AN ABSTRACT OF THE THESIS OF

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Title INHIBITION OF THE DEVELOPMENT OF LEE INFLUENZA VIRUS BY PUROMYCIN

Abstract approved (Major Professor)

The development of the Lee strain of type B influenza virus was shown to be inhibited by puromycin in tissue culture of the chick embryo chorioallantoic membrane. The compound is an antibiotic of unique structure which has been reported to possess a broad range of biological activity against unicellular and multicellular organisms. Investigations were carried out to examine the extent of inhibition induced by puromycin and to discover possible explanations for its antiviral activity.

Puromycin caused complete inhibition of Lee influenza virus development in tissue cultures at a concentration of 8.0 micrograms per ml, marked inhibition at 4.0 micrograms per ml, and significant inhibition at 2.0 micrograms per ml. The compound inhibited the formation of infectious virus in tissue cultures, as well as virus hemagglutinin.

At a concentration of 4.0 micrograms per ml it caused inhibition of cell growth, but did not block all metabolic activity. In vitro experiments indicated that the compound was not virucidal and did not influence the end point of the hemagglutination reaction. At a concentration of 4.0 micrograms per ml it did not interfere with the adsorption of virus to the chorioallantoic membrane in vitro. The combination of L-canavanine plus puromycin showed a synergistic effect on the inhibition of virus development in tissue cultures. Combinations of ribonuclease plus puromycin, benzimidazole plus puromycin and m-tyrosine plus puromycin showed additive effects on virus inhibition in tissue cultures.

None of a group of purines, pyrimidines or their nucleosides or nucleotides was able to reverse the inhibition caused by puromycin in tissue cultures. A dose of 1.0 mg of the compound administered twice daily for three days did not interfere with the development of influenza virus in mouse lung.

The amino nucleoside of puromycin not only failed to inhibit the virus but actually gave a significant enhancement of virus yield in tissue culture fluids at a concentration of 0.20 mg/ml. Concentrations of 0.40 mg and 0.10 mg per ml caused a significant increase of the virus

concentration in the culture fluid. This proved that the amino acid moiety of the molecule is necessary for the inhibition of influenza virus.

It appears from the results obtained that the inhibition of virus development induced by puromycin may be due to its interference with normal synthesis or utilization of metabolites essential for the multiplication of the host cell and of the virus, possibly the synthesis of protein.

INHIBITION OF THE DEVELOPMENT OF LEE INFLUENZA VIRUS BY PUROMYCIN

by

JOE NELSON HOBBS, JR.

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INHIBITION OF THE DEVELOPMENT OF LEE INFLUENZA VIRUS BY PUROMYCIN

INTRODUCTION

An investigation of the effects of chemical compounds, structural analogues of known metabolites, on the development of viruses is justified both by the need of chemotherapeutic agents and by the possibility that such an investigation may provide some information on the relationship between the virus and host cell.

A compound displaying antiviral activity might interfere with virus development at any of the three phases of virus infection. The compound may prevent the initial adsorption of the virus particle to the host cell. This interference might be due to the altering of surface structures of virus particles essential for their reaction or combination with the host cells, or to destruction or altering of cell receptor sites necessary for the adsorption of virus particles to the host cell. The inhibitor may interfere with substances essential to the formation of new virus material, or it may become incorporated into newly formed virus particles, and once incorporated, the cell may produce abnormal virus particles that are unable to further multiply and infect other cells. The chemical compound may exert its effect on the final phase of the infection cycle by interfering with the release of newly

formed virus particles.

The probability of finding an active antiviral agent that would selectively inhibit the development of new virus material in the intact experimental animal is indeed slight at the present time. A large number of chemical compounds have been tested for antiviral activity, but only a few have demonstrated a significant inhibitory effect on virus development without toxic effects on the host cells.

In preliminary studies of a number of organic compounds tested for their possible inhibitory effect upon the development of influenza virus in tissue culture, puromycin, a specific inhibitor of protein synthesis, was found to be highly inhibitory.

Evidence that a sufficient concentration of puromycin could be obtained in the living tissue of mice and guinea pigs to completely cure or suppress trypanosome infection, the reported cure of human sleeping sickness due to trypanosomes, and the low concentration found to inhibit the development of influenza virus in tissue culture prompted further study with this compound.

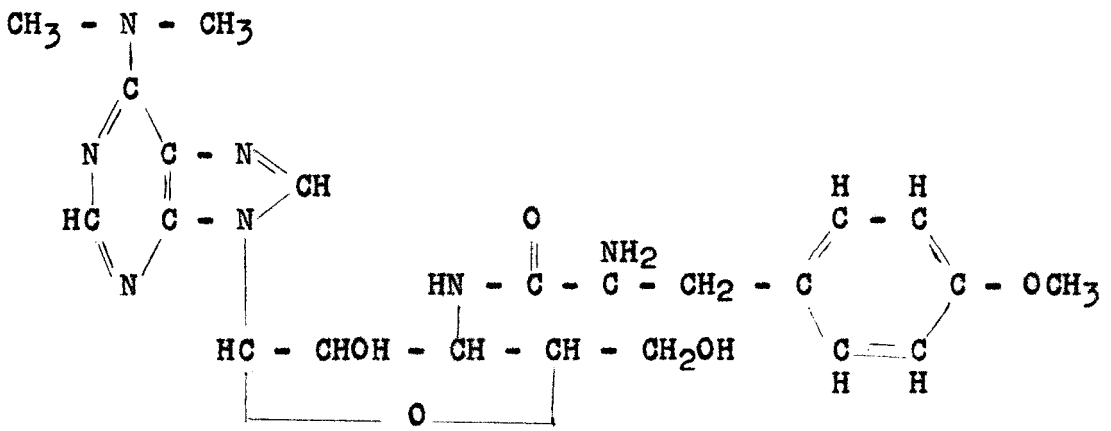
Bowen's work (3, pp. 1-154) showing the synergistic effect of combinations of several amino acid analogues, ribonuclease, and benzimidazole, in inhibiting the growth of influenza virus suggested that the mechanism here was associated with synthesis of virus protein. This led us

to look for other possible inhibitors of protein synthesis, and puromycin thus appeared worth exploring. Preliminary experiments were promising and justified the studies reported in this thesis.

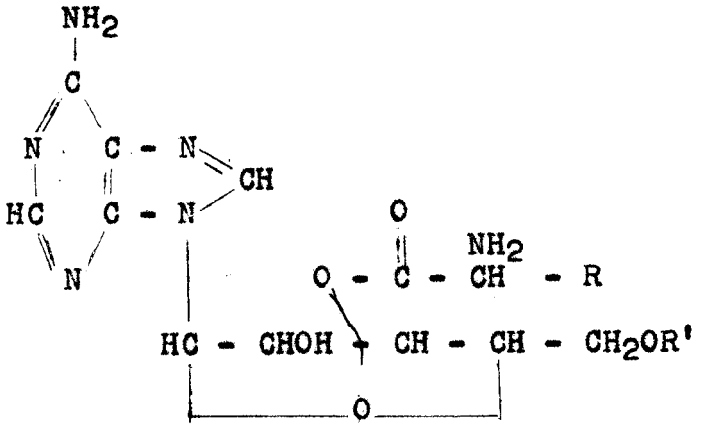
HISTORICAL REVIEW

Puromycin is an antibiotic of unique structure and biological activity. It is produced during fermentation by the actinomycete Streptomyces albo-niger as described by Porter et al. (23, pp. 409-410) and Hewitt et al. (12, pp. 254-264).

The structure of puromycin bears a close resemblance to that of the amino acid-bearing end of transfer ribonucleic acid, whose similarity can be seen in the following formulae.

