1,2-CYCLOHEXANEDIONE AND 2,8-DITHIO-6-OXYPURINE AS INHIBITORS OF LEE INFLUENZA VIRUS

by

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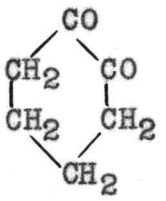
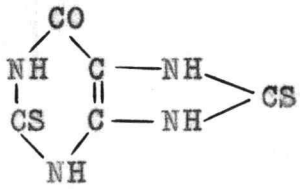
INTRODUCTION

Investigation of the effects of chemical compounds upon the development of viruses is justified both by the need for chemotherapeutic agents and by the possibility that such investigations may provide information on the chemistry of virus development in host cells.

A compound showing antiviral activity might interfere with virus development in a number of ways at any of the three phases of virus infection. The probability of finding an active antiviral agent, and being able to locate exactly the reaction responsible for virus inhibition, is slight in the present state of knowledge of virus multiplication; however, the success of this approach in related fields of microbiology offers some encouragement to continued research along these lines. The antiviral properties of 1,2-cyclohexanedione and 2,8-dithio-6-oxypurine, two compounds which had shown inhibitory activity for Lee influenza virus in chick embryo screening tests, were selected for further studies. The structures of the two compounds are shown below.



1,2-Cyclohexanedione



2,8-Dithio-6-oxypurine

The structure of 1,2-cyclohexanedione suggests a relationship to the quinones. Various quinones are known to have antibacterial activity, which has in some cases been interpreted as being due to interaction with vital thiol groups. This consideration prompted the preliminary testing of 1,2-cyclohexanedione for antiviral activity. The structure of 2,8-dithio-6-oxypurine suggests a relationship to uric acid and other naturally occurring purine derivatives. Any unnatural purine might be inhibitory because of metabolic antagonism to host systems involved in the development of virus nucleoprotein.

HISTORICAL REVIEW

There is little available information about either of the two compounds studied. No information relating to any biological activity of 1,2-cyclohexanedione has been found; although a related compound, 1,2-cyclohexanedicarboxylic acid, has been mentioned briefly by Takemoto and Robbins (10, p.141) as being inhibitory to influenza type A in the chick embryo. Urion first synthesized 1,2-cyclohexanedione in 1931 by the reduction of a cyclic compound formed during the decomposition of divinylglycol (15, pp.1662-1664). He found it to be identical to a compound isolated from the products of the distillation of wood.

No information has been found relating to properties of 2,8-dithio-6-oxypurine; however, several other purine derivatives not found naturally have been reported to possess antiviral activity. The development of Russian spring-summer encephalitis virus (4, p.151) and vaccinia virus (13, pp.530-532) has been shown to be inhibited by 2,6-diaminopurine. Inhibition of both viruses by this compound was reversed by adenine (4, p.151). It has also been reported that vaccinia virus development in tissue cultures was inhibited by 2,6-dichloro-7-methylpurine (13, pp.529-534).

EXPERIMENTAL MATERIALS AND METHODS

Virus. Four stocks of influenza virus were used. The Lee type B influenza virus, which was originally obtained from the American Type Culture Collection, was used for chick embryo, *in vitro*, and mouse experiments and in some tissue cultures. The stock of virus used in the mouse experiments had been maintained by passage in the mouse lung. Infected allantoic fluid from eggs inoculated with this mouse-adapted Lee virus was used as the inoculum in the mouse experiments. The stock of Lee virus used in the chick embryo and *in vitro* experiments and in some tissue cultures was maintained by egg passage. The strain of Lee influenza virus designated as "Lee-R" had been obtained from Dr. F. L. Horsfall. This virus had a long history of egg passages and was well adapted to the chick embryo. The Lee-R virus was used in tissue cultures. The PR8 strain of type A influenza virus was obtained from the American Type Culture Collection.

Stock virus preparations were made by inoculating 9 to 11-day old eggs with 0.1 ml volumes of a 1:1000 or 1:10,000 dilution of infected fluid from the preceding frozen stock virus. The eggs were incubated 40 to 46 hours at 35°C. The fluids were aseptically harvested, pooled, and diluted 1:10,000 in buffered saline. The

allantoic sacs of a group of 9 to 11-day old eggs were inoculated with 0.1 ml volumes of this dilution and the eggs were incubated 40 to 46 hours at 35°C. The fluids from these eggs were aseptically harvested, pooled, and centrifuged to remove red blood cells and other large particles. The fluid was then distributed in 0.7 ml volumes in small sterile cotton-plugged Pyrex tubes which were sealed with rubber stoppers and rapidly frozen in a dry ice-ethanol mixture. The tubes were stored at -60°C. The fluid was titrated for infectivity after freezing. For one stock virus the dilution causing 50 per cent infectivity varied from 5.0×10^{-7} to 4.8×10^{-7} over a three month period. This was an insignificant difference. New stocks of frozen virus usually were prepared every two months.

Eggs. Fertile chicken eggs of various breeds, chiefly New Hampshire Red, were obtained from the Oregon State College poultry farm and from Russell's Hatchery. The eggs were incubated for 9 to 11 days at 38°C. Following inoculation with virus, they were incubated at 35°C.

Compounds. The 1,2-cyclohexanedione was made available through the kindness of Dr. I. F. Halverstadt. The 2,8-dithio-6-oxypurine and the purine derivatives used

in reversal experiments were supplied by Nutritional Biochemicals Corporation. Reagent grade chemicals were used in the preparation of all solutions. Deionized water used in washing and in the preparation of solutions had a conductivity equivalent to 0.3 p.p.m. or less as sodium chloride.

Saline Solutions. Physiological saline used in the hemagglutinin titrations was 0.85 per cent sodium chloride. This saline was not sterilized. The buffered saline used in the dilution of infected fluid for inoculations into the chick embryo had the following composition:

NaCl		8.500 grams
Na_2HPO_4	(anhydrous)	0.769 grams
KH_2PO_4		0.170 grams
Deionized water to 1000 ml		

The buffered saline was sterilized for 20 minutes at 121°C. in Pyrex bottles. The pH was 7.3.

Alsever's Solution. This solution, used in the collection and storage of red blood cells, had the following composition:

Glucose	20.5	grams
Sodium chloride	4.2	grams
Citric acid	0.55	grams
Sodium citrate	8.0	grams
Deionized water to 1000 ml		

The solution was distributed in wide-mouthed jars,

autoclaved at 121°C for 15 minutes, and stored at 14°C until used.

Phosphate-Glycine Buffer. This buffer solution, used as the diluent for in vitro tests of the direct effect of compounds upon virus infectivity, was prepared by mixing equal volumes of solution A and solution B just before use. The solutions had the following compositions:

Solution A.
Glycine 3.752 grams
Sodium chloride 2.922 grams
Deionized water to 100 ml

Solution B.
Na_2HPO_4 (anhydrous) 4.000 grams
Deionized water to 100 ml

The solutions were sterilized in Pyrex tubes at 121°C for 20 minutes. The pH of this buffer solution was 8.0.

Hemagglutinin Titrations. The pattern method of Salk (9, pp.87-98) was used in the determination of hemagglutinin titers. Each week the blood of four to five chickens was collected in an equal volume of Alsever's solution. The chicken red blood cells were washed with physiological saline by three centrifugations at 1500 r.p.m. for 7 minutes. After the final washing, the cells were packed centrifugally at 1500 r.p.m. for 10 minutes and a stock suspension of cells, 10 per cent by volume, was prepared in physiological saline. The

stock suspension was stored at 4°C. The 1.0 and 0.5 per cent suspensions, used in titration of infected fluids, were prepared from this stock immediately before use.

From each of the infected fluids to be titrated, two series of dilutions were prepared. The first series progressed by serial two-fold steps in the series which begins as 1:10, 1:20, 1:40, etc.; the second series progressed in a like manner from an initial dilution of 1:15. Fluids were mixed eight times at each dilution step by being alternately withdrawn into a pipet and expelled into a serological tube. After eight such mixings, 1.0 ml of fluid was withdrawn and transferred to the next tube in the series. When the supply of pipets was unlimited, a pipet was used for mixing only one dilution and then was discarded. The volume remaining in each tube after mixing and transfer was 1.0 ml. One ml of the appropriate cell suspension was added to each tube and the tubes were thoroughly shaken to mix the cells with the diluted fluid. The final volume in each tube, after the addition of the cell suspension, was 2.0 ml and the final dilution was twice the initial dilution.

In hemagglutinin titrations of tissue culture fluids and of allantoic fluids from infectivity titrations, a 0.5 per cent suspension of cells was used for maximum sensitivity. Readings were made after 45 minutes at room

temperature. A 1.0 per cent suspension of cells was used in titrations of all other allantoic fluids. In this case, readings were made after 30 minutes. Titers were expressed as the reciprocal of the highest final dilution showing complete hemagglutination of the chicken red blood cells.

Infectivity Titrations. Infectivity titers were determined by preparing serial ten-fold dilutions of centrifuged allantoic fluids in buffered saline, and injecting 0.1 ml volumes of these dilutions into the allantoic sacs of groups of six 10 or 11-day old eggs. The eggs were incubated for 48 hours at 35°C after inoculation. A 0.5 ml volume of allantoic fluid was removed from each egg after incubation and diluted with an equal volume of physiological saline. One ml of a 0.5 per cent cell suspension was added to the diluted allantoic fluid. Fluids causing no hemagglutination at this final 1:4 dilution were considered to be uninfected. The method of Reed and Muench (8, pp.493-497) was used in calculating the EID_{50}. The EID_{50} calculated by this method represents the dose of virus infected fluid causing infection in 50 per cent of the embryos.

