.75}$
	50 EID_{50}	10	10	$10^{8.17}$
3	50 EID_{50}	0	96	$10^{8.40}$
	50 EID_{50}	10	12	$10^{7.38}$

1. IBG is isopropyl biguanide.

2. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluids causing complete hemagglutination of chicken red blood cells.

3. Treated eggs received 10 mg IBG injected into the allantoic sac. One hour later all eggs received 50 EID_{50} of virus by the same route and were incubated at 35°C.

4. Virus titrations were carried out 44-48 hours after inoculation of the eggs with 50 EID_{50} Lee influenza virus.

5. Infectivity titers were determined by injection of 0.1 ml of serial ten fold dilutions of pooled allantoic fluids into groups of 5 to 6 eggs per dilution. After 44 hours incubation at 35°C, presence or absence of infection was determined by hemagglutination at a 1:4 dilution of the allantoic fluids.

6. Experiment 3 was conducted during the more recent period when inhibition was less complete. The two series of dilutions of fluids for hemagglutinin titration were started at 1:2 and 1:3, respectively.

In Table 19 are presented results of experiments relating to the degree of inhibition occurring when decreasing doses of isopropyl biguanide were administered one hour before infection with 50 EID_{50} of Lee influenza virus.

During the early investigations with isopropyl biguanide, inhibition of hemagglutinin production was striking even at a dose of 2.5 mg per embryo when this dose preceded the virus injection of 50 EID_{50}. This inhibition at the 1 mg level, by statistical analysis, was not significant. These results emphasize the marked anti-viral activity of isopropyl biguanide during the early investigations.

The effect of a 10 mg dose of isopropyl biguanide upon the amount of virus hemagglutinin formed following infection of the chick embryo with increasing doses of virus is presented in Table 20. This data was obtained in several experiments wherein the virus dose was increased from approximately 7 EID_{50} to 1,000,000 EID_{50}. Striking inhibition was observed at the lower dose levels of 6.9 and 69 EID_{50}. This inhibition of virus development by isopropyl biguanide had decreased considerably, although the difference was still significant at dose levels of 12,000 EID_{50}. Ten milligrams of isopropyl biguanide still

Table 19

Effect of Dosage of Isopropyl Biguanide upon the Degree of Inhibition of Lee Influenza Virus

Dosage of IBG per egg	Total number of eggs treated	Number of eggs showing titers of				Geometric mean of hemagglutinin titers of all fluids
		(10 or below)	(20-80)	(120-320)	(480 or above)	
10 mg	10	9	1	0	0	11.5
5 mg	10	6	3	1	0	22.6
2.5 mg	7	4	1	1	1	27.4
1.0 mg	10	1	3	5	1	131.5
0.5 mg	10	0	2	6	2	210.0
Control	20	1	4	10	5	201.6

1. IBG is isopropyl biguanide.
2. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.
3. Treated eggs received the indicated dose of isopropyl biguanide injected into the allantoic sac. One hour later all eggs received 50 EID_{50} of virus by the same route and were incubated at 35°C.
4. Virus titrations were carried out 44-48 hours after inoculation of the eggs with 50 EID_{50} Lee virus.
5. As the lowest dilution titrated was the 1:20 dilution, all eggs recorded as zero showed no agglutination at this dilution and were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

Table 19, continued

6. When the mean values for the hemagglutinin titers of the control group and the group receiving 1.0 mg IBG are compared using Fischer's "t" test (25, pp.151-170) and using logarithms of individual titers, a "t" value of 1.02 is obtained. For 28 degrees of freedom this difference is not significant at the usually accepted .05 probability level.

Table 20

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

EID_{50} Virus	IBG dose mg per egg	Number of eggs with hemagglutinin titers of (10 or below)	(20-80)	(120-320)	(480 or above)	Hemagglutinin titers of pooled fluids	Geometric mean of individual eggs
6.9	0	2	2	5	1	160	100.8
6.9	10	10	0	0	0	0	10.0
69	0	0	0	8	2	320	248.3
69	10	5	5	0	0	30	21.7
1,200	0	0	0	4	6	480	450.1
1,200	10	1	1	5	1	160	127.1
12,000	0	1	0	7	2	320	207.5
12,000	10	1	1	8	0	160	117.2
78,000	0	0	2	6	0	160	151.3
78,000	10	1	2	3	0	120	63.2
100,000	0	5	2	3	0	10	60.0
100,000	10	2	4	1	0	10	40.0
1,000,000	0	9	1	0	0	10	11.2
1,000,000	10	8	2	0	0	10	12.8

1. IBG is isopropyl biguanide.

Table 20, continued

2. Hemagglutinin titers are expressed as the reciprocal of the highest dilution causing complete hemagglutination of chicken red blood cells.

3. Treated eggs received 10 mg IBG injected into the allantoic sac. One hour later all eggs received the indicated dose of virus as determined by infectivity titration.

4. Virus titrations were carried out 44-48 hours after inoculation of the eggs with the indicated virus dosage.

5. When the mean values for the hemagglutinin titers in the treated and control groups receiving 12,000 EID_{50} are compared by using Fischers "t" test (25, pp. 151-170) and using logarithms of individual titers, a "t" value of 2.62 is obtained. For 18 degrees of freedom this figure corresponds to a *P* value of less than .02 indicating the observed difference is unlikely to be due to chance.

seemed to exert an inhibitory effect when 78,000 EID_{50} of virus were inoculated. The same complicating factor noted previously in experiments with very large doses of virus is also evident here. Virus doses of the order of magnitude of 100,000 EID_{50} and larger seemed to cause a marked reduction of the amount of virus synthesized. This effect of self-inhibition was particularly marked when 10^6 EID_{50} of Lee influenza virus were injected and only a very small amount of virus hemagglutinin could be detected in the allantoic fluid. It appears that the maximum inhibitory effect of isopropyl biguanide could be demonstrated when smaller doses of virus were inoculated. The differences between treated and untreated control eggs were still of considerable magnitude following a dose of 69 EID_{50} of virus, becoming much less as the dose approached 1000 EID_{50}. The data suggest that any definite inhibition of virus development that could be ascribed to the action of 10 mg of isopropyl biguanide alone became insignificant as the virus dose exceeded approximately 12,000 EID_{50} of virus per egg. At lower dose levels the degree of inhibition noted appears to be inversely related to the concentration of infectious virus in the inoculum.

Hemagglutinin formation was completely suppressed when isopropyl biguanide was administered in a 10 mg dose via the yolk sac. Inoculation of the virus into the

allantoic sac followed the compound injection by one hour. The results of such an experiment are shown in Table 21 which indicates greater than a 48 fold difference between hemagglutinin titers of fluids pooled from untreated control eggs and a similar pool from a group of eggs treated by the yolk sac route with this compound. When a value of 10 was given to allantoic fluids showing no agglutination at the 1:20 dilution as was done in previous experiments reported, a 32 fold difference in the geometric means of the two groups was still observed. As with canavanine, these results suggest that the anti-viral activity of isopropyl biguanide is not very likely to be due to a direct effect of the compound upon the virus when both are present together in the allantoic sac. The data suggests that isopropyl biguanide may be absorbed from the yolk sac to be distributed to the cells of the allantoic sac wherein virus multiplication occurs. Within the cells of the allantoic sac it seems to exert its effect.

Investigations in vitro. Further experiments were conducted in vitro in an effort to more carefully determine whether isopropyl biguanide did cause any direct inactivation of the Lee influenza virus. In Table 22 are recorded the results obtained in two experiments when isopropyl biguanide at a concentration of 2 mg per ml was incubated together with allantoic fluid virus at temperatures

Table 21

Inhibition of Lee Influenza Virus in the Chick Embryo Resulting from the Yolk Sac Injection of 10 mg Isopropyl Biguanide Hydrochloride

Egg Number	Hemagglutinin in untreated control eggs	Hemagglutinin in eggs treated with 10 mg IBG
1	640	0
2	640	0
3	680	0
4	320	0
5	320	0
6	320	0
7	320	0
8	320	--
9	160	--
10	120	--
Hemagglutinin titer of pooled fluids	480	0
Geometric mean	323.8	10

1. IBG is Isopropyl Biguanide.

2. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.