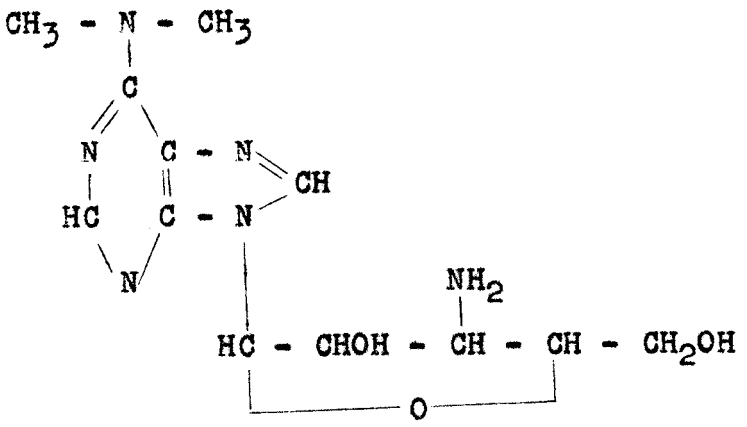


Puromycin



Transfer RNA amino acid

R represents the remainder of the amino acid residue, R' represents the remainder of the RNA polymer.



Puromycin amino nucleoside

It is seen that puromycin differs in structure from transfer ribonucleic acid at the bond between the amino acid and the pentose, and in the methyl substituents on the 6-amino nitrogen of the purine ring.

A number of investigators have shown that puromycin

possesses a broad range of biological activity, being effective against parasitic worms, protozoa, bacteria, and mammalian cells. Puromycin displayed a slight bacterostatic effect when tested against Gram positive and Gram negative bacteria *in vitro* and *in vivo* Porter *et al.* (23, pp. 409-410). Collier and Huskinson (5, pp. 146-159) found the antibiotic to be a competitive antagonist for guanine in the growth of a purine requiring strain of *Escherichia coli*. Gale and Folkes (9, pp. 507-517) observed that puromycin inhibited the incorporation of glycine-C^{14} into disrupted *Staphylococcus aureus* cells.

The antibiotic has exhibited the most interesting activity against protozoa and multicellular organism. Hewitt and associates (12, pp. 259-264) observed that puromycin readily suppressed *Trypanosoma equiperdum* infection in mice and rabbits, and was partially effective against *T. cruzi*. Trincoa *et al.* (32, pp. 505-507) observed that multiple doses of puromycin administered daily for a period of seven to 10 days prevented the development of *T. gambiense* and *T. rhosiense*. Tobie (31, pp. 853-859) in an extensive study of trypanosome infection in mice, observed that puromycin had a strong suppressive effect against five of six species if the drug was administered approximately four hours after inoculation or at the height of infection. Clinical evidence reported by

Trincao *et al.* (33, pp. 13-17; 34, pp. 784-785) suggested that the oral administration of puromycin daily for a period of seven to 10 days effectively cured human sleeping sickness due to *T. gambiense*. The work of Trincao and associates was confirmed by Heuls and co-workers (11, pp. 108-113) in the treatment of patients in the blood-lymph stage of trypanosomiasis. Similarly, Young *et al.* (37, pp. 808-812) observed that multiple doses of the drug administered daily to patients who were heavily infected or were exposed to large numbers of intestinal protozoa had a suppressive as well as curative effect against the protozoa. Further, once the infection was cleared, the continuous administration of the drug three times weekly for six weeks prevented any new infection.

The growth inhibitory effect of puromycin has also been demonstrated against other genera of protozoa. Taylor *et al.* (29, p. 4497) showed that experimentally induced amoebiasis in guinea pigs was effectively controlled by puromycin. Thompson and co-workers (30, pp. 337-350) reported that the drug had a potent effect against intestinal amoebiasis in rats, and amoebic dysentery in dogs.

The broad range of biological activity displayed by puromycin has also been shown by its effect upon multicellular organisms. Donckoster and Habibe (77, pp. 44-49)

in a clinical test against Hymenolepis nana infection in humans reported the drug had only a slight effect in reducing the infection.

In addition to the growth inhibition displayed against protozoa and multicellular organisms, puromycin has also been found to have a significant effect on a variety of experimental tumors. Troy et al. (35, pp. 186-191) observed that puromycin inhibited the growth of mammary adenocarcinoma CH_3 in mice. A range of effects from destructive or inhibitory to mild or no activity was obtained, depending upon the type of tumor and dosage. General toxicity accompanied carcinostasis, since peak activity was only apparent at maximum tolerated doses.

Owing to its chemical structure it was natural to attribute the growth inhibitory effect of puromycin to its possible role as inhibitor of purines, nucleic acid, and protein synthesis in biological systems. Yarmolinsky and de la Haba (36, pp. 1721-1729) were the first to recognize that puromycin inhibited the incorporation of L-leucine-C-14 into protein in a cell-free preparation from rat liver. It was suggested that this inhibition induced by puromycin prevented the transfer of amino acid from soluble ribonucleic acid to microsomal protein.

There is considerable evidence which suggests that puromycin is a specific inhibitor of protein synthesis.

Creaser (6, pp. 288-295) reported that a minimum bacteriostatic concentration of the antibiotic was sufficient to completely block the induced synthesis of B-galactosidase in Staphylococcus aureus. Takeda et al. (27, pp. 169-177) studying the growth inhibition induced by puromycin in Pseudomonas fluorescens suggested that the antibiotic might be selective in blocking protein synthesis. Gorski et al. (10, pp. 508-511) reported that intraperitoneal injection of puromycin hourly for four hours into living rats suppressed protein synthesis in vivo in uteri, liver, heart, kidney, and thymus as measured by the incorporation of injected radioactive glycine. Also under the same conditions ribonucleic acid synthesis was not inhibited in any of the rat tissues tested. Mueller et al. (20, pp. 164-169) in a similar test with ovariotomized rats suggested that puromycin is a highly effective agent for blocking protein synthesis in vivo in the uteri. Here again the antibiotic had no effect on ribonucleic acid synthesis. Levintow and co-workers (17, pp. 220-229) reported that upon the addition of puromycin to a tissue culture infected with polio virus both maturation and ribonucleic acid synthesis were prevented if the antibiotic was added early in the latent period, but that limited ribonucleic acid synthesis occurred if it was added near the end of the latent period.

Recent experimental data reported by several investigators augment the concept first suggested by Yarmolinsky and de la Haba. Nathans and Lipman (21, pp. 497-504) using E. Coli ribosomes as a template suggested that puromycin prohibited the final condensation of activated amino acids to peptides. Nathans et al. (22, pp. 127-133) in additional work suggested that puromycin interfered with protein synthesis by splitting off peptides from the ribosomal ribonucleic acid template by substituting itself for cellular amino-acyl soluble ribonucleic acid. As a further indication of interference with protein synthesis Rabinovitz and Fisher (24, pp. 477-481) observed that puromycin prevented the transfer of amino acid into ribosomal protein in Ehrlich Ascites tumor cells and rabbit reticulocytes in vitro. Similar results were observed by Morris and Schweet (19, pp. 415-416).

EXPERIMENTAL MATERIALS AND METHODS

The Lee strain of type B influenza virus designated as Lee-R, was used in tissue culture studies. This virus was obtained from Dr. F. L. Horsfall.

The Lee strain of influenza virus used in mouse experiments had a long history of egg passages and was originally obtained from the American Type Culture Collection. The stocks of this virus had been adapted by a series of passages in the mouse lung.

Stock virus was maintained as infective allantoic fluid taken from embryonated eggs and stored at -60°C. Stock virus preparations were made by diluting old stock virus 1:1000 in chilled phosphate buffered saline and inoculating 0.1 ml volumes of this dilution into the allantoic sac of 6-8 ten-day-old chick embryos. These eggs were incubated for 40 hours at 35°C. After such time, the eggs were chilled at -20°C. for one hour, and the allantoic fluids were aseptically harvested, pooled, and centrifuged to remove aggregates from the fluid. The pooled infective fluid was diluted 1:1000 in chilled phosphate buffered saline and 0.1 ml volume of this dilution was injected into the allantoic cavities of 30-40 ten-day-old chick embryos. The eggs were incubated for 44 hours at 35°C. The allantoic fluids were aseptically removed

from these eggs, pooled, and centrifuged to remove blood cells and other debris. The infective fluid was distributed in 1.0 ml quantities in sterile cotton plugged serological tubes fitted with sterile rubber stoppers. The tubes were rapid-frozen in a solid CO_2 ethanol mixture, and then stored at -60°C. After freezing, two tubes were thawed, pooled and the virus titer determined by infectivity titration.