Chick Embryo Experiments. In the majority of experiments in the chick embryo, both the virus inoculum

and the compound solution were injected into the allantoic sac. The period of incubation after inoculation with the virus was from 43 to 48 hours at 35°C. Allantoic fluid was aseptically removed after incubation by disinfecting the shell over the air sac with tincture of iodine, breaking away the shell over the air sac with sterile forceps, tearing off the exposed shell membrane with another pair of sterile forceps, inserting a 21 gauge needle into the allantoic fluid well away from the embryo, and drawing the fluid into a sterile 5 or 10 ml syringe.

The virus inoculum for eggs was 0.1 ml of a suitable dilution of infected allantoic fluid in buffered saline. The 1,2-cyclohexanedione was dissolved in deionized water by gentle warming and the addition of one-tenth and one-hundredth saturated solutions of reagent grade sodium hydroxide. Solutions to be injected into the allantoic sac were adjusted to between pH 7.0 and pH 9.0. The solutions were sterilized by filtration through an ultrafine porosity sintered glass filter. The 2,8-dithio-6-oxypurine, at concentrations of 25 or 12.5 mg per ml, was suspended in a 1.0 per cent solution of Hercules low viscosity carboxymethylcellulose. The suspension was sterilized in a water bath at 100°C for 25 minutes. The volume of compound solution or suspension injected into each egg

was 0.5 ml or less. The control eggs received equal volumes of the diluent.

Mouse Tests. For the toxicity test in mice, the 1,2-cyclohexanedione was dissolved in deionized water by the addition of one-tenth and one-hundredth saturated solutions of sodium hydroxide. A solution containing 60 mg of 1,2-cyclohexanedione per ml was adjusted to pH 7.1 and sterilized by filtration through an ultrafine porosity sintered glass filter. The 2,8-dithio-6-oxypurine was prepared as a suspension in a 1.0 per cent solution of Hercules low viscosity carboxymethylcellulose. The pH of the suspension was adjusted to 7.1. This suspension, containing 6 mg of 2,8-dithio-6-oxypurine per ml, was sterilized in a water bath at 100°C for 25 minutes. A group of three mice was used at each dosage level. The mice received twice daily intraperitoneal injections. Deaths occurring in the three days during which the injections were administered were recorded. The highest dosage level at which at least two of three mice survived the sixth injection was taken to be the maximum tolerated dose.

In the test for inhibition of the virus, the compounds were prepared in the same way except that the concentrations used were those calculated to contain the

maximum tolerated dose in a volume of 0.25 ml. Two experimental groups of mice received twice daily intraperitoneal injections of the maximum tolerated doses of the two compounds and two control groups of mice received intraperitoneal injections of the diluents.

All groups of mice were inoculated with the virus five hours after the first injection. The virus inoculum was 0.05 ml of a 1:1000 dilution of mouse-adapted Lee virus in buffered saline. This was administered intranasally under light ether anesthesia. The mice received a total of six injections of the compounds over the three day period. On the fourth day all mice were sacrificed. The lungs of each group were removed, pooled, washed three times by agitation in physiological saline, blotted, weighed, and ground in a mortar with Pyrex glass to give a 1:10 suspension of ground lung material in physiological saline. This suspension was centrifuged for 15 minutes at 2500 r.p.m. and held at 14°C for 24 hours. The supernatant was removed and titrated for virus hemagglutinin, using a 1.0 per cent suspension of chicken red blood cells.

Tissue Cultures Methods. The tissue culture techniques used were similar to those of Tamm, Folkers, and Horsfall (12, pp.560-561). Glassware, rubber stoppers,

and Bakelite caps were boiled in Labtone solution and were rinsed ten times in tap water and five times in deionized water. Glassware was sterilized either by dry heat at 180°C for one hour or by autoclaving at 121°C for 20 minutes. Instruments were washed in cold Labtone solution, rinsed thoroughly in tap water and in deionized water, and sterilized at 110°C by dry heat overnight.

Buffered glucosol solutions were the nutrient fluids for tissue cultures. Nutrient fluids were prepared from solutions of the following compositions:

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 A.

Na_2HPO_4 (anhydrous)	7.105 grams
KH_2PO_4	2.269 grams
Phenol red	0.020 grams
Deionized water to	1000 ml

Buffer solution B.

Na_2HPO_4 (anhydrous)	7.689 grams
KH_2PO_4	1.702 grams
Phenol red	0.020 grams
Deionized water to	1000 ml

These solutions were sterilized in Pyrex bottles with Bakelite screw caps at 121°C for 20 minutes. Glucosol was mixed with an equal volume of buffer solution immediately before use. Streptomycin and penicillin G were

added to give final concentrations of 40 micrograms per ml and 10 units per ml respectively. The initial pH of the buffered glucosol prepared with buffer solution A was 7.28; the initial pH of that prepared with buffer solution B was 7.35. Buffer solution A was used in preparing nutrient fluid for tissue cultures with 1,2-cyclohexanedione; nutrient fluid for tissue cultures with 2,8-dithio-6-oxypurine was prepared with buffer solution B.

Chorioallantoic membrane tissue was aseptically removed from 9 to 11-day old eggs. The shell over the air sac of an egg was disinfected with tincture of iodine, this portion of the shell was removed with sterile forceps, and the shell membrane covering the chorioallantoic membrane of this region was stripped away with sterile forceps. The egg contents were tipped into a sterile petri dish, the chorioallantoic membrane was gripped with sterile forceps, and the membrane was cut free from other tissues with sterile scissors. The membrane was washed three times in buffered glucosol, either by centrifugation or by agitation, and was cut into 2 cm x 2 cm squares. The pieces from all eggs were thoroughly mixed and distributed at random in sterile Pyrex culture tubes (2.5 x 15 cm) containing 2 ml of the nutrient fluid, with or without the compound to be tested. These tubes were fitted with rubber stoppers. After inoculation with 0.1

ml volumes of virus infected allantoic fluid, suitably diluted in buffered glucosol, the 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. The incubation time was usually 44 to 46 hours. After incubation the culture fluids were tested for sterility by inoculation of a portion of the culture fluid into Difco fluid thioglycollate broth. The culture fluids were titrated for virus hemagglutinin.

For use in tissue cultures, 1,2-cyclohexanedione was prepared as a concentrated solution in tissue culture buffer solution A. The solution was adjusted with one-tenth and one-hundredth saturated solutions of reagent grade sodium hydroxide to pH 7.28. The 1,2-cyclohexanedione dissolved in the buffer with the addition of base and with gentle warming. The concentrated solutions prepared in this way contained 8 mg or 4 mg of 1,2-cyclohexanedione per ml. They were sterilized by filtration through an ultrafine porosity sintered glass filter. Glucosol and the antibiotics were added to the solution just before use in tissue culture.

The 2,8-dithio-6-oxypurine was dissolved in tissue culture buffer solution B or in buffered glucosol at 1.0 or 2.0 mg per ml. The pH of the solution was adjusted to 7.35 with solutions of reagent grade sodium hydroxide.

The solution was sterilized in a water bath at 100°C for 25 minutes and then diluted with glucosol or buffered glucosol before the addition of the antibiotics.

Statistical Methods. Differences between the means of hemagglutinin titers of groups of allantoic fluids or tissue culture fluids were tested for significance by means of Student's "t" test (6, pp.153-157). This method of analysis is appropriate for samples containing less than 30 items. The arithmetic means of the logarithms of the titers were used in the calculation of "t", as is shown in Table 1. The use of the log transformation of data has been discussed by Bartlett (1, pp.39-45).

In all tables except Table 1, the means shown are geometric means of the titers. In Table 1, the means are arithmetic means of the logarithms of the titers.

As dilutions below 1:20 were not made in the hemagglutinin titrations in the majority of experiments, the true titers of fluids which did not cause complete hemagglutination at the 1:20 dilution were not determined. In many fluids there may have been no hemagglutinin; in other fluids there might have been sufficient hemagglutinin present to cause complete hemagglutination of red blood cells at the 1:10 dilution. All fluids which did not cause complete hemagglutination at the 1:20 dilution were arbitrarily assigned a titer of 10 for the

calculation of geometric means.

Table 1

Sample Analysis Used in Comparing Means of Two Experimental Groups of Virus Hemagglutinin Titers

Culture numbers	Hemagglutinin titers	Logarithms of titers$_{10}$	Deviations from mean	Deviations squared
		Group I		
1	120	2.0792	0.16895	0.028544
2	160	2.2041	0.04405	0.000194
3	160	2.2041	0.04405	0.000194
4	320	2.5052	0.25705	0.066075
		8.9926		0.095007
		Group II		
1	<20,* 10	1.0000	0.6373	0.406151
2	40	1.6021	0.0352	0.001239
3	40	1.6021	0.0352	0.001239
4	80	1.9031	0.2654	0.070337
5	120	2.0792	0.4417	0.195099
		8.1865		0.674065

* Fluids which did not cause complete hemagglutination at the lowest dilution titrated (1:20), were arbitrarily assigned a titer of 10.