3. Treated eggs received 10 mg IBG injected into the yolk sac one hour before inoculation of 50 EID_{50} into the allantoic sac and were incubated at 35°C.

4. Virus titrations were carried out 44-48 hours after inoculation of the eggs with 50 EID_{50} Lee influenza virus.

5. As the lowest dilution titrated was the 1:20 dilution, all eggs recorded as zero showed no agglutination at this dilution and were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

Table 22

Investigation of an In Vitro Effect of Isopropyl Biguanide upon the Hemagglutinin and Infectivity of Lee Influenza Virus

Experiment Number	IBG Concentration (mg per ml)	Temperature and period of incubation	Hemagglutinin titer	Infectivity titer (EID_{50} per ml)	pH of fluids after incubation
I	0	before incubation	240	$10^{6.61}$	- -
	2	before incubation	240	$10^{6.70}$	- -
	0	10°C - 20 hours	240	$10^{7.50}$	8.40
	2	10°C - 20 hours	240	$10^{7.00}$	8.37
	0	35°C - 20 hours	320	$10^{3.64}$	8.72
	2	35°C - 20 hours	320	$<10^{2}$	8.80
II	0	before incubation	160	$10^{8.50}$	- -
	0	35°C - 2 hours	160	$10^{7.16}$	- -
	2	35°C - 2 hours	160	$10^{7.55}$	- -
	0	10°C - 24 hours	120	$10^{8.20}$	8.70
	2	10°C - 24 hours	160	$10^{8.00}$	8.70
	0	35°C - 24 hours	160	$10^{2.71}$	8.70
	2	35°C - 24 hours	160	$<10^{2}$	8.74

1. IBG is isopropyl biguanide.

Table 22, continued

2. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.

3. Infectivity titers were determined by injection of 0.1 ml of serial ten fold dilutions of virus fluids into groups of six or more eggs per dilution. After 44 hours incubation at 35°C, presence or absence of infection was determined by hemagglutination at a 1:4 dilution.

4. Virus in all preparations was freshly harvested allantoic fluid.

5. All preparations were shown to be free from microorganisms by sterility tests at the end of the experiment.

of 10° and 35°C for a 20 to 24 hour period and at 35°C for a 2 hour period. No demonstrable effect upon the virus hemagglutinin was observed at either temperature. On titration of the infectivity of the virus following the 20 to 24 hour incubation, isopropyl biguanide was found to exert no detrimental effect at the 10° temperature. However, at 35°C the compound appeared to cause a reduction in the infectivity titer when compared with a similarly incubated control group containing no isopropyl biguanide. This effect was not noted at this temperature during a short incubation period of two hours. Incubation at the 35°C temperature for a two hour period may have caused some decrease in infectivity of both preparations but no significant difference can be observed between virus fluids with or without the presence of isopropyl biguanide. Apparently prolonged contact between the virus and isopropyl biguanide at 35°C is necessary for any inactivation to occur. Possibly this action may contribute to the inhibitory effect of the compound on growth of the virus in the chick embryo, but it seems unlikely that this can be the only mechanism or even the major method by which the compound effects the development of the virus. Therefore, following any virus inoculation and its subsequent rapid adsorbtion, infection will ensue. The newly released virus will again be quickly adsorbed

and the infection process will continue until the number of virus particles present in the allantoic fluid exceeds the number of available uninfected cells and the virus titer of the allantoic fluid will then increase. If isopropyl biguanide were likely to produce an effect by direct inactivation of the virus present, it would be most likely to do so here, when both the infectivity titer and the hemagglutinin titer should be fairly high. As noted in Table 22, the hemagglutinin titer is unaffected by direct contact with isopropyl biguanide; only the infective property of the virus particle is apparently destroyed by prolonged contact. However, as indicated in Table 18, in some experiments in the chick embryo, the hemagglutinin titer may show a more striking reduction following treatment with this compound than the infectivity titer. It appears as though isopropyl biguanide acts more through interference with the normal synthesis of the virus than by a direct inactivating effect upon the virus, though this effect must be considered in the end result of decreased virus production. Before leaving these arguments, it must be mentioned that the compound might be converted through metabolism of the cell to another structure which might exert a more pronounced direct effect upon the virus. Any such reaction would not be demonstrated in

an experiment of the type described in Table 22.

Investigation of resulting inhibition following administration at various time intervals. The strong antiviral activity of isopropyl biguanide was demonstrated in an experiment in which the time interval between virus and compound injections was varied. Isopropyl biguanide was administered in a 10 mg dose to groups of ten day embryos by the allantoic route one hour before the virus injection and with different groups at 2, 24, 36, or 44 hours after the virus inoculation. The allantoic fluids were harvested 48 hours after inoculation of virus. Marked inhibition of virus development was observed in those groups receiving isopropyl biguanide one hour before the virus and at 2 and 24 hours after the virus inoculation, as recorded in Table 23. A less striking, though still highly significant difference, was found on comparison of the geometric mean of the control group with the means of the group receiving the injection as late as 36 hours after the virus. When the treatment followed the virus inoculation by a 44 hour interval, no difference in degree of infection could be detected. The data again seems to suggest an interference with some essential step or steps necessary for the synthesis of intact hemagglutinating virus.

Table 23

Influence of Time Interval Between Infection and Injection of Isopropyl Biguanide on Inhibition of Lee Influenza Virus

IBG 10 mg per egg	Number of eggs showing titers of (10 or below)	(20-80)	(120-320)	(480 or above)	Geometric mean
1 hour before virus	7	3	0	0	3.5
2 hours after virus	9	1	0	0	11.2
24 hours after virus	9	7	2	0	24.0
36 hours after virus	0	3	5	0	114.8
44 hours after virus	0	2	4	4	285.3
Control, no IBG	0	1	10	9	290.2

1. IBG is isopropyl biguanide.

2. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluids causing complete hemagglutination of chicken red blood cells.

3. All eggs received 50 EID_{50} Lee influenza virus inoculated into the allantoic sac and were incubated at 35°C for 48 hours after virus inoculation.

4. As the lowest dilution titrated was the 1:20 dilution, all eggs recorded in the column "10 or below" were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

5. On comparison of mean values for hemagglutinin titers of the control groups and the group treated 36 hours after the virus inoculation by use of Fischer's "t" test (25, pp.151-170) using logarithms of individual titers, a "t" value of 3.76 was obtained. For 26 degrees of freedom, this value is highly significant at the .05 significance level with this difference very unlikely to occur by chance.

Investigations on adsorption of virus by host cells in the presence of IBG. The effect of isopropyl biguanide upon the adsorption of the virus to the tissue was investigated and the data are reported in Table 24. Freshly removed chorioallantoic membranes were washed three times in sterile buffered saline, weighed aseptically, and added at one gram per ml to 1:2 and 1:5 dilutions of allantoic fluid virus both with and without isopropyl biguanide in a concentration of 2 mg per ml. Control fluids of each virus dilution not containing tissue and both with and without isopropyl biguanide, were prepared. Hemagglutinin titrations were carried out before incubation on a control fluid and after incubation at 35°C for 30 minutes on each preparation. Adsorption of 50 to 75 per cent of the virus present, as indicated by hemagglutinin titration, occurred during this incubation period. Isopropyl biguanide was not observed to interfere with the adsorption of the virus to the cells of the chorioallantoic membrane using this method of measurement.