Eggs

Fertile chicken eggs, chiefly New Hampshire Reds, were obtained from the Mid-Valley Hatchery. Eggs were incubated from nine to 11 days in a moist atmosphere at 38°C. After inoculation, the eggs were incubated at 35°C.

Puromycin

Puromycin was supplied by Nutritional Biochemicals Corporation.

Chemicals

Reagent grade chemicals were used in preparation of all solutions. Deionized water used in preparation of solutions and washing glassware had a conductivity of 0.3ppm or less as sodium chloride.

Alsever's Solution

This solution used for the collection and storage of red blood cells had the following composition:

Glucose	20.50 grams
Sodium chloride	4.20 grams
Citric acid	0.55 grams
Sodium citrate	8.00 grams
Deionized water	to 1000 ml.

The solution was dispensed in 100 ml quantities in wide-mouth jars and autoclaved for 15 minutes at 121°C. After sterilization the solution was stored at 10°C.

Physiological Saline Solution

Physiological saline used as diluent in the hemagglutinin titrations was 0.85 per cent sodium chloride. This solution was not sterilized. Buffered saline used as diluent in preparation of virus inocula for chick embryos had the following composition:

NaCl	8.500 grams
Na_2HPO_4 (anhydrous)	0.796 grams
KH_2PO_4	0.170 grams
Deionized water to make	1000 ml.

The buffered saline solution was distributed in Pyrex bottles and sterilized by autoclaving for 15 minutes at 121°C. The pH was 7.25.

Hanks Balanced Saline Solution

This solution was the nutrient fluid used in tissue cultures. The nutrient solution was prepared as a 10X concentration stock without glucose and stored in a glass stoppered bottle at 10°C. Prior to use the stock solution was diluted ten-fold and 1.0 gram per liter of glucose was added. The diluted solution had the following composition:

NaCl	8.000 grams
KCl	0.400 grams
$MgSO_4.7H_2O$	0.200 grams
$CaCl_2.2H_2O$	0.186 grams
NA_2HPO_4, anh.	0.060 grams
KH_2PO_4	0.060 grams
Glucose	1.000 grams
Phenol red	0.020 grams
Deionized water to make	1000 ml.

The solution was dispensed in 50 ml quantities into Pyrex bottles with screw caps and autoclaved for 10 minutes at 121°C. The pH was 6.7 after sterilization.

Phosphate-Glycine Buffer

This buffer solution, used as diluent for *in vitro* studies for the direct effect of puromycin on virus infectivity, was prepared by mixing equal volumes of solution A and solution B just before use. The solution had the following composition:

Solution A:

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

Solution B:

Na_2HPO_4, anh. 4.000 grams
Deionized water to 100 ml.

The two solutions were sterilized separately by filtration through an ultrafine porosity siniter glass filter and distributed in sterile Pyrex screw cap tubes. PH of the final solution was 8.00.

Infectivity Titration

The infectivity titer was determined by preparing serial ten-fold dilutions of infective fluid in chilled phosphate buffered saline. 0.1 ml volume of these dilutions were inoculated into the allantoic cavities of groups of six 9-11 day-old chick embryos and incubated for 40 hours in a moist atmosphere at 35°C. After incubation, the eggs were chilled for one hour at -20°C., and a 0.5 ml volume of allantoic fluid was removed from each egg. This quantity of allantoic fluid was diluted with an equal volume of physiological saline and 1.0 ml volume of 1.0 per cent red blood cell suspension was added to give a final dilution of 1:4. Allantoic fluid from eggs showing hemagglutination at this dilution was considered infected. The EID_{50} infectivity titer was calculated according to the method of Reed and Muench (25, pp. 493-497). The EID_{50} represents the dose of virus infected fluid causing infection in 50 per cent of the embryos.

Hemagglutinin Titration

Fresh blood from four or five chickens was collected weekly in sterile Alsever's solution. The chicken red blood cells were filtered through coarse cheesecloth and washed with physiological saline by three centrifugations at 1500 r.p.m. for seven minutes. The washed cells were packed in a graduated centrifuge tube by a ten minute centrifugation at 1500 r.p.m. A stock 10 per cent suspension of the washed cells was prepared in physiological saline. The stock was stored at $4^{o}C$. The red cell suspensions used in hemagglutinin titrations were diluted with physiological saline to the appropriate concentration immediately before use.

Serial 2-fold dilutions of the infected fluids were prepared in physiological saline, beginning with a 1:5 dilution and progression through a 1:320 dilution. Similarly, serial dilutions were prepared, beginning with a 1:15 dilution and progressing through a 1:480 dilution. The fluids were mixed by alternately withdrawing the fluid in a 1.0 ml pipette and expressing it back and forth into serological tube eight times. After mixing, 1.0 ml of the dilution was withdrawn and transferred to the next tube in the series, mixed, and etc. 1.0 ml of the mixture was discarded from the final dilution of each series. The final volume remaining in each tube after mixing and

transfer was 1.0 ml. A 1.0 ml volume of 0.5 per cent red cell suspension was added to each tube and the tubes were thoroughly shaken. Readings were made after the cells were allowed to settle for 30 minutes at room temperature. Hemagglutinin titers were determined using the pattern method of Salk (26, pp. 87-98). Titers were expressed as the reciprocal of the highest virus dilution showing complete hemagglutination of the red blood cells.

Tissue Culture Techniques

Tissue culture techniques used were similar to those of Tamm, Folkers, and Horsfall (28, pp. 559-567). All glassware, rubber stoppers, and caps were boiled in a Labtone solution and were rinsed 10 times in taped water and five times in deionized water. Glassware was sterilized either by dry heat at 180°C. for two hours or by autoclaving at 121°C. for 25 minutes. Instruments were washed in a hot Labtone solution, rinsed thoroughly in tap water and deionized water and sterilized at 110°C. overnight.

The puromycin solution used in tissue cultures was prepared by diluting one tube of stock solution of puromycin in nutrient solution. The pH of such solutions was adjusted to 7.5 by the addition of sterile 1.4 per cent $NaHCO_3$ solution or sterile 0.03N HCl. All tissue culture solutions received sufficient penicillin G and streptomycin

to give a final concentration of 10 units per ml of penicillin G and 40 micrograms per ml of streptomycin. Sterile Pyrex culture tubes were fitted with sterile #4 rubber stoppers and the nutrient solution, Hanks' BSS, with or without puromycin was added in volumes of 2.0 ml per tube.

Chorioallantoic membrane tissue was aseptically removed from 9-11 day-old eggs in the following manner: The portion of shell over the air sac was disinfected with strong tincture of iodine and carefully removed with sterile forceps. The air sac membrane was stripped away with sterile forceps and the egg contents were tipped into a sterile petri dish. The chorioallantoic membrane was gently freed from other structures with sterile forceps. The membranes were aseptically washed by agitation in three changes of sterile nutrient solution, and cut into 2 cm x 2 cm squares. The tissue fragments from each membrane were pooled and thoroughly mixed to assure random distribution. The tissue pieces were distributed one per tube in the culture tubes containing nutrient solution. The cultures were inoculated with 0.1 ml volume of an appropriate dilution of stock virus in nutrient solution, and then, the stoppers were sealed on with cellophane tape. The culture tubes were incubated for 44-46 hours and the culture fluids were titrated for virus

hemagglutinin.