Mean of Group I: 8.9926/4 ..... 2.24815
Mean of Group II: 8.1865/5 ..... 1.6373
Sum of squared deviations, Group I: 0.095007
Sum of squared deviations, Group II: 0.674065
Combined sum of squares: 0.769072
Sum of degrees of freedom: (4-1) + (5-1) 7
Estimated standard deviation:

$$\pm\sqrt{\frac{0.769072}{7}} \quad \ldots\ldots\ldots\ldots\ldots\ldots \pm 0.1048$$

Standard error of difference between means:

$$\pm\sqrt{\left(\frac{0.1048}{\sqrt{4}}\right)^2 + \left(\frac{0.1048}{\sqrt{5}}\right)^2} \quad \ldots\ldots\ldots\ldots\ldots \pm 0.070216$$

Difference between means: 0.61085
Difference between means, divided by standard error of difference, gives the quotient which is "t": 8.69

Table 1, continued

To enter Fisher's Table of "t", "n" (degrees of freedom) for this analysis is 7, and for this value of "n" the table gives:

P (probability)	0.05	0.02
"t"	2.365	2.998

The value of "t" calculated from the data is 8.699, and exceeds the value of "t" given in Fisher's table for the 0.05 probability level; it also exceeds the value of "t" given in the table for the 0.02 probability level. The difference between the means of Groups I and II is therefore judged to be highly significant.

EXPERIMENTAL RESULTS

Inhibition Studies Involving 1,2-cyclohexanedione

Inhibition Studies in the Chick Embryo. The antiviral activity of 1,2-cyclohexanedione, a compound which had shown inhibitory activity for Lee influenza virus in chick embryo screening tests, was investigated by further studies in the chick embryo. The minimum inhibitory dose of the compound was determined by injecting increasing amounts into the allantoic sacs of groups of 10-day old eggs and injecting, one hour later, a minimal dose of virus by the same route. Following virus inoculation, the eggs were incubated 45 hours at 35°C and the allantoic fluids were removed and individually titrated for virus hemagglutinin. A dose of 3.5 mg per egg was found to exert a marked inhibitory effect on the growth of Lee influenza virus type B, as measured by the amount of hemagglutinin produced in eggs receiving injections of the compound. The mean of the titers of the control eggs, which did not receive injections of the compound, was approximately six times greater than the mean of the titers of the 3.5 mg dose group, as shown in experiment I in Table 2. The 3.5 mg dose was effective also in inhibiting the development of the PR8 strain of type A influenza virus, as shown in Table 3. A dose of 7.0 mg

Table 2

Relation of Dosage of 1,2-Cyclohexanedione to the Degree of Inhibition of Lee Influenza Virus in the Chick Embryo

Experiment	Dosage of 1,2-cyclohexanedione per egg	Total number of eggs treated	Per cent of eggs showing titers of (<20)	(20-80)	(120-320)	(480 or above)	Geometric mean of all fluids
I	3.5 mg	19	53	16	21	10	35
	control	16	0	19	50	31	225
II	2.5 mg	10	10	10	30	50	210
	control	11	0	0	9	81	556

1. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of allantoic fluids causing complete hemagglutinin of chicken red blood cells.

2. The indicated dose of 1,2-cyclohexanedione was injected into the allantoic sac. One hour later all eggs received 50 EID_{50} of Lee influenza virus by the same route. The eggs were incubated 45 hours at 35°C.

3. As the lowest dilution titrated was the 1:20 dilution, all eggs which did not show complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

4. Comparison of the mean values for the hemagglutinin titers of the control and 3.5 mg groups of experiment I by means of the "t" test (6, pp.153-157), using the logarithms of the individual titers, gives a "t" value of 4.24. For the 33 degrees of freedom, this indicates that the difference between the means is

Table 2, continued

significant at the 0.01 probability level. Comparison of the mean values of the control and 2.5 mg groups of experiment II gives a "t" value of 1.81, which is not significant at the 0.05 probability level for the 19 degrees of freedom.

Table 3

Relation of Dosage of 1,2-Cyclohexanedione to the Degree of Inhibition of PR8 Influenza Virus in the Chick Embryo

Dosage of 1,2-cyclo-hexanedione per egg	Total number of eggs treated	Per cent of eggs showing titers of (<20)	(20-80)	120-320)	(480 or above)	Geometric mean of all fluids
7.0 mg	7	43	0	0	57	111
3.5 mg	15	27	13	0	60	163
control	18	0	0	0	94	671

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

2. The indicated dose of 1,2-cyclohexanedione was injected into the allantoic sac. One hour later all eggs received 100 EID_{50} of PR8 influenza virus by the same route. The eggs were incubated 45 hours at 35°C.

3. As the lowest dilution titrated was the 1:20 dilution, all eggs which did not show complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

4. Comparison of the mean values for the titers of the control and 3.5 mg groups by means of the "t" test (6, pp.153-157), using the logarithms of the individual titers, gives a "t" value of 2.72. For the 31 degrees of freedom, this value of "t" indicates that the difference between the means is significant at the 0.02 probability level and is unlikely to be due to chance. A similar comparison of the means of the control and 7.0 mg group gives a "t" value of 3.29. With 23 degrees of freedom, this value of "t" indicates that the difference of the means is significant at the 0.01 probability level.

per egg was slightly more inhibitory to the PR8 virus than the 3.5 mg dose; the former, however, was considerably more toxic and killed the majority of chick embryos. A dose of 2.5 mg per egg did not cause sufficient inhibition of Lee influenza virus to be significant with the number of eggs employed, as shown by the results in experiment II in Table 2. Titers shown in Tables 2 and 3 are those of allantoic fluid from eggs surviving 46 hours after injection of the compound.

When the 3.5 mg dose of 1,2-cyclohexanedione was injected into the yolk sac and the virus inoculum was injected into the allantoic sac, there did not appear to be a marked inhibition of virus development. The results of the yolk-sac experiment are shown in Table 4. This apparent decrease in inhibition resulting from yolk sac injection of the compound, as compared to inhibition resulting from allantoic sac injection of the same dose, suggested that at least a part of the inhibitory activity of 1,2-cyclohexanedione depended upon the direct contact of the compound with the virus inoculum or with the chorioallantoic membrane tissue, or with both.

Virucidal Effect. The direct virucidal effect of 1,2-cyclohexanedione was measured by determining the effect of the compound upon the infective property of the Lee virus. A mixture composed of 2 parts of freshly

Table 4

Inhibition of Lee Influenza Virus Growth in the Chick Embryo Resulting from Yolk Sac Injection of 1,2-Cyclohexanedione

Dosage of 1,2-cyclohexanedione per egg	Number of eggs treated	Per cent of eggs showing titers of (<20)	(20-80)	(120-320)	(480 or above)	Geometric mean of all fluids
3.5 mg	19	63	16	16	5	25
controls	24	14	57	29	0	56

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

2. The indicated dose of 1,2-cyclohexanedione was injected into the yolk sac. One hour later all eggs received 50 EID_{50} of Lee influenza virus by allantoic sac injection. Eggs were incubated 45 hours at 35°C.

3. As the lowest dilution titrated was the 1:20 dilution, all eggs which did not show complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

4. Comparison of the means of the control and 3.5 mg groups by means of the "t" test, (6, pp.153-157), using the logarithms of the individual titers, gives a "t" value of 1.88. At 31 degrees of freedom, this value for "t" is not significant at the 0.05 probability level.

harvested infective allantoic fluid, 7 parts of phosphate-glycine buffer, and 1 part of concentrated 1,2-cyclohexanedione solution was prepared. The 1,2-cyclohexanedione was present in the mixture at a final concentration of 0.8 mg per ml. A control mixture, composed of 2 parts of infective allantoic fluid, 7 parts of phosphate-glycine buffer, and 1 part of water, was prepared also. The control mixture was titrated for infectivity immediately after it was prepared. Aliquots of the control mixture were titrated after incubation for 3 hours at 35°C and 0°C. The mixture containing the compound was titrated after incubation at 35°C and 0°C. The results of this experiment are shown in Table 5. During incubation at 0°C, the infectivity titer of the control mixture dropped only 0.1 logarithm unit. Knight (5, pp.487-495) has demonstrated that a difference of less than 0.62 logarithm units between two infectivity titers cannot be considered significant at the 0.05 probability level when five embryos are used at each dilution. A variation of 0.1 logarithm unit is not significant. The mixture containing 1,2-cyclohexanedione contained infective allantoic fluid in the same concentration as that in the control preparation and was assumed to have had the same initial infectivity titer as the unincubated control. After incubation at 0°C, the titer of the preparation

Table 5

Measurement of the Direct Virucidal Effect of 1,2-Cyclohexanedione for Lee Influenza Virus

Preparation	Incubation time and temperature	pH before incubation	pH after incubation	EID_{50} per ml
Virus, buffer, and water	Before incubation	8.10	--	$10^{7.3}$
Virus, buffer, and 0.8 mg of 1,2-cyclohexanedione per ml	3 hours, 35°C	8.07	8.10	$<10^{2.0}$
Virus, buffer, and water	3 hours, 35°C	8.10	8.15	$10^{3.8}$
Virus, buffer, and 0.8 mg of 1,2-cyclohexanedione per ml	3 hours, 0°C	8.07	8.08	$10^{4.3}$
Virus, buffer, and water	3 hours, 0°C	8.10	8.12	$10^{7.2}$

1. 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 incubation for 48 hours at 35°C, the presence or absence of infection was determined by testing the allantoic fluids for hemagglutinin at a 1:4 dilution. The EID_{50} was calculated from this data.
2. Virus in all preparations was freshly harvested infected allantoic fluid.
3. All preparations were shown to be free from contaminating microorganisms at the end of the experiment.

containing 1,2-cyclohexanedione dropped from the assumed initial titer of $10^{7.3}$ to a titer of $10^{4.3}$, a difference of 3.0 logarithm units. This is a loss of titer significantly greater than that which occurred in the control preparation incubated at the same temperature and indicates that over 99% of the virus had been inactivated. The difference between the titers of the two preparations incubated at 35°C was significant also. The 1,2-cyclohexanedione appeared to have had a directly virucidal effect upon Lee influenza virus under the conditions of this experiment.