Information accumulated at this point in the investigation with respect to inhibition of Lee influenza virus by isopropyl biguanide shows that a striking effect on virus development in the chick embryo was regularly obtained with a dose of 10 mg of the compound per egg.

Table 24

Investigation of the Effect of Isopropyl Biguanide Upon the Adsorption of Lee Influenza Virus on to Cells of Chorioallantoic Membrane Tissue

Virus Dilution	IBG concentration	Tissue concentration	Time of incubation at 35°C	Hemagglutinin titer
1:2	0	0	Before incubation	160
1:2	0	0	30 minutes	120
1:2	2 mg per ml	0	30 minutes	160
1:2	0	1 gm per ml	30 minutes	40
1:2	2 mg per ml	1 gm per ml	30 minutes	40
1:5	0	0	Before incubation	40
1:5	0	0	30 minutes	60
1:5	2 mg per ml	0	30 minutes	40
1:5	0	1 gm per ml	30 minutes	15
1:5	2 mg per ml	1 gm per ml	30 minutes	20

1. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.
2. The tissue used was freshly harvested chorioallantoic membrane tissue which had been washed three times in sterile saline and weighed using aseptic conditions.
3. Incubation was carried out at 35°C in a water bath with occasional agitation.

Ratios of hemagglutinin titers of pooled fluids of controls to those of treated eggs range from 40 to 160 and greater. Ratios of geometric means of the control to treated groups usually fell between 10 and 64. Isopropyl biguanide does not seem to exert this anti-viral effect by interfering with the adsorption of the virus to the cell although it may cause some degree of direct inactivation of the infectivity of the virus during incubation at 35°C over a 20 to 24 hour period. The data at this point seems to suggest that isopropyl biguanide may more likely interfere with some intracellular step involved in the biosynthesis of the virus particle.

Investigation on the effect of IBG on the growth of other viruses. It was felt that isopropyl biguanide might cause an inhibition of the development of Lee influenza virus by interfering with some cellular reaction which was also essential to the multiplication of other viruses. The effect of isopropyl biguanide upon the multiplication of the PR-8 strain of influenza A virus in the chick embryo was investigated by methods similar to those used with the Lee strain of influenza B. An injection of 10 mg of the compound preceeded 50 EID_{50} of PR-8 virus by one hour. After 48 hours incubation at 35°C, it was noted that isopropyl biguanide was able to cause a definite

inhibition of the PR-8 strain but a much less marked effect than that noted with Lee influenza virus. Results illustrating the degree of inhibition are recorded in Table 25. A two to three fold difference in titers between the control and treated groups is noted on observing either the hemagglutinin titer of the fluids pooled from each egg or the actual calculation of the geometric means of each group. It appears that isopropyl biguanide is unable to inhibit the PR-8 strain of influenza virus as completely as the Lee strain of this virus. This difference may be a function of the concentration of the compound necessary for the inhibition of these two strains. The Lee strain appears to be somewhat more sensitive to the inhibitor than the PR-8 strain.

Mumps virus inoculated into the allantoic sac of eight day old embryos in a dose of 50 EID_{50} and followed 24 hours later with 10 mg of isopropyl biguanide by the same route was observed to be definitely inhibited when allantoic fluids were titrated four days after inoculation. Results of an experiment of this type are indicated in Table 26. Greater inhibition might have been possible if more than a single injection had been used. Mumps virus is a relatively slow growing virus in comparison with the influenza viruses, taking four to five days for maximum

Table 25

Inhibition of the Development of PR-8 Influenza Virus in the Chick Embryo by 10 mg Isopropyl Biguanide Hydrochloride

Egg Number	Hemagglutinin in untreated control eggs	Hemagglutinin in eggs treated with 10 mg IBG
1	480	320
2	320	120
3	240	80
4	240	80
5	240	60
6	160	60
7	160	60
8	160	60
9	120	40
10	120	0
Hemagglutinin titer of pooled fluids	320	120
Geometric mean	190.4	66.5

1. IBG is isopropyl biguanide.
2. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.
3. Treated eggs received 10 mg IBG injected into the alalantoic sac. One hour later all eggs received 50 EID_{50} of virus by the same route and were incubated at 35°C.
4. Virus titrations were carried out 44-48 hours after inoculation of the eggs with 50 EID_{50} PR-8 influenza virus.
5. As the lowest dilution titrated was the 1:20 dilution, all eggs recorded at zero showed no agglutination at this dilution and were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

Table 26

Inhibition of the Development of Mumps Virus in the Chick Embryo by 10 mg Isopropyl Biguanide Hydrochloride

Egg Number	Hemagglutinin in untreated control eggs	Hemagglutinin in eggs treated with 10 mg IBG
1	160	30
2	80	20
3	60	20
4	60	20
5	60	10
6	40	10
7	40	10
8	40	0
9	30	0
10	0	0
Hemagglutinin titer of pooled fluids	60	0
Geometric mean	54.2	12.8

1. IBG is isopropyl biguanide.
2. Hemagglutinin titers are expressed as the reciprocal of the highest dilution of allantoic fluid causing complete hemagglutination of chicken red blood cells.
3. Treated eggs received 10 mg IBG injected into the allantoic sac 24 hours after inoculation with 50 EID_{50} mumps virus.
4. Virus titrations were carried out 4 days after inoculation of the eggs with 50 EID_{50} of mumps virus.
5. As the lowest dilution titrated was a 1:20 dilution, all eggs recorded as zero showed no agglutination at this dilution and were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.
6. A titer of 10 indicates the presence of some hemagglutinin at the 1:20 dilution but the endpoint was below that dilution. The value of 10 was arbitrarily assigned to these fluids.

development. In using a single 10 mg injection, it is much more difficult to maintain an inhibitory level of isopropyl biguanide in the allantoic fluid over this longer period. However, it appears that the compound also interferes with the development of mumps virus in the chick embryo.

Observation of decreased effect of isopropyl biguanide. After about nine months of fairly intensive study with isopropyl biguanide, this phase of the problem was interrupted while investigations with other compounds and various problems were begun. When our attention was again returned to isopropyl biguanide, it was noted that the amount of inhibition resulting from a 10 mg dose of the compound was much less and more variable within a given experiment than in the period before the interruption. The inhibition of the virus development, however, was still evident but much less complete. Ratios of titers of hemagglutinin from pooled fluids of control and treated groups were of the order of two to eight. It appeared that one of three changes might have occurred. First, the supply of isopropyl biguanide, though in the dry form, might have undergone some change such as a decomposition or structural alteration. A fresh quantity of isopropyl biguanide was received from the American

Cyanamid Company and another sample synthesized by N. V. Philips-Roxane Weesp of the Netherlands was received from Dr. J. Brug of that firm. When the two new samples were compared with the old, no one preparation demonstrated any more marked effect than the others. The inhibition was still variable from egg to egg and was not complete. Ratios of hemagglutinin titers of pooled fluids from control and treated groups were equal to three or four in these tests. It appeared that the difference could not be accounted for as due to a decomposition of the isopropyl biguanide as fresh preparations from two sources did not display the earlier degree of inhibition.

The second factor that might have accounted for the difference in the degree of inhibition was a change in the virus used in the tests. Since the first observation of the anti-viral activity of isopropyl biguanide, the virus had been transfered through approximately twenty egg passages and had become somewhat better adapted to growth in the chick embryo as indicated by higher hemagglutinin and infectivity titers. It might have been possible that concurrently with this better adaptation to development in the cells of the chick embryo, some change in certain metabolic pathways used by the virus in biosynthesis could have occurred. Perhaps some cellular

reactions sensitive to isopropyl biguanide were no longer specifically required for virus multiplication. This was investigated by use of virus from two stock preparations; one stock prepared from an early transfer of virus and used in the early studies with isopropyl biguanide, the other from the latest preparation of stock virus.