The tissue culture techniques designed to determine the toxicity of puromycin for the growth of host cells were similar to those of Lum and Smith (18, pp. 281-293). Chorioallantoic membranes were harvested from 9-11 day-old chick embryos in the manner described above, washed three times in sterile nutrient solution, and finely minced with sterile scalpels. The fragments were washed in four changes of nutrient solution by centrifugation. After the final washing the supernatant was replaced with sterile nutrient solution to give a dilution of three parts tissue fragments to two parts nutrient solution. One tenth ml volumes of this tissue suspension was distributed into sterile Pyrex roller tubes fitted with #0 rubber stoppers. The tubes were rotated and gently tapped to distribute the fragments as uniformly as possible over the lower third of the tubes. The cultures were incubated in a horizontal position for two hours at 35°C. to allow adherence of the fragments to the glass surfaces. Nutrient solution with or without puromycin was distributed in 0.9 ml volumes into the tubes. The cultures were then placed in a roller drum operating at 2 r.p.m. and incubated for 44 to 46 hours at 35°C. After incubation the fragments were examined microscopically for growth of new cells around the fragments.

Sterility Tests

All preparations were tested for sterility before and after experiments by inoculation of a small amount of each preparation into a tube of thioglycollate broth. These sterility controls were incubated for one week at 35°C. Tubes showing no growth after this period were considered sterile.

Statistical Methods

Differences between means of hemagglutinin titers of groups of tissue culture fluids were tested for significance using Student's "t" test (8, pp. 153-157). This method of analysis is appropriate for samples containing less than 30 items.

The means shown in all tables are geometric means of the titers.

As dilutions below 1:10 were not made in the experiments, the true titers of fluids which did not cause complete hemagglutination at this dilution were not determined. In some fluids there may have been no hemagglutinin; in other fluids there might have been sufficient hemagglutinin present to cause complete hemagglutination at the 1:5 or 1:2 dilution. Fluids which did not show complete hemagglutination at the 1:10 dilution, but did show partial hemagglutination were arbitrarily assigned a titer of

10 for the purpose of calculating geometric means. Fluids showing negligible hemagglutination were arbitrarily assigned a titer of 5 for the purpose of calculating geometric means. The following example shows a representative statistical comparison of two means.

Table I. Statistical comparison of virus present in tissue cultures containing 2.0 micrograms per ml of puromycin with cultures containing no puromycin. (A sample statistical analysis)

Culture numbers	Hemagglutinin titer	$\log_{10}$ of titer	Deviation from mean	Deviation squared
	Group I (treated)			
1	40	1.60206	0.25086	0.06293
2	20	1.30103	0.05017	0.00252
3	20	1.30103	0.05017	0.00252
4	20	1.30103	0.05017	0.00252
5	20	1.30103	0.05017	0.00252
6	20	1.30103	0.05017	0.00252
	Sum	8.10721		
	Group II (untreated)			
1	160	2.20412	0.45155	0.20389
2	80	1.90309	0.15052	0.02266
3	40	1.60206	0.15051	0.02265
4	80	1.90309	0.15052	0.02266
5	40	1.60206	0.15051	0.02265
6	20	1.30103	0.45154	0.20388
	Sum	10.51545		

Table I. (cont'd)

Mean of Group I = 1.35120
Mean of Group II = 1.75257
Sum of squared deviations, Group I = 0.07553
Sum of squared deviations, Group II = 0.49839
Combined sum of squares = 0.57392
Sum of degrees of freedom (6-1) + (6-1) = 10
Estimated standard deviation =

$$\pm \frac{0.57392}{10} = \pm 0.13829$$

Standard error of difference between means =

$$\pm \frac{0.23956^2}{6} + \frac{0.23956^2}{6} = \pm 0.13829$$

Difference between means = 0.40137

$$\frac{\text{Difference between means}}{\text{Std. Error of Difference}} = \text{"t"} = 2.902$$

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

2. The value of "t" calculated from the data is 2.902. Fisher's table of "t" lists a value of 2.228 for 10 degrees of freedom at the 0.05 probability level, indicating that the difference between the means of Groups I and Groups II are significant at this level.

EXPERIMENTAL RESULTS

Minimum Inhibitory Concentration of Puromycin in Tissue Culture

The inhibitory activity of puromycin for Lee influenza virus was noted in preliminary studies in tissue cultures and was further investigated in this system. The minimum inhibitory concentration of puromycin was determined by preparing groups of tissue cultures containing varying concentrations of puromycin in Hanks BSS and inoculating them with an appropriate dilution of virus. The culture tubes were incubated for 44 to 46 hours at 35°C. and the culture fluids were titrated for virus hemagglutinin. The results of five experiments are shown in Table II. Puromycin was found to be completely inhibitory for the development of Lee-R influenza virus at a minimum concentration of 8.0 micrograms per ml, while a concentration of 4.0 micrograms per ml caused significant, but not complete, inhibition. The mean virus hemagglutinin titer of culture fluid containing puromycin at concentrations of 8.0 and 4.0 micrograms per ml was reduced to 1.9 per cent and 8.7 per cent of the control value, respectively. The mean titer of culture fluid containing puromycin at a concentration of 2.0 micrograms per ml was reduced to 46.7 per cent of the control value.

Table II. Relation of concentrations of puromycin to the degree of inhibition of Lee-R influenza virus in tissue cultures.

Experiment	EID_{50} of virus per ml in tissue cultures	Culture number	Virus hemagglutinin titers of tissue culture fluids containing the following concentrations of puromycin			
			8.0*	4.0*	2.0*	Control
I	4.7×10^6	1	10	10	40	160
		2	10	40	20	80
		3	10	20	20	40
		4	10	10	20	80
		5	10	10	20	40
		6	10	10	20	20
		Geo. Mean	5	5	22	57
II	4.7×10^6	1	10	10	40	20
		2	10	10	20	60
		3	10	10	10	40
		4	10	10	20	80
		5	10	10	10	40
		6	10	10	10	80
		Geo. Mean	5	5	16	48
III	4.7×10^6	1	10	10	20	80
		2	10	10	30	60
		3	10	10	60	60
		4	10	40	80	60
		5	10	30	80	60
		6	10	20	80	60
		Geo. Mean	5	15	65	63
IV	4.7×10^6	1	10	10	20	80
		2	10	20	20	80
		3	10	20	20	80
		4	10	10	10	80
		5	10	10	20	80
		6	10	10	20	80
		Geo. Mean	5	10	22	80

Table II. (cont'd)

Experiment	EID_{50} of virus per ml in tissue cultures	Culture number	Virus hemagglutinin titers of tissue culture fluids containing the following concentrations of puromycin			
			8.0*	4.0*	2.0*	Control
V	4.7×10^6	1	10	10	60	80
		2	10	10	80	120
		3	10	10	80	80
		4	10	10	60	120
		5	10	10	40	120
		6	10	20	60	120
		Geo. Mean	5	5	52	105

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 1:10, all which did not show complete hemagglutination, but did show partial hemagglutination at this dilution were arbitrarily assigned a titer of 10 for the purpose of calculating the geometric means. All fluids showing negligible hemagglutination at the 1:10 dilution were arbitrarily assigned a titer of 5 for the purpose of calculating the geometric means.

* Micrograms per ml.

It was desirable to determine whether puromycin at minimum inhibitory concentration as measured by hemagglutination would also cause a significant reduction of infectious virus in tissue culture. Groups of tissue cultures containing inhibitory concentrations of puromycin in Hanks BSS were prepared in the manner described above. All cultures received a virus inoculum sufficient to give

a final virus concentration of 4.7×10^6 EID_{50} per ml of tissue fluid. After incubation for 44 to 46 hours at 35°C., the culture fluids were pooled, and centrifuged to remove aggregates and other debris. The pooled infected culture fluids were titrated for virus infectivity and hemagglutinin. Table III shows the results of this experiment. Infected culture fluid containing 4.0 micrograms of puromycin per ml showed a significant reduction in the infectivity titer and hemagglutinin titer, while fluid containing 2.0 micrograms of puromycin per ml showed no significant decrease in the virus infectivity and hemagglutinin. The infectivity titer of culture fluid containing 4.0 micrograms of puromycin per ml was 2.1 logarithm units below that of culture fluid containing no puromycin. The infectivity titer of culture fluid containing 2.0 micrograms of puromycin per ml was 0.50 log units below that of the control titer. Knight (16, 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. The difference between the titers of culture fluids containing 4.0 micrograms per ml of puromycin and no puromycin is highly significant. The results indicate that puromycin inhibits formation of infectious virus in tissue cultures as well as virus

hemagglutinin.