Effect on Adsorption of Virus by Host Cells. The effect of 1,2-cyclohexanedione upon the adsorption of influenza virus to chorioallantoic membrane was tested. Freshly harvested chorioallantoic membranes were washed in buffered saline, coarsely minced, and pooled. The minced tissue was placed in tared centrifuge tubes and centrifuged. The supernatant fluid was removed and the wet weight of the tissue was obtained by difference. Two control preparations, containing infected allantoic fluid at dilutions of 1:2 and 1:5, were prepared by mixing the infected allantoic fluid with normal allantoic fluid and water. Two mixtures containing 0.8 mg of 1,2-cyclohexanedione per ml were prepared by mixing infected

allantoic fluid with normal allantoic fluid and a concentrated solution of 1,2-cyclohexanedione. The dilutions of infected allantoic fluid in these preparations correspond to the dilutions in the control preparations. Both control preparations were titrated for hemagglutinin before incubation, after incubation, and after incubation with the tissue. Both preparations containing the compound were titrated after incubation, and after incubation with tissue. The infective allantoic fluid preparations were added to the tissue at the rate of one ml per gram of tissue. Table 6 shows the results of the adsorption experiment. In the control and compound preparations containing the 1:2 dilutions of infected fluid, there was no significant loss of hemagglutinin during incubation, as was demonstrated by the comparison of their hemagglutinin titers to that of the control preparation which had not been incubated. Comparison of the titers of preparations incubated with tissue to those of preparations incubated without tissue indicates that approximately 50 to 75 per cent of the hemagglutinin was adsorbed to the tissue in both the control preparation and the preparation containing the compound. In the 1:5 preparations the degree of adsorption appears to have been between 85 and 90 per cent. In neither case was there any indication that the compound had a significant effect upon the

Table 6

Investigation of the Effect of 1,2-Cyclohexanedione upon the Adsorption of Lee Influenza Virus to the Chorioallantoic Membrane in Vitro

Dilution of the infected fluid	Concentration of 1,2-cyclohexanedione	Incubation time	Tissue present	Hemagglutinin titer
1:2	0	Before incubation	0	240
1:2	0	30 minutes	0	240
1:2	0.8 mg per ml	30 minutes	0	160
1:2	0	30 minutes	1.0 gm per ml	60
1:2	0.8 mg per ml	30 minutes	1.0 gm per ml	80
1:5	0	Before incubation	0	80
1:5	0	30 minutes	0	80
1:5	0.8 mg per ml	30 minutes	0	80
1:5	0	30 minutes	1.0 gm per ml	10
1:5	0.8 mg per ml	30 minutes	1.0 gm per ml	10

1. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of preparations causing complete hemagglutination of chicken red blood cells.

2. The tissue was freshly harvested chorioallantoic membrane which had been washed five times in sterile buffered saline and had been aseptically weighed.

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

adsorption process.

Inhibition in Tissue Cultures. Table 7 shows the results of an experiment in which several concentrations of 1,2-cyclohexanedione were tested for inhibitory effect against two virus inocula in tissue culture. A tenfold difference in the concentration of the virus inoculum appeared to have had only a very slight effect on the titers obtained from the control tissue cultures. Concentrations of 0.4 and 0.8 mg of 1,2-cyclohexanedione per ml in tissue culture fluids produced significant inhibition of virus development. At between 15 and 24 hours incubation of the tissue in these concentrations of the compound, the tissue began to assume a somewhat abnormal appearance. After 45 hours incubation the tissue appeared yellow and flaccid and the blood vessels were no longer visible. In control cultures the tissue retained its pink, curled appearance and the blood vessels were still visible after 45 hours incubation.

It seemed not unlikely that these gross morphological changes in the tissue might be accompanied by permanent cellular changes which rendered the tissue incapable of supporting normal development of the virus. Table 8 shows the results obtained when tissue was exposed to the minimum inhibitory concentration of the

Table 7

Relation of 1,2-Cyclohexanedione Concentration to the Degree of Inhibition of Lee-R Influenza Virus in Tissue Culture

EID_{50} per ml in culture fluids before incubation	Culture number	Virus hemagglutinin titers of tissue culture fluids containing the following concentrations of 1,2-cyclohexanedione: 0.8 mg per ml	0.4 mg per ml	0.2 mg per ml	control
2.0×10^5	1	<20	<20	20	30
	2	<20	20	40	30
	3	<20	20	40	40
	4	<20	30	40	40
	5	--	30	40	60
	6	--	30	40	120
	7	--	30	40	--
	8	--	40	40	--
	Geometric mean	10	25	37	47
2.0×10^6	1	<20	<20	30	30
	2	<20	<20	30	40
	3	<20	<20	30	40
	4	30	20	40	80
	5	--	30	40	80
	6	--	40	60	160
	7	--	40	120	--
	8	--	60	240	--
	Geometric mean	13	22	55	61

1. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of culture fluids causing complete hemagglutination of chicken red blood cells.

2. As the lowest dilution titrated was the 1:20 dilution, all fluids which did not

Table 7, continued

show complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

3. Tissue cultures were inoculated with virus and then incubated 45 hours at 35°C on a reciprocating shaker.

4. Comparison of the mean values of the titers of each of groups inoculated with 2.0 x 10^5 EID_{50} with the mean values for the corresponding group inoculated with 2.0 x 10^6 EID_{50} by means of the "t" test (6, pp.153-157), using the logarithms of the individual titers, gives "t" values between 0.35 and 1.56. None of these values of "t" is significant at the 0.05 probability level with the number of degrees of freedom allowed in each case.

5. Comparison of the mean of the control titers with the means of the titers of the 0.8, 0.4, and 0.2 mg groups by means of the "t" test gives "t" values of 3.74, 2.73, and 0.00 for the groups inoculated with 2.0 x 10^6 EID_{50} per ml. For the degrees of freedom allowed in each case, the "t" values of the 0.8 and 0.4 mg group comparisons are significant at the 0.05 probability level; that of the 0.2 mg group is not significant at this probability level. In a similar comparison for the groups inoculated with 2.0 x 10^5 EID_{50} per ml the 0.8 mg "t" value is 5.67, the 0.4 mg "t" value is 2.53, and the 0.2 mg "t" is 1.13. Again the values of "t" for the 0.8 and 0.4 mg groups are significant, while that of the 0.2 mg group is not.

Table 8

Effect of 1,2-Cyclohexanedione upon Chorioallantoic Tissue as Measured by the Ability of the Tissue to Support Virus Growth When the Compound Is Removed

Group	Treatment of tissue cultures	Hemagglutinin titers
1	Tissues were incubated for 23 hours at 35°C in nutrient fluid containing no 1,2-cyclohexanedione, washed three times in nutrient fluid, and placed in fresh nutrient fluid without 1,2-cyclohexanedione. The tissue cultures were inoculated with 4.8 x 10^5 EID_{50} of Lee-R influenza virus per ml. Tissue cultures were then incubated for 45 hours at 35°C.	40 80 80 120 120 120 120 160 320 320
	Geometric mean	124
2	Tissues were incubated for 23 hours at 35°C in nutrient fluid containing 0.4 mg of 1,2-cyclohexanedione per ml, washed three times in nutrient fluid, and placed again in fresh nutrient fluid containing 0.4 mg of 1,2-cyclohexanedione per ml. The tissue cultures were inoculated with 4.8 x 10^5 EID_{50} of Lee-R influenza virus per ml and then incubated for 45 hours at 35°C.	<20 <20 <20 <20 <20 <20 <20 <20 <20 <20
	Geometric mean	10
3	Tissues were incubated for 23 hours at 35°C in nutrient fluid containing 0.4 mg of 1,2-cyclohexanedione per ml, washed three times in nutrient fluid, and placed in fresh nutrient fluid without 1,2-cyclohexanedione. The tissue cultures were inoculated with 4.8 x 10^5 EID_{50} of Lee-R influenza virus per ml and then incubated for 45 hours at 35°C.	<20 <20 <20 <20 <20 <20 <20 <20 <20 <20
	Geometric mean	10

1. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of nutrient fluids causing complete hemagglutination of chicken red blood cells.

Table 8, continued

2. As the lowest dilution titrated was the 1:20 dilution, all fluids which did not cause complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

compound for 23 hours and then was well washed before virus inoculation. No virus hemagglutinin was detected in such cultures 45 hours after inoculation, even though the compound was not present in the culture fluid during this second incubation period. Control cultures similarly treated, but not exposed to the compound during either incubation period, produced normal yields of virus hemagglutinin. The 23 hour exposure to 1,2-cyclohexanedione appeared to have interfered with the ability of the tissue to support virus growth.