Following treatment with isopropyl biguanide and infection with either one of these two preparations of virus, no significant difference in the degree of inhibition could be detected between eggs inoculated with early passage Lee virus or the latest passage virus which was somewhat better adapted to growth in the chick embryo. The resulting inhibition again was more variable and less complete than that observed a year earlier. Differences in titers of the hemagglutinin of pooled fluids of treated and untreated control groups still showed only a three to four fold difference.

To eliminate the possibility that a change may have occurred in our stock virus, perhaps even contamination with the PR-8 strain of influenza A, a new specimen was ordered from the American Type Culture Collection. Isopropyl biguanide at 10 mg per egg demonstrated only a very slight degree of inhibitory activity against a virus inoculum of 50 EID_{50} of this virus. The ratio of hemagglutinin of pooled fluids of control to treated groups was slightly

greater than two.

The third factor that might be responsible for the difference and which was difficult to control was possible differences between lots of eggs. In the earliest studies involving isopropyl biguanide, White Leghorn eggs were made available through the kindness of Mr. J. A. Hansen of Hansen's Leghorn Farm. Later investigations were carried out using eggs from a breed of New Hampshire Red hens from both the Oregon State College Poultry Department and from Russell's Hatchery. It was therefore necessary to carry out several inhibition studies using eggs from both breeds of chickens and with New Hampshire Red eggs from the Oregon State College flock and from Russell's Hatchery. These investigations were carried out as far as practical and disclosed no striking differences that could be ascribed to the breed of chicken producing the eggs. It was suggested that dietary differences of the hens involved might be responsible for this failure to demonstrate a consistent degree of inhibition. The most striking change in diet would possibly involve feeding of various antibiotics, aureomycin and streptomycin, and of vitamin B_{12} to different groups of chickens. It was not possible to obtain sufficient information regarding the diets actually fed these chickens to assess the possible importance of this factor. It is known that neither

aureomycin nor streptomycin has any effect upon influenza infection and if it were possible for some amount of these antibiotics to be passed into the egg previous to hatching, it is not likely that this might effect the results of the inhibition studies. Vitamin B_{12} was also tested in tissue culture at concentrations of 100, 10 and 1 γ per ml against the complete inhibition resulting from isopropyl biguanide at 0.2 mg per ml. No suggestion of reversal of the inhibition was noted at any concentration of the vitamin tested.

However, the most logical variable appears to be in the eggs. This conclusion derives from the fact that the compound and the virus have both been eliminated as possible variables accounting for the decreased effect of IBG and from the fact that in tissue cultures of chick embryo membranes and Lee virus, no such change in the effect of the compound has been noted. In more recent tests it has produced the same marked inhibition of growth of the Lee virus as in early experiments, as will be described in the next section.

At this point it is not possible to designate what specific factor in the egg may be responsible for this variation in inhibition. However, it is known that isopropyl biguanide does inhibit to some degree the development

of the Lee strain of influenza virus in the embryonated chicken egg and causes marked inhibition of the virus in tissue culture.

Inhibition studies in tissue culture with respect to virus titers in the tissue. Isopropyl biguanide at 0.2 mg per ml in tissue culture causes a very definite decrease in the yield of Lee influenza virus from cells of chorioallantoic membrane (48, pp.27-34). The degree of inhibition definitely varies with the size of the virus inoculum. Frozen virus, in which 90 per cent of the virus is likely to be inactivated upon freezing, storage and thawing, when used at a 1:100 dilution and in a volume of 0.1 ml as the inoculum for 2.0 ml of tissue culture fluid, may be completely inhibited by 0.2 mg per ml of isopropyl biguanide and inhibited to a slightly less extent by 0.1 and 0.05 mg of isopropyl biguanide per ml. When freshly harvested virus was used as the inoculum at 1:100 dilution, virus hemagglutinin titers of the control fluids were considerably higher and inhibition, though very marked at 0.2 mg per ml, was not always complete.

Although tissue culture studies of inhibition of the Lee virus by isopropyl biguanide were carried out by Trosper (48, pp.27-34), it appeared of interest subsequently to examine the effect of the compound on the virus within the tissue itself. The earlier studies concerned

only the tissue culture fluids. A number of experiments were carried out by the tissue culture techniques previously described. In Table 27 the results of several experiments are given wherein isopropyl biguanide was added to tissue cultures at concentrations ranging from 0.4 to 0.05 mg per ml. The nature and dilution of the virus inoculum employed in each case is indicated. The geometric mean of the hemagglutinin titers of tissue culture fluids from each tube are calculated. As described earlier, a value of 10 is used for this calculation when no agglutination was observed at the 1:20 dilution. The membranes from each culture tube in the given experimental groups were pooled, rinsed three times in saline and ground with Pyrex glass with mortar and pestle to a measured dilution in buffered saline. The amount of virus contained within the cells was released in this manner and determined by hemagglutinin titration and infectivity titration. It was found that a considerable amount of virus was present in these cells of the control membranes as well as in the membranes of cultures receiving the 0.05 mg per ml dose of isopropyl biguanide. In three of the four experiments, the hemagglutinin titer of the membranes from cultures receiving the 0.2 mg dose of isopropyl biguanide indicated striking inhibition of virus development. It should be noted that the virus inoculum in each of these three experiments was a dilution of frozen

Table 27

The Effect of Isopropyl Biguanide upon the Amount of Virus Developing in Tissue Culture as Determined by Titration of the Fluids and the Tissue

Experiment Number	Virus Preparation	IBG Concentration (mg per ml)	Geometric mean of hemagglutinin titers of individual culture fluids	Hemagglutinin titer of pooled membranes	Infectivity titer of pooled membrane (EID_{50} per gm)
I	Frozen virus	0.2	0	10	$10^{5.20}$
	1:100 dilution	Control	167.8	480	$10^{6.50}$
II	Frozen virus	0.4	10	40	- - -
	1:100 dilution	0.2	10	40	- - -
		0.05	19.7	3840	- - -
		Control	161.3	3840	- - -
III	Frozen virus	0.2	10	20	- - -
	1:1000 dilution	0.1	10	60	$10^{4.30}$
		0.05	25.5	80	$10^{7.00}$
		Control	105.0	160	$10^{7.36}$
IV	Fresh virus	0.2	11.5	2560	$10^{6.40}$
	1:10 dilution	0.1	41.0	7680	$10^{6.54}$
		0.05	168.6	10,240	- - -
		Control	277.1	15,360	$10^{6.50}$

Table 27, continued

1. IBG is isopropyl biguanide.

2. Hemagglutinin titers are expressed as the reciprocal of the highest dilution causing complete hemagglutination of chicken red blood cells.

3. As the lowest dilution titrated was the 1:20 dilution, all eggs showing incomplete or no agglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

4. Incubation of cultures was carried out at 35°C for 46-48 hours on a reciprocating shaker.

5. Membranes from each group of culture tubes were removed and rinsed three times in sterile saline and ground in a sterile mortar and pestle with sterile ground Pyrex in buffered saline to a definite dilution. Hemagglutinin and infectivity titrations were carried out after centrifuging.