Table III. Inhibition of formation of infectious Lee-R influenza virus in tissue culture by puromycin.

Preparation	Time of incubation at 35°C.	pH after incubation	EID_{50} per ml	Hemagglutinin titer
Culture fluid--no puromycin	44 hrs.	6.4	$10^{7.50}$	160
Culture fluid containing 4.0 μg of puromycin per ml	44 hrs.	6.7	$10^{5.40}$	10
Culture fluid containing 2.0 μg of puromycin per ml	44 hrs.	6.4	$10^{7.0}$	80

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

2. Infectivity titers were determined by injection of a 0.10 ml volume of serial 10-fold dilutions of culture fluids into the allantoic cavities of groups of six 10-day-old chick embryos. After incubation for 40 hours at 35°C., the allantoic fluids of individual eggs were tested for the presence of virus hemagglutinin at the 1:4 dilution. This data were used for the calculation of the EID_{50}.

Cytotoxicity Test

Another phase of this investigation consisted of determining the toxicity of puromycin for the growth of host cells. Toxicity was determined by adding puromycin, prepared in Hanks BSS, to chorioallantoic membrane tissue fragments in roller tubes. After incubation for 44 to 46 hours at 35°C., the tubes were examined microscopically for the growth of new cells around the tissue fragments. Puromycin at concentrations of 8.0 micrograms per ml and 4.0 micrograms per ml completely inhibited the growth of new cells, while a concentration of 2.0 micrograms per ml had no effect upon cellular growth. Although cellular growth was completely suppressed at inhibitory concentrations, the cells still remained metabolically active as indicated by the change in pH.

Virucidal Effect

The direct virucidal effect of puromycin was measured by determining the effect of the compound on the Lee-R influenza virus infectivity property. A mixture of equal parts of freshly harvested infective allantoic fluid and phosphate-glycine buffer of pH 8.00 containing a final concentration of 4.0 micrograms of puromycin per ml was prepared. A control mixture of equal parts of infective allantoic fluid and phosphate-glycine buffer without

puromycin was prepared also. The control mixture was titrated for virus infectivity and hemagglutinin immediately after preparation. The mixture containing puromycin and infective allantoic fluid was assumed to have had the same initial infectivity titer and hemagglutinin titer as the unincubated control mixture. Infectivity and hemagglutinin titrations were made on both the control and puromycin preparation after incubation for 24 hours at 35°C. The results of this experiment are shown in Table IV. Puromycin caused no greater loss in infectivity than occurred in the control virus during the 24 hours exposure. The infectivity titer of the mixture containing puromycin was 0.428 log units higher than the control mixture. The difference between the titers of the two preparations is considered not significant when five eggs are used for each dilution (16, pp. 487-495). The results indicate that puromycin had no direct virucidal effect on the virus infectivity and hemagglutinin at the concentration tested under the conditions set up in this experiment.

Effect on Hemagglutination

The effect of puromycin on the hemagglutination reaction was measured by testing for any effect on a titration of the hemagglutinin of Lee-R virus. Two series of 2-fold dilutions of frozen infective allantoic fluid were

Table IV. *In vitro* effect of puromycin on Lee-R influenza virus.

Preparation	Time of incubation at 35°C.	pH before incubation	pH after incubation	EID_{50} per ml	Hemagglutinin titer
Virus + buffer	before	8.0	--	$10^{9.75}$	640
Virus + buffer	24 hrs.	8.0	8.15	$10^{7.00}$	640
Virus+buffer with puromycin 4.0 μg per ml	24 hrs.	8.0	8.21	$10^{7.428}$	640

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

2. The virus used was in the form of infective allantoic fluid harvested on the day the experiment was carried out.

3. Infectivity titers were determined by injecting a 0.1 ml volume of serial 10-fold dilutions of the test and control mixtures into the allantoic cavities of groups of six 10-day-old chick embryos. After incubation for 40 hours at 35°C., the allantoic fluids of individual eggs were tested for the presence of virus hemagglutinin at the 1:4 dilution. These data were used for the calculation of the EID_{50}.

prepared in physiological saline containing a final concentration of 8.0 micrograms of puromycin per ml, beginning with a 1:10 and 1:15 dilution and progressing through to 1:2560 and 1:1920 dilutions, respectively. Serial 2-fold dilutions of the same frozen infective allantoic fluid were prepared in physiological saline, without puromycin,

beginning with 1:10 and 1:15 dilutions and progressing through to 1:2560 and 1:1920 dilutions, respectively. The fluids were mixed and a 1.0 ml volume of 0.5 per cent red cell suspension was added to each tube and the tubes were thoroughly shaken. Readings were made after the cells were allowed to settle for 30 minutes at room temperature. The results of two experiments are shown in Table V. A comparison of the two hemagglutinin titers indicates that the presence of puromycin did not influence the endpoint of the hemagglutination reaction. In other words the compound did not itself cause agglutination, nor did it interfere with the reaction caused by the virus.

Effect on Adsorption of Virus by Host Cells

The effect of puromycin upon the adsorption of Lee-R influenza virus by host cells was tested. Chorioallantoic membranes were harvested, washed three times in Hank's Balance Saline solution and pooled. The pooled tissue was lightly blotted on sterile filter paper and weighed into sterile petri dishes. Two control preparations, containing infective allantoic fluid at 1:2 and 1:5 dilutions, were prepared by diluting freshly harvested infective allantoic fluid in phosphate buffered saline. Two identical mixtures of infective fluid and phosphated buffered saline but containing puromycin in a final

Table V. Tests for possible effect of puromycin on the hemagglutination of chicken erythrocytes by influenza virus.

Experiment	Preparation	Hemagglutinin titer
I	virus - saline	640
	virus - puromycin 4.0*	640
	puromycin - saline 4.0*	---
II	virus - saline	640
	virus - puromycin 4.0*	640
	puromycin - saline 4.0*	---

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

* Micrograms per ml.

concentration of 4.0 micrograms were prepared also. The dilutions of these preparations were the same as that of the control. Each preparation was divided into two equal volumes in which one half received tissue at the rate of 1.0 gram per ml of fluid and the other half received no tissue. The control preparations were titrated for virus hemagglutinin before incubation. After incubation for 30 minutes at 37°C., both the control and compound preparations, with and without tissue, were titrated for virus hemagglutinin. The results of the adsorption experiment

are shown in Table VI. In the control and compound preparations, containing the 1:2 dilutions of infective 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. There was no apparent difference in the adsorption of virus to the tissue whether puromycin was present or not. A comparison of the titers of preparations incubated with tissue to those of preparations incubated without tissue indicated that approximately 60 per cent of the virus hemagglutinin was adsorbed to the tissue in both the control and compound preparations based on the initial titer before incubation. In the preparations containing the 1:5 dilutions of infective allantoic fluid about 80 per cent of the virus hemagglutinin was adsorbed to the tissue. There was no apparent difference in the adsorption of the virus to the tissue whether puromycin was present or not. There was no indication that the compound exerted any significant effect upon the adsorption process.

Combined Effect of Puromycin and Amino Acid Analogs on the Development of Influenza Virus

It was of major interest in the course of this investigation to determine whether puromycin would exert a

Table VI. The effect of puromycin upon the adsorption of Lee influenza virus to Chorioallantoic membrane tissue *in vitro*.

Dilution of infected fluid	Concentration of puromycin	Incubation time	Tissue present	Hemagglutinin titer
1:2	0	Before incubation	0	640
1:2	0	30 minutes	0	480
1:2	4.0*	30 minutes	0	480
1:2	0	30 minutes	1.0**	240
1:2	4.0*	30 minutes	1.0**	240
1:5	0	Before incubation	0	320
1:5	0	30 minutes	0	240
1:5	4.0*	30 minutes	0	240
1:5	0	30 minutes	1.0**	60
1:5	4.0*	30 minutes	1.0**	60

* Microgram per ml
** Gram per ml

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 three times in Hanks balance saline solution and had been aseptically weighed.