Mouse Tests. The maximum tolerated intraperitoneal dose of 1,2-cyclohexanedione was found to be 1.2 gm per kilogram, indicating a very low degree of acute toxicity for the mouse. This was the dose of compound which, when administered twice daily for a period of three days, was not lethal to more than one of the three mice treated. This dose of compound was tested for ability to inhibit growth of Lee influenza virus in the lungs of infected mice. Briefly, the five mice were injected with the maximum tolerated dose of compound and inoculated a few hours later with virus. The mice were then injected with the same dose twice daily during the remainder of the three day period. Details of the method have been given previously. The five mice in the control group received

injections of water and were inoculated with the same amount of virus. On a weight/volume basis, the pooled lung material from the control mice had a hemagglutinin titer of 80; that of the mice which received the compound had a titer of 160. There was no detectable inhibition of virus development by 1,2-cyclohexanedione under the conditions of this test. Peak concentrations of the compound in the mouse tissues following an injection could have been as high as 1.2 mg per gram of tissue. In the egg, assuming that all of the compound remained in the allantoic fluid after injection, the concentration of the compound probably rarely exceeded 1.0 mg per ml when the dose given was 3.5 mg per egg.

Inhibition Studies Involving 2,8-dithio-6-oxypurine

Inhibition in the Chick Embryo. The dosage of 2,8-dithio-6-oxypurine found to cause a significant degree of inhibition of Lee influenza virus development in the chick embryo was 12.5 mg per egg. There appeared to be some inhibition at the 6.25 mg dosage level, but this was not significant with the number of eggs used. The results of an experiment, in which increasing amounts of the compound were tested for inhibitory effect upon Lee influenza virus, are shown in Table 9. In this experiment the compound was injected into the allantoic

Table 9

Relation of Dosage of 2,8-Dithio-6-oxypurine to the Degree of Inhibition of Lee Influenza Virus in the Chick Embryo

Dosage of 2,8-dithio-6-oxypurine per egg	Total number of eggs treated	Per cent of eggs showing titers of (<20)	(20-60)	(80-240)	(320 or above)	Geometric mean of all fluids
12.50 mg	10	50	0	40	10	38
6.25 mg	10	10	10	20	60	174
3.12 mg	10	0	0	10	90	602
control	8	0	0	13	87	554

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

2. As the lowest dilution titrated was the 1:20 dilution, all eggs which did not show complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

3. The indicated dose of 2,8-dithio-6-oxypurine was injected into the allantoic sac. One hour later all eggs received 100 EID_{50} of Lee influenza virus by the same route. All eggs were incubated 45 hours at 35°C.

4. Comparison of the means of the control and 12.5 mg groups by means of the "t" test (6, pp.153-157), using the logarithms of the individual titers, gives a "t" value of 5.15. For the 16 degrees of freedom, this value of "t" indicates that the difference between the means is significant at the 0.01 probability level. Comparison of the mean of the control group with the means of the 6.25 mg and 3.12 mg groups gives "t" values which are not significant at the 0.05 probability level.

sac. This was followed, one hour later, by an injection of virus into the allantoic sac. The eggs were then incubated 45 hours at 35°C. After incubation, the allantoic fluids were removed and individually titrated for hemagglutinin. The geometric mean of the titers of the control group eggs was approximately 15 times greater than the mean of the titers of eggs which received the 12.5 mg dose of the compound.

The results shown in Table 10 indicate that the 12.5 mg dose of the compound was less effective in inhibiting virus development when administered by the yolk-sac route than when administered by the allantoic route. The mean of the titers of eggs receiving 12.5 mg of the compound by the yolk-sac route and virus by the allantoic route was 266; the mean of titers of eggs receiving both the virus inoculum and the 12.5 mg dose by the allantoic route was 55. The failure of this dose of compound to inhibit virus development significantly when administered by the yolk-sac route suggested that the presence of the compound in the allantoic sac might be a necessary condition for inhibition, either because of a directly virucidal effect of the compound or because of a direct effect of the compound upon the chorioallantoic tissue.

Effect upon Adsorption of Virus by Host Cells. The procedure used in testing the effect of 2,8-dithio-6-

Table 10

Investigation of the Effect of Allantoic and Yolk Sac Injection of 2,8-Dithio-6-oxypurine upon the Growth of Lee Influenza Virus in the Chick Embryo

Dosage of 2,8-dithio-6-oxypurine per egg	Total number of eggs treated	Per cent of eggs showing titers of (<20)	(20-80)	(120-320)	(480 or above)	Geometric mean of all fluids
Yolk sac 12.5 mg	9	0	0	89	11	266
Allantoic sac 12.5 mg	7	43	0	57	0	55
Control	8	0	0	75	25	330

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

2. As the lowest dilution titrated was the 1:20 dilution, all eggs which did not show complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

3. The eggs were injected with 2,8-dithio-6-oxypurine either by the yolk sac or allantoic sac route. One hour later all eggs received 100 EID_{50} of Lee influenza virus by the allantoic route. The eggs were incubated for 45 hours at 35°C.

4. Comparison of the means of the allantoic 12.5 mg group and the control group by means of the "t" test (6, pp.153-157), using the logarithms of the individual titers, gives a "t" value of 3.07. For 13 degrees of freedom, this value of "t" is significant at the 0.01 probability level. Comparison of the yolk sac 12.5 mg group mean with the mean of the control group gives a "t" value of 1.06. For the 15 degrees of freedom, this value of "t" indicates that the difference between the means of these groups is not significant at the 0.05 probability level.

oxypurine upon the adsorption of virus to chorioallantoic tissue was similar in most respects to that used in the adsorption experiment with 1,2-cyclohexanedione, except that in the experiment with 2,8-dithio-6-oxypurine the concentrated solution of the compound was sterilized by boiling and was added to the mixture of normal and infected allantoic fluids to give a final concentration of 2.5 mg per ml. Where the 1:2 dilution of virus was used, in both the control preparation and the preparation containing the compound, the adsorption of virus hemagglutinin to the tissue approached 80 per cent. Results of this experiment are shown in Table 11. In the same experiment, where the 1:5 virus dilution was used, in both the control preparation and that containing the compound, the adsorption of hemagglutinin to the tissue exceeded 90 per cent. There was no indication in either case that the compound interfered with the adsorption of virus hemagglutinin to the tissue when the compound was present at a concentration of 2.5 mg per ml.

Virucidal Effect. The effect of 2,8-dithio-6-oxypurine on the infectivity of influenza virus was used as a measure of the virucidal activity of the compound. A control preparation containing one part of Lee virus infected allantoic fluid, seven parts of phosphate-glycine

Table 11

Investigation of the Effect of 2,8-Dithio-6-oxypurine upon the Adsorption of Lee Influenza Virus to the Chorioallantoic Membrane in Vitro

Dilution of the infected fluid	Concentration of 2,8-dithio-6-oxypurine	Incubation time	Tissue present	Hemagglutinin titer
1:2	0	Before incubation	0	160
1:2	0	30 minutes	0	160
1:2	2.5 mg per ml	30 minutes	0	120
1:2	0	30 minutes	1.0 gm per ml	30
1:2	2.5 mg per ml	30 minutes	1.0 gm per ml	30
1:5	0	Before incubation	0	40
1:5	0	30 minutes	0	40
1:5	2.5 mg per ml	30 minutes	0	30
1:5	0	30 minutes	1.0 gm per ml	<4
1:5	2.5 mg per ml	30 minutes	1.0 gm per ml	<4

1. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of preparations causing complete hemagglutination of chicken red blood cells.

2. The tissue was freshly harvested chorioallantoic membrane which had been washed five times in sterile buffered saline and had been aseptically washed.

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

buffer, and two parts of water was prepared. This was titrated for infectivity before incubation. Aliquots of this preparation were titrated again for infectivity after incubation for one hour at 35°C and 0°C. A preparation containing one part of infected allantoic fluid, seven parts of phosphate-glycine buffer, and two parts of concentrated solution of the compound was also prepared. The final concentration of the compound in the mixture was 2.5 mg per ml. The mixture was titrated for infectivity after incubation at 35°C and 0°C. The results of this experiment are shown in Table 12. Comparison of the infectivity titers of the control preparation and the preparation containing compound, which were both incubated for one hour at 35°C, indicates that the presence of the compound did not reduce the infectivity titer of the fluid. In the preparation incubated at 0°C there appeared to have been no loss of infectivity which could be attributed to the presence of the compound. As the 2,8-dithio-6-oxypurine did not appear to have affected the infectivity of the virus during a one-hour exposure period, the effect of the compound upon infectivity during a longer incubation period was investigated. Two parts of Lee-R virus infected allantoic fluid mixed with six parts of phosphate-glycine buffer and one part of water served as the control preparation. A mixture

Table 12

Measurement of Direct Virucidal Activity of 2,8-Dithio-6-oxypurine for Lee and Lee-R Influenza Virus

Preparation	Incubation time and temperature	pH before incubation	pH after incubation	EID_{50} per ml
Lee virus, buffer, and water	Before incubation	8.02	--	$10^{5.0}$
Lee virus, buffer, and 2.5 mg of 2,8-dithio-6-oxypurine per ml	1 hour, 35°C	8.01	8.02	$10^{6.5}$
Lee virus, buffer, and water	1 hour, 35°C	8.02	8.03	$10^{6.2}$
Lee virus, buffer, and 2.5 mg of 2,8-dithio-6-oxypurine per ml	1 hour, 0°C	8.01	8.02	$10^{8.0}$
Lee virus, buffer, and water	1 hour, 0°C	8.02	8.02	$10^{7.2}$
Lee-R virus, buffer, and water	Before incubation	8.03	--	$10^{8.8}$
Lee-R virus, buffer, and 2.5 mg of 2,8-dithio-6-oxypurine per ml	26 hours, 35°C	8.06	8.08	$<10^{3.0}$
Lee-R virus, buffer, and water	26 hours, 35°C	8.03	8.05	$10^{4.6}$

Table 12, continued

1. 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 incubation at 35°C for 48 hours, the presence or absence of infection was determined by hemagglutination at a 1:4 dilution. The EID_{50} was calculated from this data.