6. A 1:100 dilution of frozen virus contained approximately $10^{4.5}$ EID_{50} per culture in a 0.1 ml inoculum. A 1:1000 dilution of frozen virus contained approximately $10^{3.5}$ EID_{50} per culture in a 0.1 ml inoculum. The 1:10 dilution of freshly harvested allantoic fluid virus contained approximately 10^{7} to 10^{8} EID_{50} per culture in a 0.1 ml inoculum.

virus. When a more concentrated preparation of fresh allantoic fluid virus was used as the inoculum, titration of the tissue culture fluids indicated a less complete inhibition of virus development, though a still highly significant inhibition was obtained at the 0.2 mg per ml level. The hemagglutinin titers of the pooled membranes of control cultures indicated a high virus content in all four groups of this experiment. This difference observed between experiment four and the first three experiments tabulated is very likely a reflection of the amount of virus in the inoculum. The infectivity titers, where indicated in experiments one and three, show a reduction in the amount of infective virus present when isopropyl biguanide was present in the culture at a concentration of 0.2 mg per ml. This reduction in infectivity is not noted in experiment four and is possibly related again to the large virus inoculum. Isopropyl biguanide at 0.2 mg per ml caused a reduction in the amount of virus formed within the cell, though this effect was much less apparent than by titration of the culture fluids. It appears that the effect is retardation of the growth of the virus. The most interesting observation is that the infectivity titer in most instances is below the titer that would be assumed to accompany any hemagglutinin figures of these magnitudes. With hemagglutinin titers of the order of 1000 and 10,000 infectivity values of 10^9 and

10^{10} might be expected. This data suggests that at this stage of development a large amount of "incomplete" virus is present in the tissue; that is, virus possessing the properties of hemagglutination but as yet incapable of inducing infection.

Effect of isopropyl biguanide on respiration of tissue. As with the series of studies involving inhibition of virus multiplication by L-canavanine, it was necessary to perform a series of Warburg respirometer studies to see whether isopropyl biguanide exerted any effect on the respiration of the tissue. Chorioallantoic membrane tissue from ten day old embryonated chicken eggs was washed three times in nutrient glucosol solution of pH 7.2 identical to that used in tissue cultures. After blotting of the membrane to remove excess moisture, pieces of membrane approximating 250 mg wet weight of tissue were weighed out and added to sterile Warburg flasks containing a given volume of nutrient glucosol plus isopropyl biguanide at the desired concentration. Isopropyl biguanide was incorporated at 0.2 mg per ml, the dose used in tissue culture for demonstrating maximum inhibition of hemagglutinin formation, and at 0.4 mg per ml, twice the tissue culture dose. A set of control flasks contained no isopropyl biguanide. A 0.4 ml volume of 20 per cent KOH was added to the center well to maintain a CO_2-free atmosphere over the extended period. The total

volume contained in each flask was 3.2 ml. Incubation was carried out at 37°C and the flasks were allowed to equilibrate for 15 minutes. Manometric readings were recorded every half hour for seven hours. At the end of this period, the manometers were opened to the atmosphere and the flasks allowed to incubate while shaking in the water bath at this same temperature for an additional ten hours. After 17 hours total incubation time, the manometers were again closed off and readings taken every 30 minutes during the 17 to 24 hour period. When the 24 hour period ended, the tissues from each flask were removed, rinsed and dried. The rate of oxygen uptake was calculated in microliters of oxygen per milligram of dry weight of tissue per hour. In Table 28 results obtained in Warburg respiration studies are reported for 0.2 and 0.4 mg of isopropyl biguanide per ml of nutrient medium. On comparison with control flasks containing no isopropyl biguanide, the respiration of the tissue was definitely impaired at the 0.4 mg per ml concentration. At both 0.2 and 0.4 mg per ml the effect of the compound on the respiration of the tissues appeared to increase with time so that a rather marked inhibition of respiration was evident after six hours incubation. When this incubation period was extended to 17 to 24 hours, respiration of the tissue was very noticeably effected. A concentration of isopropyl biguanide of 0.4 mg per ml almost

Table 28

The Effect of Isopropyl Biguanide upon the Rate of Oxygen Uptake by Chorioallantoic Membrane Tissue in the Presence of Glucose

Period of incubation	Rate of oxygen uptake in microliters per milligram of dry weight of tissue per hour in a CO_2-free atmosphere		
	Control, no IBG	IBG at 0.2 mg per ml	IBG at 0.4 mg per ml
2 hours	5.9	5.9	4.7
4 hours	5.9	5.0	3.8
6 hours	5.9	4.4	3.2
17 hours incubation plus			
2 hours	4.1	1.0	0.3
4 hours	4.1	1.2	0.3
6 hours	3.9	1.3	0.4

1. IBG is isopropyl biguanide.

2. Respiration of chorioallantoic membrane tissue was measured in a CO_2-free atmosphere in nutrient glucosol solution buffered at pH 7.2. The formula of the nutrient glucosol solution is identical to that used in tissue culture studies.

3. Each flask contained a total fluid volume of 3.2 ml and approximately 250 mg wet weight of chorioallantoic membrane which, upon completion of the experiment, was dried at 110°C for 20 hours and yielded roughly 10 mg dry weight of tissue.

4. Oxygen uptake was calculated on the basis of microliters per milligram of dry weight of membrane.

completely inhibited respiration of the cells, as respiration decreased to less than 10 per cent of that of the control flask. As the concentration of compound found to inhibit virus development in tissue culture also inhibits respiration after 17 hours incubation, this effect may be a very important factor in the mechanism of inhibition of virus development.

It was of interest to investigate whether this same inhibition of respiration of the cells of the chorioallantoic membrane resulted on injection of 10 mg of isopropyl biguanide into the allantoic sac. Following this injection of 10 mg, the effective maximum concentration of isopropyl biguanide in the allantoic fluid would be approximately 2 mg per ml, ten times the dose inhibitory in respiration studies <u>in vitro</u>. This was investigated by injection of a 10 mg dose of the compound into the allantoic sac of several ten day old chick embryos. A similar group received no injection. All eggs were reincubated for an additional 46 hour period. At the end of this period of incubation, the chorioallantoic membranes from each group were removed and prepared for respiration studies. Respiration was followed in a CO_2-free atmosphere under the same experimental conditions as outlined for Table 28. One set of control flasks contained isopropyl biguanide at the 0.2 mg per ml concentration, while another

set contained no isopropyl biguanide. The third set of flasks contained no compound in the Warburg flask, but the membrane present was removed from eggs treated 46 hours earlier with 10 mg of isopropyl biguanide per egg. Respiration was followed as oxygen uptake during the first eight hours of incubation and, after incubation overnight, during the 18 to 25 hour period. Respiration rates as microliters of oxygen per milligram of dry weight of tissue per hour are recorded in Table 29. It was indicated from this experiment that chorioallantoic membrane tissue removed from eggs treated 46 hours earlier with a 10 mg dose of isopropyl biguanide does not show a reduced rate of respiration when compared with membranes from untreated control eggs. Isopropyl biguanide at the concentration used in tissue culture of 0.2 mg per ml, however, caused a marked depression of respiration when present in culture in Warburg flasks. We might speculate that the inhibition of respiration is not a permanent impairment in the intact chick embryo, but that by the end of the 48 hour period of incubation the effective concentration of the compound is greatly reduced and the respiration again returns to its normal level. Another possible explanation is that following injection, the compound may be rapidly metabolized by the embryo or may undergo decomposition. In this way,

Table 29

Investigation of the Effect of Isopropyl Biguanide Upon the Respiration of Chorioallantoic Membranes Removed from Eggs Injected 46 Hours Earlier with a Ten Milligram Dose of the Compound

Period of incubation	Rate of oxygen uptake in microliters per milligram of dry weight of tissue per hour in a CO_2-free atmosphere		
	Contol, no IBG	0.2 mg per ml IBG in flask	10 mg IBG, 48 hours before removal of membrane; no IBG in Warburg flask
2 hours	6.4	4.5	6.3
3 hours	6.2	4.1	6.4
6 hours	5.8	3.4	6.2
8 hours	5.6	3.0	6.2
18 hours incubation plus			
2 hours	3.6	0.9	4.1
4 hours	3.6	0.9	4.0
6 hours	3.5	0.8	3.9

1. IBG is isopropyl biguanide.

2. Respiration of chorioallantoic membrane tissue was measured in a CO_2-free atmosphere in nutrient glucosol solution buffered at pH 7.2. The formula of the nutrient glucosol solution is identical to that used in tissue culture studies.