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

combined inhibitory effect upon virus development in chorioallantoic membrane tissue cultures with various amino acids analogs. The various inhibitors were selected and tested at concentrations which gave some degree of inhibition but had no toxic effect upon the tissue.

L-Canavanine and Puromycin

Combinations of L-canavanine, a structural analog of arginine, and puromycin were examined for inhibitory activity against the development of influenza virus in tissue cultures. The two compounds were tested both singly and in combination in the tissue culture-virus system in the manner previously described. The results of the combined experiments are shown in Figure 1. It can be seen that canavanine, at a concentration of 0.0125 mg/ml in the tissue culture fluid, reduced the virus hemagglutinin titer to 74, compared to a control value of 114. The difference between these values is not significant. The virus hemagglutinin titer of tissue culture fluid containing puromycin at a concentration of 0.001 mg/ml was 98, which likewise was not significantly different from the control. The virus hemagglutinin titer of culture fluid containing the two concentrations combined was reduced to 28 or 25 per cent of the control value. This more than 4-fold difference has been repeatedly found to be significant ($P = 0.05$) when the values are the geometric means from eight cultures in both compound and control groups. Jawetz (15, p. 95) has described four effects which may be noted when two antimicrobial agents act simultaneously on the same microbial population: (1) The effect is indifference when the combined action

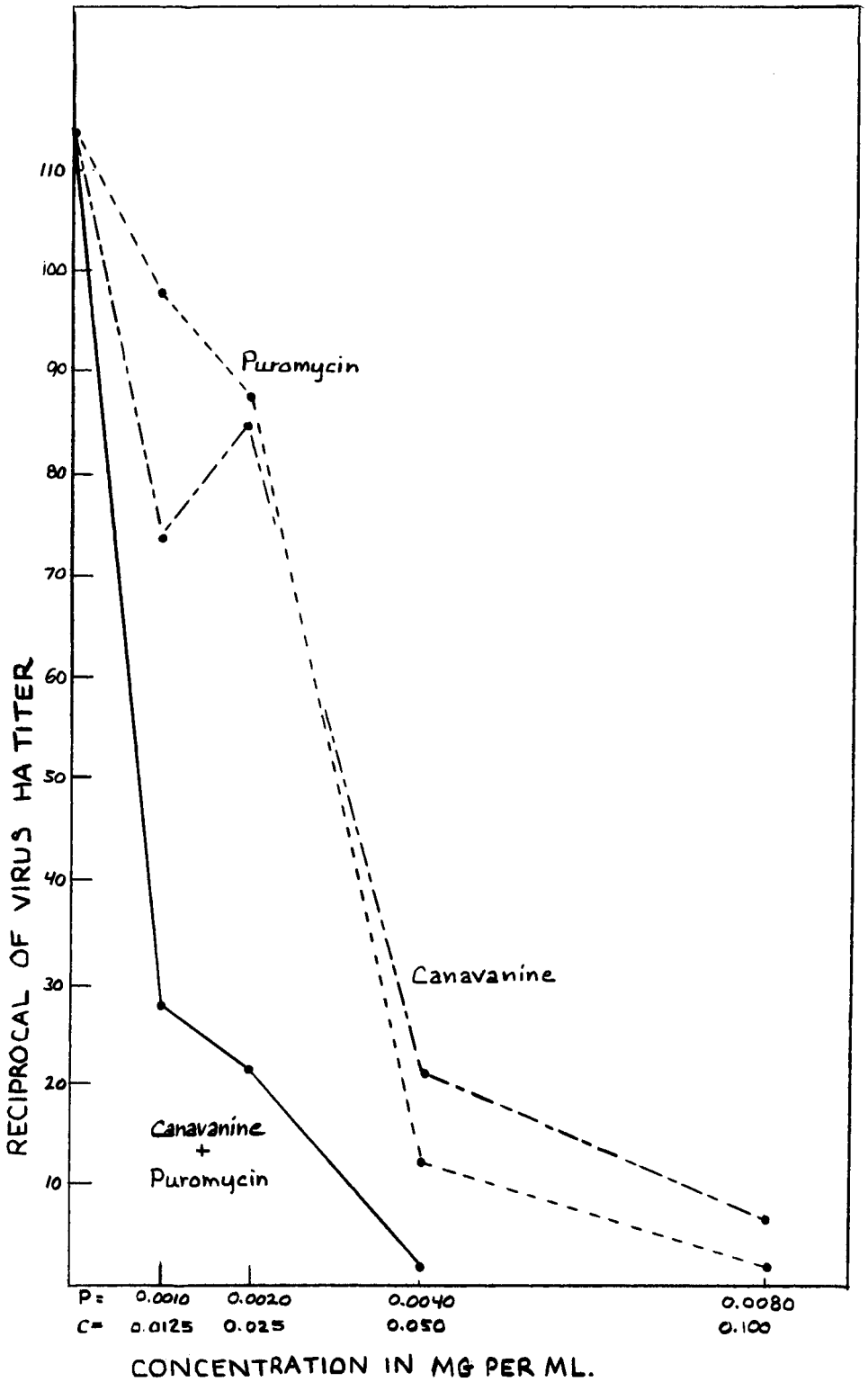
FIGURE 1

Combined effect of L-canavanine and puromycin on the development of Lee influenza virus in tissue culture

1. Hemagglutinin titers are expressed as the highest dilutions of tissue culture fluids giving complete agglutination of chicken erythrocytes.

2. Each point represents the geometric mean of the hemagglutinin titers of eight tissue cultures in the compound groups, and twelve tissue cultures in the control.

FIGURE 1.


RECIPROCAL OF VIRUS HA TITER
110
100
90
80
70
60
50
40
30
20
10
Puromycin
Canavanine
Canavanine
+
Puromycin
P= 0.0010 0.0020 0.0040 0.0080
C= 0.0125 0.025 0.050 0.100
CONCENTRATION IN MG PER ML.

is no greater than that of the more effective agent when used alone. (2) The effect is addition when the combined action is equivalent to the sum of the actions of each agent when used alone. (3) The effect is synergism when the combined action is significantly greater than the sum of both individual effects. (4) The effect is antagonism when the action is less than that of the more effective agent when used alone. These criteria can also be used in the study of the combined action of antiviral inhibitors. The reduction in titer noted with the combinations represent a clear cut synergistic activity of the two compounds. The combination of ineffective levels of the two compounds gave a marked degree of inhibition. The reduction in titer observed with the combination is greater than that obtained by doubling the concentration of either compound alone. This criterion is a second one sometimes used in testing for synergism.

Ribonuclease and Puromycin

Ribonuclease, an enzyme inhibitory for the development of influenza virus in chick embryo tissue culture, and puromycin were studied in a similar manner. The results are shown in Figure 2. Ribonuclease at a concentration of 0.20 mg/ml in the culture fluid reduced the hemagglutinin titer to 40, compared with a control value