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

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

4. Inspection of the infectivity titers of the control fluid in experiment I, before and after incubation, shows that the initial titer was apparently too low. The reason for this is unknown.

containing two parts of virus infected allantoic fluid, six parts of phosphate-glycine buffer, and two parts of a concentrated solution containing 12.5 mg of the compound per ml was also prepared. The control mixture was titrated for infectivity before incubation and after incubation at 35°C for 26 hours. The mixture containing the compound was titrated after incubation at 35°C for 26 hours. During incubation the titer of the control preparation dropped 4.2 logarithm units, while the titer of the preparation containing compound dropped more than 5.8 logarithm units. The difference of more than 1.6 logarithm units between the control and compound mixtures is considered significant when five or more eggs are used for each dilution in the titration (5, pp.487-495). From the results of these two experiments it appeared that 2,8-dithio-6-oxypurine was not rapidly virucidal but that prolonged exposure of the virus to the compound did result in a loss of infectivity greater than that in the control preparation.

Inhibition in Tissue Cultures. The 2,8-dithio-6-oxypurine was tested for inhibitory activity for Lee and Lee-R influenza viruses in tissue cultures. The results of two tissue culture experiments are shown in Table 13. In experiment I each tissue culture was inoculated with

Table 13

Relation of Concentration of 2,8-Dithio-6-oxypurine to the Degree of Inhibition of Lee and Lee-R Influenza Virus in Tissue Cultures

Experiment	EID_{50} of virus per ml in tissue culture	Culture number	Virus hemagglutinin titers of tissue culture fluids containing the following concentrations of 2,8-dithio-6-oxypurine		
			2.0 mg per ml	1.0 mg per ml	control
I	Lee virus 4.8×10^4	1	<20	<20	20
		2	<20	<20	20
		3	<20	<20	30
		4	<20	<20	40
		5	<20	<20	40
		6	<20	40	60
		7	<20	60	80
		8	<20	60	80
		Geometric mean	10	19	42
II	Lee-R virus 1.5×10^6	1	<20	80	320
		2	20	80	320
		3	40	80	480
		4	40	120	640
		5	60	160	640
		6	60	160	640
		Geometric mean	32	108	484

1. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of tissue culture fluids causing complete hemagglutination of chicken red blood cells.

2. As the lowest dilution titrated was the 1:20 dilution, all fluids which did not

Table 13, continued

cause complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

3. Tissue cultures were inoculated with virus and then incubated for 45 hours at 35°C in experiment I. In experiment II the incubation period was 46 hours.

4. Comparison of the means of the 2.0 mg and the control group in experiment I by means of the "t" test (6, pp.153-157), using the logarithms of the individual titers, gives a "t" value of 9.84. For the 14 degrees of freedom, this value of "t" indicates that the difference between the means is significant at the 0.01 probability level. Comparison of the means of the 1.0 mg and the control group gives a "t" value of 1.62 which is not significant at the 0.05 probability level. In experiment II the comparison of the mean of the control group with that of the 2.0 mg and 1.0 mg groups gives "t" values of 8.51 and 8.02 respectively. For the 10 degrees of freedom in each of these cases, the values obtained for "t" indicate that the differences between the mean of the control group and those of the 1.0 and 2.0 mg groups are significant at the 0.01 probability level.

4.8×10^4 EID_{50} of Lee influenza virus per ml. A significant inhibition of virus growth was noted in tissue cultures containing 2.0 mg of the compound per ml but not in those containing 1.0 mg per ml. In experiment II, also shown in Table 13, each tissue culture was inoculated with 1.5×10^6 EID_{50} of Lee-R influenza virus per ml. In this experiment there was significant inhibition at both the 2.0 and 1.0 mg concentration levels. The results of these two experiments illustrate that the measurable inhibition produced by a given concentration of the compound is not necessarily constant when other conditions are varied.

In a third experiment four levels of Lee-R virus inoculum were tested for their effect upon the degree of inhibition produced by the 1.0 mg per ml concentration of the compound. The results of this experiment are shown in Table 14. At a concentration of 1.0 mg per ml, the compound produced a significant inhibition at all four of the virus inoculum levels. There did not appear to be a greater degree of inhibition in tissue cultures receiving a dilute virus inoculum than in tissue cultures receiving a concentrated inoculum.

The ability of the chorioallantoic membrane tissue to support virus development, after exposure of the tissue to the compound, was investigated. The tissues were

Table 14

The Effect of Virus Inoculum Concentration upon the Inhibitory Activity of 2,8-Dithio-6-oxypurine for Lee-R Influenza Virus in Tissue Culture

Concentration of 2,8-dithio-6-oxypurine in the tissue culture fluid	Tissue culture number	Hemagglutinin titers of tissue cultures which received the following virus inocula:			
		1.5×10^6 EID_{50} per ml	1.5×10^5 EID_{50} per ml	1.5×10^4 EID_{50} per ml	1.5×10^3 EID_{50} per ml
1.0 mg per ml	1	30	30	20	<20
	2	60	40	40	20
	3	60	40	40	30
	4	60	40	40	40
	5	80	60	60	40
	6	80	80	80	40
	Geometric mean	59	46	41	27
control	1	80	60	40	60
	2	80	80	60	60
	3	160	80	80	60
	4	240	120	80	80
	5	240	120	120	80
	6	320	120	120	120
	Geometric mean	163	93	78	74
Ratios of control titer means to means of titers of cultures containing 2,8-dithio-6-oxypurine		2.8	2.0	1.9	2.7

Table 14, continued

2. As the lowest dilution titrated was the 1:20 dilution, all fluids which did not cause complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

3. Following inoculation with the designated concentration of virus, the tissue cultures were incubated 45 hours at 35°C on a reciprocating shaker.

4. Comparison of the means of the tissue culture titers of the groups containing 2,8-dithio-6-oxypurine with the means of the tissue culture titers of the corresponding controls by means of the "t" test (6, pp.153-157), using the logarithms of the individual titers gave the following values for "t": (1.5×10^6 EID_{50}) 3.60, (1.5×10^5 EID_{50}) 3.86, (1.5×10^4 EID_{50}) 2.72, (1.5×10^3 EID_{50}) 4.70. For the number of degrees of freedom allowed in each case, these values for "t" indicate that the differences between the means were significant at the 0.05 probability level.

incubated in nutrient fluid containing 1.0 mg of the compound per ml. After 12 hours, the tissues were washed thoroughly, placed in fresh nutrient fluid without compound, and inoculated with the virus. After 45 hours incubation the fluids were removed, pooled, and the pools titrated for hemagglutinin. The pools of fluids of two control groups of tissue cultures were also titrated. One group of control tissue cultures was exposed to the minimum inhibitory concentration of the compound during both incubation periods; the other control group was not exposed to the compound. The results of this experiment are shown in Table 15. The titers of the pool of control fluids from tissue cultures incubated without the compound is the same as that of the pool of fluids from tissue cultures exposed to the compound during the preliminary incubation period. These titers were four times greater than that of the pool of fluids exposed to the compound during both incubation periods. These results suggested that the compound did not rapidly destroy the ability of the tissue to support virus development. In a second experiment of this type, the ability of the tissue to support virus growth, after a 24 hour exposure to the compound, was investigated. The results of this experiment, shown in Table 16, suggest that the tissue was not able to support normal virus growth after this

Table 15

Effect of 2,8-Dithio-6-oxypurine upon Chorioallantoic Tissue as Measured by the Ability of the Tissue to Support Virus Growth When the Compound Is Removed

Group	Treatment of tissue cultures	Hemagglutinin titers
I	Tissues were incubated for 12 hours at 35°C in nutrient fluid containing no 2,8-dithio-6-oxypurine, washed three times in nutrient fluid, and placed in fresh nutrient fluid without 2,8-dithio-6-oxypurine. Tissue cultures were then inoculated with 1.5 x 10^6 EID_{50} of Lee-R influenza virus per ml and incubated 45 hours at 35°C. Pool of fluids from 8 cultures:	160
II	Tissues were incubated for 12 hours at 35°C in nutrient fluid containing 1.0 mg of 2,8-dithio-6-oxypurine per ml, washed three times in nutrient fluid, and placed in fresh nutrient fluid containing 1.0 mg of 2,8-dithio-6-oxypurine per ml. The tissue cultures were inoculated with 1.5 x 10^6 EID_{50} of Lee-R influenza virus per ml and incubated 45 hours at 35°C. Pool of fluids from 8 cultures:	40
III	Tissues were incubated 12 hours at 35°C in nutrient fluid containing 1.0 mg of 2,8-dithio-6-oxypurine per ml, washed three times in nutrient fluid, and placed in fresh nutrient fluid containing no 2,8-dithio-6-oxypurine. The tissue cultures were inoculated with 1.5 x 10^6 EID_{50} of Lee-R influenza virus per ml and incubated for 45 hours at 35°C. Pool of fluids from 8 cultures:	160
	Dummy tube containing fluid only, inoculated with 1.5 x 10^6 EID_{50} of Lee-R influenza virus per ml, titrated before incubation:	< 20