3. Each flask contained a total fluid volume of 3.2 ml and approximately 250 mg wet weight of chorioallantoic membrane, which upon completion of the experiment was dried at 110°C for 20 hours and yielded roughly 10 mg dry weight of tissue.

4. Oxygen uptake was calculated on the basis of microliters per milligram of dry weight of membrane.

no inhibition may be noted. The first suggestion seems the most probable, with isopropyl biguanide causing an initial decrease in respiration in the embryo. This effect is then lost either through the metabolism or detoxification of the compound or because the tissue has been removed to an environment free from the compound. In either case, the respiration of the tissue may return to normal. However, this early effect in the chick embryo may be sufficient to cause a retardation in virus development which is observed in an experiment titrated at the 48 hour interval. In an experiment wherein titrations were performed three and four days after treatment with the compound, the hemagglutinin was slightly lower in the treated group when compared with the control on the third day. On the fourth day, no difference in hemagglutinin could be noted between the two groups of eggs titrated.

Whatever the explanation may be, it seems probable that the mechanism of the inhibition is probably due to a combination of several factors. The complete inhibition of virus development at 0.2 mg per ml in tissue culture may be chiefly a result of inhibition of respiration of the tissue. Some of the inhibition noted in the chick embryo may be a result of interference with some metabolic reaction, wherein isopropyl biguanide plays

the role of an antimetabolite. If respiration is inhibited by action of the compound shortly after its injection into the egg, this in itself may allow for enough retardation of virus development to make some difference in hemagglutinin titers of the treated and untreated groups detectable.

Investigations on the effect of Lee influenza virus infection in mice. The effect of isopropyl biguanide upon the prognosis of Lee influenza virus infection in mice was examined. Two groups of mice with weight ranges of 27.0 to 33.0 grams and 24.6 to 27.0 grams respectively, were selected containing 20 mice in each group. The average weights of all mice in each group was 30 grams and 26 grams, respectively. The mice of the lower weight range were held as the untreated control mice while the heavier mice received daily injections of isopropyl biguanide. Two dose levels of virus, equivalent to $10^{3.75}$ and $10^{2.75}$ egg 50 per cent infectious doses, were inoculated by intranasal instillation in a 0.05 ml volume. Following sterilization in the dry form at 110°C for ten hours, isopropyl biguanide was prepared as a suspension in sesame oil containing two per cent aluminum monostearate. The concentration of isopropyl biguanide in oil was 300 mg per ml. Ten mice of each virus dose group received daily intramuscular injections

of 15 mg of the compound, equivalent to a dose of approximately 500 mg per kilogram of body weight per day. After ten days of daily injections during which time deaths of any mice were recorded and graded as 4+, the remaining mice were sacrificed and the lungs removed and graded as to extent of infection by observation of the area of lung consolidation as described previously in this section of the canavanine studies.

It was observed that ten days of daily injections of isopropyl biguanide had no effect upon the infection of mice by Lee influenza virus. All mice in each infected group died, or showed marked pulmonary lesions. Isopropyl biguanide apparently was unable to inhibit the development of the virus in the lung. This inability to exert a protective effect in mice may be a result of several factors. The compound may be detoxified or degraded fairly rapidly in the body. It may thus be eliminated rapidly in the urine without providing a sufficiently high blood level to cause the inhibition of the virus. Some sort of enzymatic degration of isopropyl biguanide may occur thus producing a compound inactive as a virus inhibitor. Perhaps virus inhibition might be demonstrated if it were possible to treat the mice with a larger dose of isopropyl biguanide or to give several repeated doses during each day of treatment and thus maintain a higher blood level.

DISCUSSION

Two chemical compounds, isopropyl biguanide hydrochloride and L-canavanine, have been shown to exert an inhibitory effect upon the development of the Lee strain of the type B influenza virus. These two compounds, one being an amino acid, the other a guanidine derivative, do have in common the guanidino grouping. It is interesting to note that several compounds possessing considerable structural similarity to both compounds were not observed to exert any inhibitory activity against the Lee strain of the influenza virus. Some of these compounds tested were ethoxyguanidine sulfate, ethoxybiguanide bisulfate, n-propoxybiguanide and 3-methyl butoxyguanidine nitrate. The only other biguanide compound found active was the 1,1-dimethyl biguanide with a structure almost identical to isopropyl biguanide. The only difference noted is the substitution of the dimethyl grouping for the isopropyl group on one of the terminal nitrogen atoms of the biguanide. Both the isopropyl and dimethyl biguanides showed almost identical anti-viral activity in tissue culture and in the chick embryo with respect to dosage required for inhibition. No compounds of close structural similarity to canavanine, except the desamino canavanine

and arginine, were investigated for anti-viral activity. Neither preparation caused any inhibition of virus multiplication in the chick embryo. In tissue culture, arginine, as well as lysine and ornithine, have been shown by Eaton (7, pp.506-507) to inhibit the Lee strain of influenza.

It appears rather definite that canavanine and isopropyl biguanide inhibit virus development by different mechanisms. Canavanine, as might be expected, seems to interfere directly with some step in which the amino acid arginine plays a role in virus multiplication. This was shown by the fact that arginine was able to cause a complete reversal of the inhibition of virus development induced by canavanine at low concentrations. It was impossible to determine whether this inhibition by canavanine was of a competitive nature or not due to the definite limitations of tissue culture and the demonstration of virus inhibition by arginine, the apparent metabolite, at higher concentrations. The fact that canavanine has been shown to inhibit a variety of organisms stimulates some speculation on whether the inhibitor influences a step common for the development of all of the organisms thus inhibited. Data accumulated in studies with Escherichia coli (50, pp.894-899), in which strains of both wild type and arginine requiring mutants were

inhibited, and with Neurospora strains (21, pp.371-376) capable of arginine synthesis, suggest the interference to be in a step involving the utilization of arginine. However, variations in degree of inhibition by canavanine among Neurospora strains studied, as well as separate reports of incidents of both competitive and non-competitive reversal by arginine (21, pp.371-376; 50, pp.894-899; 42, p.10,116), might indicate some variation with respect to the definite metabolic pathway interfered with. At this point it is impossible to say exactly where canavanine is involved in the inhibition of virus development. Arginine has been shown to be present in the protein of the Lee virus (24, pp.125-126) and it is reasonable to speculate that canavanine might interfere with incorporation of the amino acid into virus protein. This, of course, is only one of many possibilities. In any case, the compound appears to interfere with the intracellular development of the virus. This theory of an inhibition of virus synthesis is also supported by an accumulation of negative data which showed that canavanine neither causes an effect on the adsorption of the virus to the host tissue, nor does it cause direct inactivation of free virus in vitro. The compound causes no depression of respiration of the tissue of the chorioallantoic membrane in

concentrations inhibitory to the virus. Furthermore, it was found to cause a marked reduction in the yield of virus contained within the cells of the chorioallantoic membrane as well as in the allantoic fluid virus, and hence did not appear to be interfering with the release of newly formed virus from the cell. Canavanine inhibition of virus infection was limited to the chick embryo or tissue culture among the host systems studied. Infection with Lee influenza virus in mice was apparently unaffected by treatment with this compound. This may be related to the method and route of injection; a sesame oil suspension of the compound was administered intramuscularly. The destruction of canavanine in the liver by the enzyme arginase is very likely, as the compound is slowly absorbed and distributed by the blood system. The large dose of virus necessary to infect mice is another unfavorable factor.