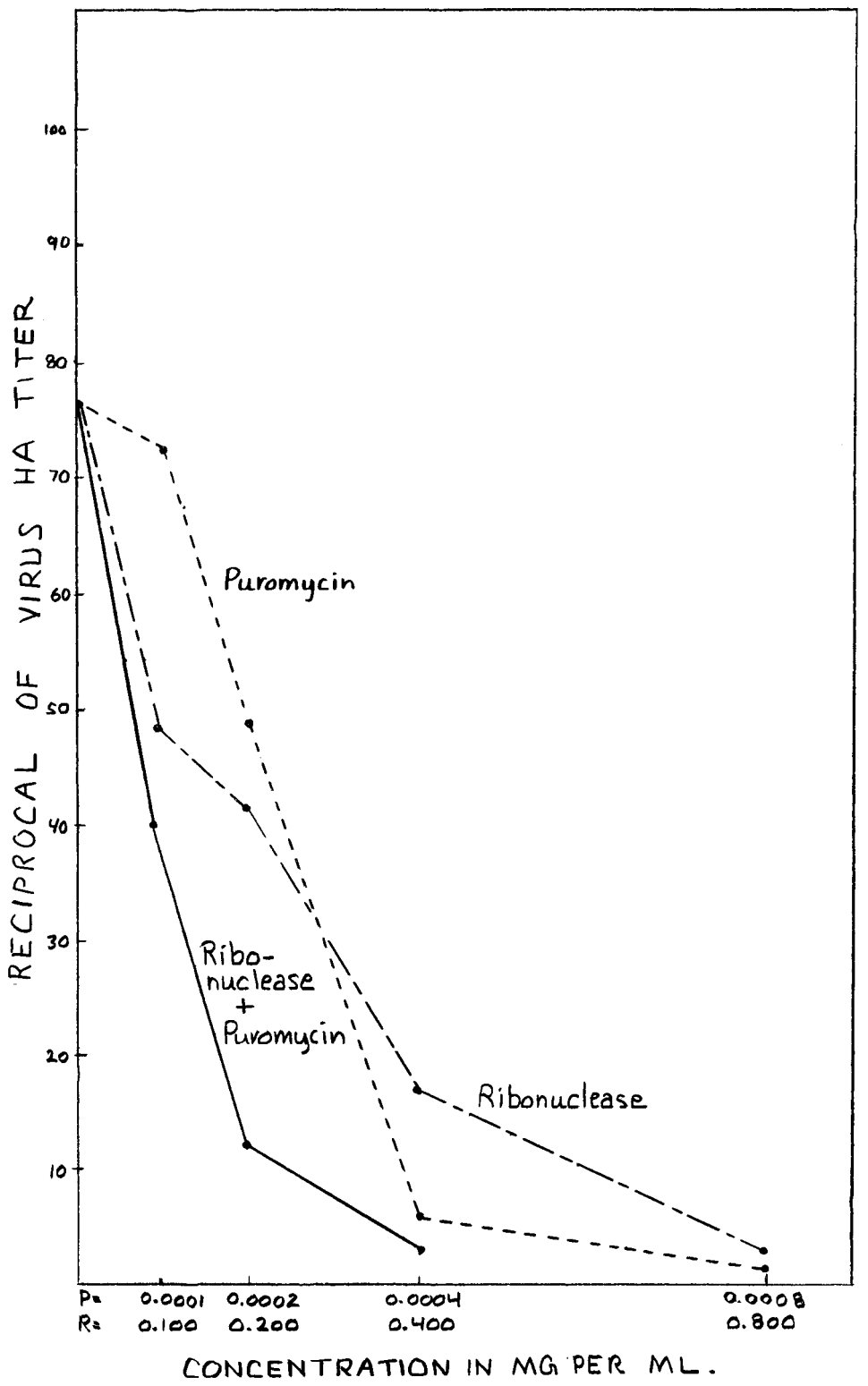
FIGURE 2

Combined effect of ribonuclease and puromycin on the development of Lee influenza virus in tissue culture

1. Hemagglutinin titers are expressed as the highest dilutions of tissue culture fluids giving complete agglutination of chicken erythrocytes.

2. Each point represents the geometric mean of the hemagglutinin titers of eight tissue cultures in the compound groups, and twelve tissue cultures in the control.

FIGURE 2


RECIPROCAL OF VIRUS HA TITER
100
90
80
70
60
50
40
30
20
10
Puromycin
Ribo-
nuclease
+
Puromycin
Ribonuclease
P= 0.0001 0.0002 0.0004 0.0008
R= 0.100 0.200 0.400 0.800
CONCENTRATION IN MG PER ML.

of 77. This reduction in titer is probably not significant and may be considered as threshold inhibition. Puromycin at a concentration of 0.002 mg/ml in the culture fluid reduced the hemagglutinin titer to 49, a titer not significantly different from the control. A mixture of the two concentrations in the culture fluid reduced the hemagglutinin titer to 12 or 15 per cent of the control value. The combined threshold levels of the two compounds thus had a significant inhibitory effect upon virus development. Doubling the concentration of the more effective compound reduced the hemagglutinin titer to a value not significantly different statistically from that obtained with the mixture. The nature of the combined effect of these two compounds appears to be one of marked addition.

DL-p-Fluorophenylalanine and Puromycin

DL-p-fluorophenylalanine, an antagonist of phenylalanine, and puromycin were examined for possible combined inhibitory effect upon virus development. The results of these experiments are shown in Figure 3. Here it can be seen that a mixture of the two compounds caused no greater reduction in the hemagglutinin titer than that of the more effective compound when used alone. Tissue cultures containing 0.0016 mg of DL-p-fluorophenylalanine per ml of fluid gave a hemagglutinin titer of 64, compared

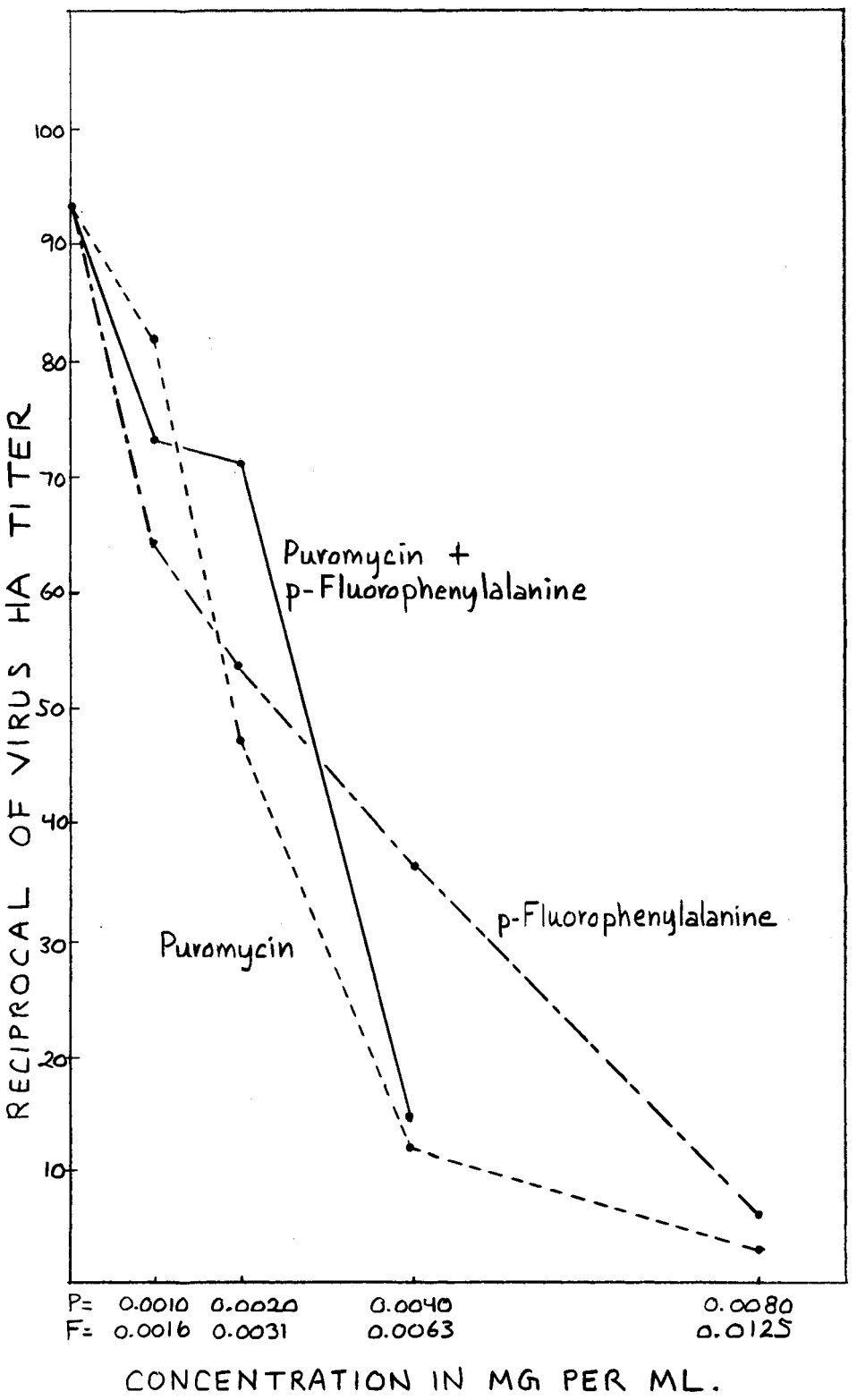
FIGURE 3

Combined effect of DL-p-fluorophenylalanine and puromycin on the development of Lee influenza virus in tissue culture

1. Hemagglutinin titers are expressed as the highest dilutions of tissue culture fluids giving complete agglutination of chicken erythrocytes.