Table 16

Effect of 2,8-Dithio-6-oxypurine on Chorioallantoic Tissue as Measured by the Ability of the Tissue to Support Virus Growth When the Compound Is Removed

Group	Treatment of tissue cultures	Hemagglutinin titers
I	Tissues were incubated for 24 hours at 35°C in nutrient fluid containing no 2,8-dithio-6-oxypurine, washed three times in nutrient fluid, and placed in fresh nutrient fluid without 2,8-dithio-6-oxypurine. The tissue cultures were then inoculated with 1.5 x 10^6 EID_{50} of Lee-R influenza virus per ml and then incubated 45 hours at 35°C.	<20
		<20
		40
		40
		60
		60
		80
		120
	Geometric mean	39
II	Tissues were incubated for 24 hours at 35°C in nutrient fluid containing 1.0 mg of 2,8-dithio-6-oxypurine per ml, washed three times in nutrient fluid, and then placed in fresh nutrient fluid containing 1.0 mg of 2,8-dithio-6-oxypurine per ml. The tissue cultures were inoculated with 1.5 x 10^6 EID_{50} of Lee-R influenza virus per ml and then incubated 45 hours at 35°C.	<20
		<20
		<20
		<20
		<20
		<20
		<20
		<20
	Geometric mean	10
	Tissues were incubated 24 hours at 35°C in nutrient fluid containing 1.0 mg of 2,8-dithio-6-oxypurine per ml, washed three times in nutrient fluid, and then placed in fresh nutrient fluid containing no 2,8-dithio-6-oxypurine. The tissue cultures were inoculated with 1.5 x 10^6 EID_{50} of Lee-R influenza virus per ml and incubated 45 hours at 35°C.	<20
		<20
		<20
		<20
		<20
		30
		30
		30
	Geometric mean	20

1. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of tissue culture fluids causing complete hemagglutination of chicken red blood cells.

2. As the lowest dilution titrated was the 1:20 dilution, all fluids which did not cause complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

prolonged exposure to the minimum inhibitory concentration of the compound.

Attempts to Reverse Inhibition. Although the ability of the compound to slowly inactivate the infective property of the virus and to decrease, within 24 hours, the ability of the tissue to support virus growth would perhaps, in itself, explain the entire inhibitory effect of the compound, the structure of the compound and its relationship to naturally occurring purine derivatives presented the possibility that a part of the inhibitory activity of the compound might be the result of an antagonism to natural purine derivatives possibly involved in the metabolism of viral nucleic acids. To investigate this possibility, naturally occurring purine derivatives were added to tissue culture fluids containing the minimum inhibitory concentration of 2,8-dithio-6-oxypurine. In each case, the maximum concentration of the compound tested in tissue cultures was approximately one-half the maximum solubility of the compound in glucosol adjusted to between pH 6.8 and 7.1. These concentrated glucosol solutions containing purine compounds were sterilized by filtration through ultrafine porosity sintered glass filters. Suitable dilutions were prepared in glucosol after filtration. The 2,8-dithio-6-oxypurine was prepared

as a concentrated solution of 2.0 mg per ml in buffer. This was sterilized in a water bath at 100°C for 25 minutes. Just before use, the concentrated 2,8-dithio-6-oxypurine solution was mixed with an equal volume of the glucosol solution containing the purine compound. Two control fluids were: (A) nutrient fluid only and (B) nutrient fluid containing 1.0 mg of 2,8-dithio-6-oxypurine per ml. The results of several experiments in which attempts were made to reverse, by the use of purine derivatives, the inhibition of virus development caused by 2,8-dithio-6-oxypurine are shown in Tables 17, 18, and 19. Neither adenine nor adenosine, at the concentrations tested, gave any evidence of reversing the inhibition. The results of experiment I in Table 18 seemed to indicate that adenylic acid might have exerted some reversing effect but this was not confirmed by the results of experiment II. A third experiment with adenylic acid gave results similar to those of experiment II. Because of its relative insolubility, guanine was not tested for reversing activity; guanosine and guanylic acid were tested. As the results shown in Table 19 indicate, neither of these compounds, in the concentrations tested, appeared to reverse the activity of 2,8-dithio-6-oxypurine to any significant degree. The final pH of the tissue culture fluids containing purine compounds was very

Table 17

Inhibition of Lee-R Influenza Virus Growth by 2,8-Dithio-6-oxypurine: Attempted Reversal by Adenine and Adenosine

	Hemagglutinin titers of tissue culture fluids inoculated with 1.5×10^5 EID_{50} of Lee-R influenza virus per ml							
	Control A	Control B	Nutrient fluids containing 1.0 mg of 2,8-dithio-6-oxypurine per ml plus the following:					
			Adenine			Adenosine		
Number	Nutrient fluid	Nutrient fluid plus 1.0 mg of 2,8-dithio-6-oxypurine per ml	1.0 mg per ml	0.5 mg per ml	0.25 mg per ml	5.0 mg per ml	2.5 mg per ml	1.25 mg per ml
1	120	40	<20	<20	20	<20	<20	<20
2	120	40	<20	20	40	<20	30	20
3	160	40	<20	20	40	<20	30	40
4	160	60	<20	30	60	<20	30	40
5	160	60	<20	40	60	<20	40	40
6	320	80	20	60	60	<20	60	80
Geometric mean	162	51	11	26	44	10	29	32

1. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of fluids causing complete hemagglutination of chicken red blood cells.

2. As the lowest dilution titrated was the 1:20 dilution, all fluids which did not cause complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

3. After inoculation with the virus, the tissue cultures were incubated for 45 hours at 35°C on a reciprocating shaker.

Table 18

Inhibition of Lee-R Influenza Virus Growth by 2,8-Dithio-6-oxypurine: Attempted Reversal by Adenylic Acid

Experiment	Culture number	Hemagglutinin titers of tissue culture gluids inoculated with 1.5×10^5 EID_{50} of Lee-R influenza virus per ml: Control A: Nutrient fluid only	Control B: Nutrient fluid plus 1.0 mg of 2,8-dithio-6-oxypurine per ml	Nutrient fluids containing 1.0 mg of 2,8-dithio-6-oxypurine per ml plus: Adenylic Acid 5.0 mg per ml	Adenylic Acid 2.5 mg per ml	Adenylic Acid 1.25 mg per ml
I	1	120	40	40	40	60
	2	120	40	40	60	60
	3	160	40	40	60	60
	4	160	60	40	80	80
	5	160	60	60	80	120
	6	320	80	80	120	120
	Geometric mean	163	51	49	70	79

Experiment	Culture number	Control A	Control B	Adenylic Acid 1.25 mg per ml	Adenylic Acid 0.63 mg per ml	Adenylic Acid 0.32 mg per ml
II	1	120	60	60	40	60
	2	120	60	60	80	60
	3	160	60	60	80	80
	4	160	80	60	80	80
	5	160	80	80	80	80
	6	240	80	80	120	120
	Geometric mean	155	69	66	68	78

1. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of fluids causing complete hemagglutination of chicken red blood cells.

Table 18, continued

2. As the lowest dilution titrated was the 1:20 dilution, all fluids which did not cause complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

3. After inoculation with the virus, the tissue cultures were incubated for 45 hours at 35°C on a reciprocating shaker.

Table 19

Inhibition of Lee-R Influenza Virus Growth by 2,8-Dithio-6-oxypurine: Attempted Reversal by Guanosine and Guanylic Acid

	Hemagglutinin titers of tissue culture fluids inoculated with 1.5×10^5 EID_{50} of Lee-R influenza virus per ml							
	Control A	Control B	Nutrient fluids containing 1.0 mg of 2,8-dithio-6-oxypurine per ml plus:					
		Nutrient fluid plus 1.0 mg of 2,8-dithio-6-oxypurine per ml	Guanosine			Guanylic Acid		
Culture number	Nutrient fluid only		1.0 mg per ml	0.5 mg per ml	0.25 mg per ml	2.5 mg per ml	1.25 mg per ml	0.63 mg per ml
1	240	80	40	80	60	<20	20	80
2	320	80	80	80	80	<20	40	120
3	320	80	80	120	120	20	40	120
4	320	120	160	160	120	20	60	160
5	480	160	160	160	240	30	60	240
6	480	160	240	240	240	30	80	240
Geometric mean	349	108	108	130	126	18	46	148

1. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of fluids causing complete hemagglutatination of chicken red blood cells.

2. As the lowest dilution titrated was the 1:20 dilution, all fluids which did not cause complete hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the calculation of the geometric mean.

3. After inoculation with the virus, the tissue cultures were incubated for 45 hours at 35°C on a reciprocating shaker.

nearly the same as that of the control fluids after incubation, except in the case of tissue culture fluids containing adenosine. In all tissue culture fluids containing adenosine the final pH was the same as the initial pH. The inhibitory activity of 2,8-dithio-6-oxypurine was not reversed by natural purine derivatives under the conditions of these experiments.

Mouse Tests. The maximum tolerated intraperitoneal dose of 2,8-dithio-6-oxypurine in mice was 57 mg per kilogram. This was the dose of the compound which when administered twice daily for three days was lethal to no more than one of three mice. This dose of the compound was tested for inhibitory effect upon the development of Lee influenza virus in the mouse lung. Seven mice, weighing 16-19 grams each, were given one injection of the maximum tolerated dose and were then inoculated with the virus. Eight control mice received injections of the diluent before virus inoculation. Injections were continued twice daily over a three day period. At the end of this time the lungs were removed, ground in a mortar, diluted with saline, and titrated for hemagglutinin. Details of the procedure have been given previously. The lung material from mice receiving 2,8-dithio-6-oxypurine had a titer of 80; that of control mice had a titer of 1:120 on a weight/volume basis. The difference between

the titers of the two pools of lung material is not significant.