The mechanism by which isopropyl biguanide produces its inhibition of virus multiplication can not be as clearly delineated as in the case of canavanine. Isopropyl biguanide, in concentrations estimated to be present within the allantoic sac of the chick embryo following injection of 10 mg, in <u>in vitro</u> experiments, demonstrated some direct inactivating effect upon the infectivity of the virus only during prolonged contact at

35°C. It is most likely that the concentration in the chick embryo decreases rather steadily on incubation due to metabolism or enzymatic degradation of the compound. Furthermore, the virus inoculated into the allantoic sac probably is not exposed for a very long period to the same high concentration of compound present in the in vitro experiments due to rapid adsorption of the virus to the cells and its subsequent penetration into the cells. The concentration of isopropyl biguanide used in tissue culture was only one-tenth of that used in the in vitro experiments and the likelihood that this concentration would still exert a noticeable direct effect on virus infectivity is rather slight.

Isopropyl biguanide was noted in a series of respiration studies to cause a significant reduction of oxygen uptake by chorioallantoic tissue after 17 to 24 hours at concentrations used in tissue culture. This interference with respiration could cause the inhibition of virus production in tissue cultures containing isopropyl biguanide.

This compound was not observed to interfere with the adsorption of the virus to the cells of the chorioallantoic membrane nor to inhibit the release of virus from these cells after its synthesis.

Investigations by Trosper (48, pp.35-45) in which reversal of isopropyl biguanide inhibition was attempted by several metabolites failed to show that any one of these compounds is involved in the mechanism of action of isopropyl biguanide. The metabolites tested were L-arginine, L-lysine, adenine, desoxyribonucleic acid, p-aminobenzoic acid and folic acid. However, the observation of the decreased amount of inhibition of virus infection in the chick embryo during the second year's work and the subsequent elimination of changes in the virus and in the compound as possible causes, seem to suggest the presence of some factor in the eggs able to influence the virus inhibitory activity of isopropyl biguanide. This factor might be a dietary factor related to the mechanism of the anti-viral activity of the compound and, of course, antagonistic to this mechanism. It might also be an enzyme capable of attacking the compound.

It is suggested by the results of the above mentioned experiments that the mechanism by which isopropyl biguanide exerts its inhibitory activity is an interplay of several factors. The inhibition of tissue respiration appears to be a definite factor in the inhibition of virus in tissue culture at concentrations of the compound of 0.2 mg per ml and higher. This may well be the most important factor in the mechanism. The direct effect of

isopropyl biguanide upon the infective property of the virus may contribute to the inhibition, especially in the chick embryo, when the concentration of the compound is at 2.0 mg per ml or greater in eggs incubated at 37°C for 40 to 48 hours. Furthermore, it is also possible that isopropyl biguanide may interfere with the normal synthesis or utilization of a metabolite essential for development of the cell and for multiplication of the virus. No evidence for the latter hypothesis exists, however.

The anti-viral effect of canavanine appears to be due to a direct interference with some step involving arginine which is essential for normal development of the Lee influenza virus. Its influence on virus multiplication appears to be a specific one. It is clearly seen that canavanine and isopropyl biguanide, though both possessing a guanidino grouping and both exhibiting activity as inhibitors of the multiplication of Lee influenza virus, produce their respective effects by a different mechanism of action.

SUMMARY

1. L-Canavanine and isopropyl biguanide hydrochloride have been shown to inhibit the development of Lee influenza virus in the chick embryo and tissue culture.

2. Twenty milligrams of L-canavanine injected into the allantoic sac of the chick embryo strongly inhibited multiplication of 50 EID_{50} of influenza virus as indicated by titration of hemagglutinin and infectivity of the allantoic fluids. This same dose of canavanine caused significant inhibition with an inoculum as large as 4500 EID_{50} of virus. Development of 50 EID_{50} was significantly retarded by as little as 2 mg canavanine.

3. Optimal inhibitory activity of canavanine was observed when injection of the compound slightly preceded the virus inoculum or followed its injection within a two hour period.

4. Canavanine exerted no direct inactivating effect upon the hemagglutinin or the infectivity of the virus when incubated with the compound at 10°C or 35°C for a 24 hour period. No effect on adsorption of the virus to the cells of the chorioallantoic membrane was observed when canavanine was incubated with the virus and the tissue.

5. L-Arginine hydrochloride effectively reversed the virus inhibiting effect of canavanine. However, no investigations as to the competitive or non-competitive nature of the inhibition could be satisfactorily carried out.

6. Canavanine exerted no inhibitory effect upon the respiration of chorioallantoic membrane tissue over a 50 hour incubation period.

7. Canavanine showed no inhibition of development of mumps virus or the PR-8 strain of influenza A virus in the chick embryo, though the PR-8 influenza virus was inhibited in tissue culture.

8. Isopropyl biguanide hydrochloride strikingly inhibited the development of Lee influenza virus as indicated by hemagglutinin and infectivity titrations in the chick embryo in early investigations. Later experiments showed a much decreased inhibition of virus multiplication in this same system. No such change was noted in the inhibitory activity of the compound for the virus in tissue culture.

9. Ten milligrams of isopropyl biguanide effectively inhibited development of an inoculum as great as 12,000 EID_{50} of Lee influenza virus. Fifty EID_{50} were strongly inhibited by 2.5 mg of the compound.

10. Isopropyl biguanide in a concentration of 2 mg per ml exhibited a direct effect upon the infectivity of Lee influenza virus when the two were incubated together at 35°C for 20 to 24 hours. This same concentration of the compound was shown to have no effect upon the adsorption of the virus to the cells of the chorioallantoic membrane.

11. Isopropyl biguanide administered as long as 36 hours after the virus inoculation significantly inhibited virus development in the chick embryo.

12. Both mumps virus and the PR-8 strain of influenza virus were inhibited by isopropyl biguanide in the chick embryo, though to a lesser degree than in the case of Lee influenza virus.

13. Investigation of the virus content of the membranes of tissue cultures infected with Lee influenza virus following treatment with isopropyl biguanide were carried out. The compound reduced the amount of intracellular virus as indicated by both lowered hemagglutinin and infectivity titers. Isopropyl biguanide does not appear to interfere with the release of virus from the infected cell.

14. Respiration of chorioallantoic membrane tissue was significantly inhibited by concentrations of isopropyl biguanide found to interfere with virus multiplication, during a 24 hour period.

15. No influence on the course of infection by Lee influenza virus in mice was noted by daily administration of maximum tolerated doses of either canavanine or isopropyl biguanide by the intramuscular route.

BIBLIOGRAPHY

1. Ackermann, W. W. Concerning the relation of the Krebs' cycle to virus propagation. Journal of biological chemistry 189:421-428. 1951.

2. Bonner, James. Limiting factors and growth inhibitors in the growth of Avena coleoptile. American journal of botany 36:323-332. 1949.

3. Bonner, James. Relation of respiration and growth in the Avena coleoptile. American journal of botany 36:429-436. 1949.

4. Brown, G. C. The influence of chemicals on the propagation of poliomyelitis virus in tissue culture. Journal of immunology 69:441-450. 1952.

5. Cushing, R. T. and H. R. Morgan. Effects of some metabolic analogues on growth of mumps and influenza viruses in tissue culture. Proceedings of the society of experimental biology and medicine 79:497-500. 1952.

6. Eaton, Monroe, Chi-To Huang and C. J. Levenson. Effect of nitro compounds and aldehyde semicarbazones on virus of atypical pneumonia. Proceedings of the society for experimental biology and medicine 77:422-425. 1951.

7. Eaton, Monroe et al. Inhibition of influenza and mumps virus in tissue culture by basic amino acids. Proceedings of the society for experimental biology and medicine 77:505-508. 1951.

8. Falco, E. A. et al. Antimalarials as antagonists of purines and pteroylglutamic acid. Nature 164: 107-108. 1949.

9. Fazekas de St. Groth, S. and H. J. F. Cairns. Quantitative aspects of influenza virus multiplication. IV. Definition of constants and general discussion. Journal of immunology 69:173-181. 1949.

10. Fisher, R. A. Statistical methods for research workers. Edinburgh, Scotland, Oliver and Boyd. 1946. 354p.

11. Friend, Charlotte. Effect of 2,6-diamino purine in virus of Russian spring-summer encephalitis in tissue culture. Proceedings of the society of experimental biology and medicine 78:150-153. 1951.

12. Garcia, E. Y. "Fluamine", a new synthetic analgesic and anti-flu drug. Journal of the Philippine medical association 26:287-293. 1950.

13. Ginsberg, H. S., W. F. Goebel and F. L. Horsfall. Inhibition of mumps virus multiplication by a polysaccharide. Proceedings of the society for experimental biology and medicine 66:99-100. 1947.

14. Green, Maurice and M. A. Stahman. Inhibition of mumps virus multiplication by a synthetic polypeptide. Proceedings of the society for experimental biology and medicine 83:852-858. 1953.

15. Green, Maurice, M. F. Stahman and A. F. Rasmussen. Protection of embryonated eggs infected with infectious bronchitis or Newcastle disease virus by polypeptides. Proceedings of the society for experimental biology and medicine 83:641-642. 1953.

16. Green, R. H. and P. W. Woolley. Inhibition by certain polysaccharides of hemagglutination and of multiplication of influenza virus. Journal of experimental medicine 86:55-64. 1947.

17. Hamre, Dorothy, K. A. Brownlee and R. Donovick. Studies in the chemotherapy of vaccinia virus. II. The activity of some thiosemicarbazones. Journal of immunology 67:305-312. 1951.

18. Henle, W. W. Studies on host-virus interactions in the chick embryo-influenza virus system. I. Adsorption and recovery of seed virus. Journal of experimental medicine 90:1-11. 1949.

19. Hood, Donald W. and Carl M. Lyman. The role of glutamic acid in arginine synthesis by Lactobacillus arabinosus. Journal of biological chemistry 185: 39-44. 1950

20. Horowitz, N. H. and Marguerite Fling. Private communication pertaining to isolation procedure for canavanine. 1952.

21. Horowitz, N. H. and A. M. Srb. Growth inhibition of Neurospora by canavanine and its reversal. Journal of biological chemistry 174:371-378. 1948.

22. Jones, J. H., C. Foster and W. Henle. Effect of oxythiamine in infection of mice with the Lansing strain of poliomyelitis virus. Proceedings of the society for experimental biology and medicine 69:454-458. 1948.

23. Knight, C. A. Titration of influenza virus in chick embryos. Journal of experimental medicine 79: 487-495. 1944.

24. Knight, C. A. Amino acid composition of highly purified viral particles of influenza A and B. Journal of experimental medicine 86:125-129. 1947.

25. Mainland, Donald B. The treatment of clinical and laboratory data. Edinburgh, Scotland, Oliver and Boyd, 1938. 333p.

26. Merck Index of Chemicals and Drugs. Rahway, N. J. Merck and Co., Inc., 1952. 1167p.

27. Miller, E. J. and J. S. Harrison. Growth inhibition of a yeast by uracil and its reversal by arginine. Nature 166:1035. 1950.

28. Morgan, H. R. Factors related to the growth of psittacosis virus (strain 6 BC). I. Pteroylglutamic acid, vitamin B_{12}, and citrovorum factor. Journal of experimental medicine 95:269-276. 1952.

29. Morgan, H. R. Factors related to the growth of psittacosis virus (strain 6 BC). II. Purines, pyrimidines and other components related to nucleic acid. Journal of experimental medicine 95:277-283. 1952.

30. Morgan, H. R. Factors related to the growth of psittacosis virus. III. Uracil and related compounds. Proceedings of the society of experimental biology and medicine 81:448-450. 1952.

31. Pardee, Arthur. Measurement of oxygen uptake under controlled pressures of carbon dioxide. Journal of biological chemistry 179:1085-1090. 1949.

32. Pearson, H. E., D. L. Lagerborg and R. J. Winzler. Effects of certain amino acids and related compounds on propagation of mouse encephalomyelitis virus. Proceedings of the society for experimental biology and medicine 79:409-411. 1952.

33. Reed, L. J. and H. Muench. A simple method of estimating fifty per cent endpoints. American journal of hygiene 27:493-497. 1938.

34. Robbins, M. L., A. R. Bourke and P. K. Smith. The effect of certain chemicals on Rickettsia typhi infections in chick embryos. Journal of immunology 64:431-446. 1950.

35. Salk, Jonas. A simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. Journal of immunology 49:87-98. 1944.

36. Sallach, H. James, Roger E. Koeppe and William C. Rose. The in vivo conversion of glutamic acid into proline and arginine. Journal of the American chemical society 73:4500. 1951.

37. Scalfi, L. and A. Bozzo. Action in vitro of N-1-p chlorophenyl-N-5-isopropyl biguanide on development of certain mycetes. Bolletino della society Italiana di biologia aperimentala 25: 978. 1949. (Abstracted in Chemical abstracts 44(19):8996a. 1950)

38. Shattuck, G. C. Diseases of the tropics. New York, Appleton-Century-Crofts Inc., 1951. 803p.

39. Sirsi, M. and N. N. De. Tuberculostatic properties of some antimalarial compounds. Current science 20:159-160. 1951.

40. Suzuki, T. and S. Muraoka. Antimetabolites of amino acids. II. Antagonism of canavanine to arginine with Lactobacillus fermenti. Journal of the pharmocological society of Japan 74:534-537. (Abstracted in Chemical abstracts 48(17):19, 116f. 1954)

41. Suzuki, T., S. Muraoka and K. Konobu. Antimetabolites of amino acids. III. Antagonism of canavanine and canaline to arginine by Lactobacillus arabinosus. Ibid 74:537-540. 1954. (Abstracted in Chemical abstracts 48(17):10, 116h. 1954)

42. Suzuki, T., S. Muraoka and K. Konobu. Antimetabolites of amino acids. IV. Relation of canavanine inhibition to arginine requirement of microorganisms. Ibid 74:540-543. (Abstracted in Chemical abstracts 48(17):10,116i. 1954)

43. Tamm, Igor, Karl Folkers and F. L. Horsfall Jr. Inhibition of influenza virus multiplication by 2,5 dimethylbenzimidazole. Yale journal of biology and medicine 24:559-567. 1952.

44. Thompson, Randall L. et al. Effects of purine derivatives and analogues on multiplication of vaccinia virus. Journal of immunology 65:529-534. 1950.

45. Thompson, Randall L. and Marian L. Wilkin. Inhibition of growth of vaccinia virus by β-2 thienylalanine and its reversal by phenylalanine. Proceedings of the society for experimental biology and medicine 68:434-436. 1948.

46. Thompson, Randall L. et al. Effect of heterocyclic and other thiosemicarbazones on vaccinia infection on the mouse. Journal of immunology 70: 229-234. 1953.

47. Thompson, Randall L. et al. Protection of mice against vaccinia virus by administration of benzaldehyde thiosemicarbazones. Proceedings of the society for experimental biology and medicine 78:11-13. 1951.

48. Trosper, Frances. Tissue culture studies of the inhibition of Lee influenza virus by isopropyl biguanide. Master's thesis. Corvallis, Oregon state college, 1954. 52 numb. leaves.

49. Umbreit, Wayne, W. R. H. Burris and J. F. Stauffer. Mamometric techniques and tissue metabolism. Second edition. Minneapolis, Burgess Publishing Co., 1949. 227p.

50. Volcani, B. E. and E. E. Snell. The effects of canavanine, arginine and related compounds on the growth of bacteria. Journal of biological chemistry 174:893-902. 1948.

51. Walker, J. B. Canavanine and homoarginine as antimetabolites of arginine and lysine in yeast and algae. Journal of biological chemistry 212:207-215. 1955.