DISCUSSION

Results obtained in the chick embryo experiments with 1,2-cyclohexanedione indicated that the compound caused a significant inhibition of Lee influenza virus development in the chorioallantois when the compound was administered by the allantoic route but not when it was administered by the yolk-sac route. At a concentration approximating that present in the allantoic fluid of treated eggs, the compound caused a significant decrease in the infectivity titer of infected allantoic fluid mixed with phosphate-glycine buffer during a three hour incubation period in vitro. The concentration of virus, in terms of EID_{50} per ml, which was employed in vitro was about a million times greater than the concentration of virus in the allantoic fluid of eggs inoculated with 50 or 100 EID_{50}. Results of the in vitro test for virucidal activity of the compound indicated that more than 99 per cent of the virus infectivity was destroyed within three hours when the virus was exposed to the compound in a mixture containing phosphate-glycine buffer. If 99 per cent of the infectivity of an inoculum of 10 to 50 EID_{50} per ml were destroyed in vivo by the compound, this could well explain the observed inhibition. Conditions in the allantoic fluid of the inoculated egg and in the phosphate-glycine buffer are not strictly comparable; it is

entirely possible that the allantoic fluid contains substances which would protect the virus from the effects of the compound better than would the constituents of the buffer. The results of the chick embryo experiments and the test for virucidal activity do suggest, however, that the compound may inhibit virus development in the chick embryo by inactivating the infective property of the virus.

From the results of adsorption experiment with 1,2-cyclohexanedione, it appears unlikely that the compound interfered with adsorption of the virus by the tissue, at least not within a short period of exposure. The results obtained in tissue culture experiments indicated that the tissue was changed, in some way that prevented normal virus development, when the period of exposure to the compound was prolonged. The physical appearance of the tissues after a 23 hour exposure to the minimum inhibitory concentration of the compound suggested rather extensive damage to the tissue, although the exact nature of the change was not determined. How much an effect of the compound upon the tissue contributed to the inhibition observed in the chick embryo is uncertain; the fact that the embryos survived the injection of the inhibitory dose of the compound tends to exclude the possibility that the chorioallantoic tissue was severely damaged or dead.

Results of the toxicity test in mice indicate that the compound has a remarkably low acute toxicity. The peak concentrations of the compound in the mouse tissue, at least momentarily, may well have been as high as the concentration obtained in allantoic fluid; despite this, the compound did not appear to have inhibited the development of the virus in the mouse lung.

The ability of the compound to inhibit virus development in the chorioallantois when administered by the allantoic route, its failure to inhibit when administered by the yolk sac route, and the observed virucidal effect of the compound in vitro suggest that the inhibition in the chick embryo may be largely accounted for by the direct effect of the compound upon virus infectivity. The inability of the chorioallantoic membrane tissue to support virus development after exposure of the tissue to the compound in tissue culture suggests that under tissue culture conditions the effect of the compound upon the tissue is at least as important as a virucidal effect of the compound. In summary, 1,2-cyclohexanedione appears to inhibit virus development both by an effect upon the virus and by an effect upon the tissue, and the relative importance of the two effects in any given case probably depends upon the conditions under consideration.

When administered by the allantoic route,

2,8-dithio-6-oxypurine caused a significant inhibition of Lee influenza virus development in the chorioallantois; no significant inhibition was observed when the compound was administered by the yolk-sac route. These results suggested that the observed inhibition might be attributable to a direct effect of the compound upon the virus or upon the membrane tissue. In vitro, at a concentration approximating that present in the allantoic fluid of treated eggs, the compound appeared to have no effect upon the ability of the tissue to adsorb virus hemagglutinin, nor did it appear to cause a rapid loss of infectivity in virus infected allantoic fluids. When the tissue was exposed to the compound in vitro at 35°C for 26 hours the loss of infectivity was significantly greater than the loss of infectivity of the control virus. The effect of the compound upon the tissue, in decreasing the ability of the tissue to support virus development, was also delayed. A 12 hour exposure to the minimum inhibitory concentration of the compound apparently caused no marked decrease in the ability of the tissue to support virus development; when the tissue was exposed to the compound for 26 hours the virus hemagglutinin produced was much less than that produced in control cultures not exposed to the compound. Although the difference was not statistically significant, the

results do suggest that the ability of the tissue to support virus development was decreased by prolonged exposure of the tissue to the compound.

In tissue culture, the concentration of virus inoculum employed, within a range of 1.5×10^6 to 1.5×10^3 EID_{50} per ml, did not appear to have had an effect upon the degree of inhibition produced by the compound. A concentration of the compound inhibitory with a dilute virus inoculum appeared to be just as effective when a concentrated virus inoculum was used. With the information available it is not possible to advance an accurate explanation for this observation; however, it has been a common observation, with both virus inhibitors and bacteriostatic agents, that the degree of inhibition or bacteriostasis was markedly influenced by the concentration of inoculum. The lack of such an effect in the case of 2,8-dithio-6-oxypurine would appear to be consistent with idea that the activity of the compound was directed primarily against the host cell rather than against the virus.

The inhibitory activity of 2,8-dithio-6-oxypurine was not reversed in tissue culture by adenine, adenosine, adenylic acid, guanosine, or guanylic acid under the test conditions. The results do not absolutely rule out the possibility that 2,8-dithio-6-oxypurine acts as an

antagonist for nucleic acid constituents, but they lend no support to this idea.

The compound was not well tolerated by mice; the maximum tolerated intraperitoneal dose of the compound, 57 mg per kilogram, failed to inhibit the development of Lee influenza virus in the mouse lung. The great majority of compounds found to be inhibitory for the development of virus in the chick embryo and in tissue culture have shown no inhibitory activity in mice or other laboratory animals.

There are a number of possible explanations for this. Mice are less sensitive to virus than chick embryos; massive doses of virus are required to infect mice and minimal doses of virus are required to infect chick embryos. The kidneys of the mouse can remove substances from the blood stream, and quite possibly the mouse liver inactivates foreign substances. The chick embryo has no way to completely eliminate foreign substances unless the substances can be completely degraded to volatile gases and water. For this reason a more uniform concentration of compound can be maintained in the chick embryo than can be maintained in the mouse. It is also possible that the embryonic cells of the chorioallantoic membrane are more permeable to certain substances than are the cells of the mouse. These differences between the chick embryo

and the mouse probably account for the failure of compounds, known to be inhibitory in the chick embryo, to inhibit virus development in the mouse.

The inhibitory effect of 2,8-dithio-6-oxypurine can probably be explained in part by its slow virucidal effect and in part by its ability to render the tissue incapable of supporting virus development.

SUMMARY

1. The inhibitory activity of 1,2-cyclohexanedione and 2,8-dithio-6-oxypurine for Lee influenza virus was investigated.

2. A dose of 3.5 mg of 1,2-cyclohexanedione per egg was found to inhibit the development of Lee influenza virus in the chorioallantois of the chick embryo when administered by the allantoic route but not when administered by the yolk-sac route.

3. At a concentration of 0.8 mg per ml, 1,2-cyclohexanedione exerted a virucidal effect upon Lee influenza virus in vitro.

4. A concentration of 0.4 mg of 1,2-cyclohexanedione per ml was found to inhibit the development of Lee-R influenza virus in tissue culture when an inoculum of 2.0×10^6 EID_{50} per ml was employed.

5. The maximum tolerated intraperitoneal dose of 1,2-cyclohexanedione in mice was found to be 1.2 gm per kilogram. This dose of the compound, when administered twice daily for a three days failed to inhibit the development of Lee influenza virus in the mouse lung.

6. In tissue culture, exposure of the tissue to the minimum inhibitory concentration of 1,2-cyclohexanedione for 23 hours markedly decreased the ability of the tissue to support virus development.

7. In vitro, a concentration of 0.8 mg of 1,2-cyclohexanedione per ml did not interfere with the adsorption of virus hemagglutinin by chorioallantoic membrane tissue.

8. A dose of 12.5 mg of 2,8-dithio-6-oxypurine per egg was found to inhibit the development of Lee influenza virus in the chorioallantois when the compound was administered by the allantoic route but not when it was administered by the yolk-sac route.

9. At a concentration of 2.5 mg per ml in vitro, 2,8-dithio-6-oxypurine had no virucidal effect during a one hour exposure; when the exposure period was extended to 26 hours this concentration of the compound was virucidal.

10. In tissue culture a concentration of 1.0 mg of 2,8-dithio-6-oxypurine per ml was inhibitory to the development of Lee-R influenza virus when the virus inoculum was between 1.5×10^6 and $1.5 \times 10^3 EID_{50}$ per ml.

11. The inhibitory activity of 2,8-dithio-6-oxypurine for Lee-R virus in tissue culture was not reversed by

adenine, adenosine, adenylic acid, guanosine, or guanylic acid.

12. The maximum tolerated intraperitoneal dose of 2,8-dithio-6-oxypurine in mice was 57 mg per kilogram. This dose of the compound, when administered twice daily for three days, failed to inhibit the development of Lee influenza virus in the mouse lung.

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