2. Each point represents the geometric mean of the hemagglutinin titers of eight tissue cultures in the compound groups, and twelve tissue cultures in the control.

FIGURE 3


RECIPROCAL OF VIRUS HA TITER
100
90
80
70
60
50
40
30
20
10
Puromycin +
p-Fluorophenylalanine
p-Fluorophenylalanine
Puromycin
P= 0.0010 0.0020 0.0040 0.0080
F= 0.0016 0.0031 0.0063 0.0125
CONCENTRATION IN MG PER ML.

with a control value of 92. This reduction in titer is not statistically significant. Puromycin at a concentration of 0.001 mg/ml in tissue culture fluid lowered the hemagglutinin titer to 80, an insignificant degree of inhibition. These two concentrations combined only reduced the virus hemagglutinin titer to 72 or 78 per cent of the control value. Also it is clear from the dose-response curves of the combination and the individual compounds that inhibition is no greater with the combinations than with the more effective compound when used alone. Jawetz describes such combined effect as indifferent.

DL-m-Tyrosine and Puromycin

Combinations of puromycin and DL-m-tyrosine, a structural analog of tyrosine and phenylalanine, were similarly examined for inhibitory activity against the development of influenza virus. The results of the experiment are shown in Figure 4. Culture fluid containing 0.063 mg of tyrosine per ml gave a hemagglutinin titer of 98, compared with a control value of 132. Puromycin at a concentration of 0.002 mg/ml in tissue culture fluid lowered the hemagglutinin titer to 116. These two concentrations combined reduced the virus hemagglutinin titer to 46 or 35 per cent of the control value. This three fold reduction in titer is on the borderline of significance with

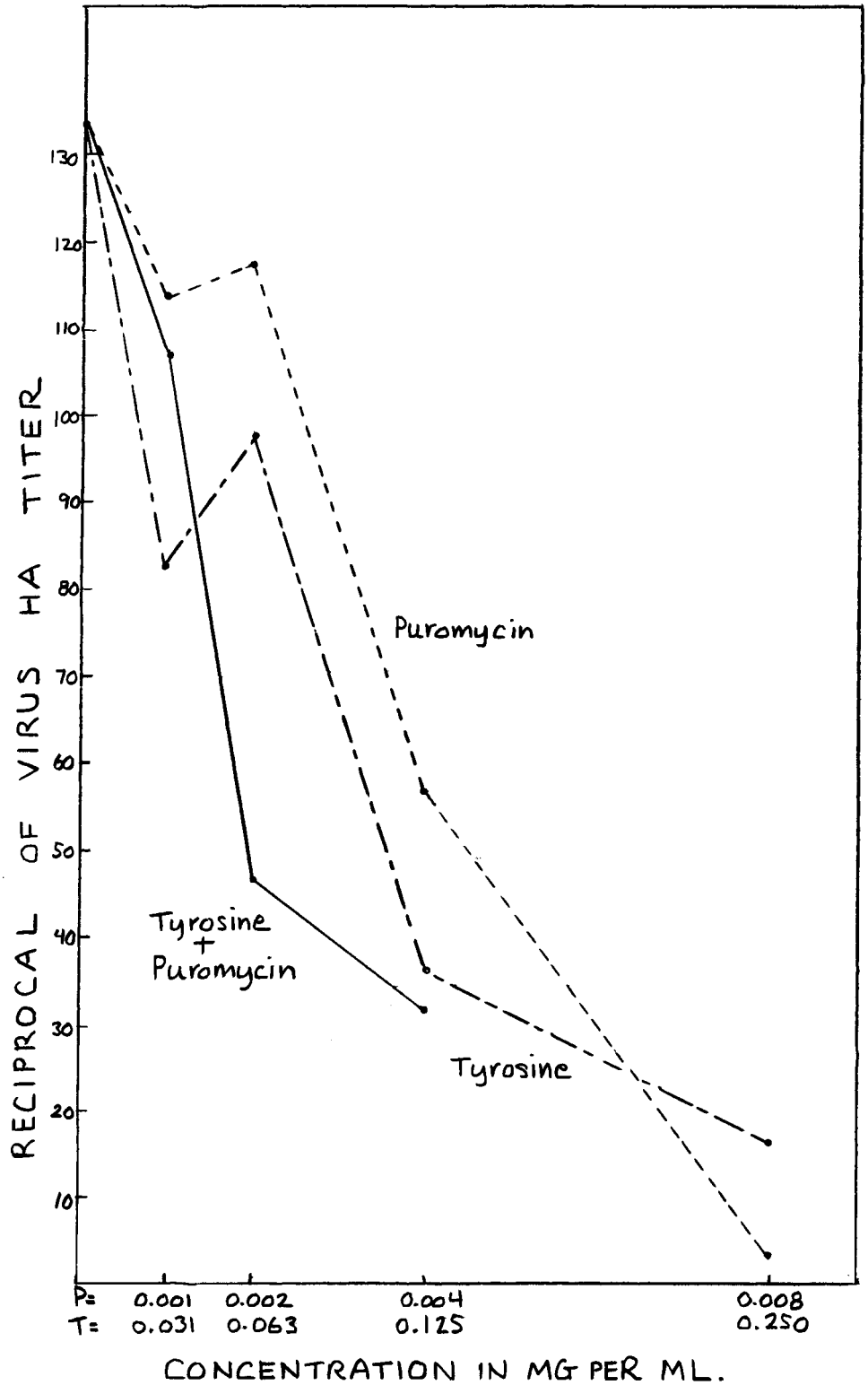
FIGURE 4

Combined effect of DL-m-tyrosine and puromycin on the development of Lee influenza virus in tissue culture

1. Hemagglutinin titers are expressed as the highest dilutions of tissue culture fluids giving complete agglutination of chicken erythrocytes.

2. Each point represents the geometric mean of the hemagglutinin titer of four tissue cultures in the compound group and twelve cultures in the control.

FIGURE 4.


RECIPROCAL OF VIRUS HA TITER
130
120
110
100
90
80
70
60
50
40
30
20
10
Puromycin
Tyrosine
+
Puromycin
Tyrosine
P= 0.001 0.002 0.004 0.008
T= 0.031 0.063 0.125 0.250
CONCENTRATION IN MG PER ML.

respect to the control value, and significantly different from the tyrosine value. Therefore, it appears from these results that the nature of the combined effect of these two compounds is addition.

Benzimidazole and Puromycin

Figure 5 shows the results obtained when puromycin is combined with benzimidazole, an antagonist of purines, in tissue cultures inoculated with influenza virus. Here it can be seen from the dose response curves of the combination and individual compounds that inhibition is somewhat higher with the combinations than with the individual compounds. Benzimidazole at a concentration of 0.15 mg/ml in the culture fluid reduced the hemagglutinin titer to 63, compared to a control value of 95. This reduction in titer is not significantly different from the control. Tissue cultures containing 0.002 mg/ml of puromycin per ml of fluid gave a hemagglutinin titer of 32, a significant degree of inhibition. A mixture of these two concentrations in culture fluid reduced the hemagglutinin titer to 20 or 21 per cent of the control value, a further decrease from the above value for puromycin alone. The combined effect of the two concentrations was a little greater than either of the individual effects, and also, a threshold effect resulted when two ineffective

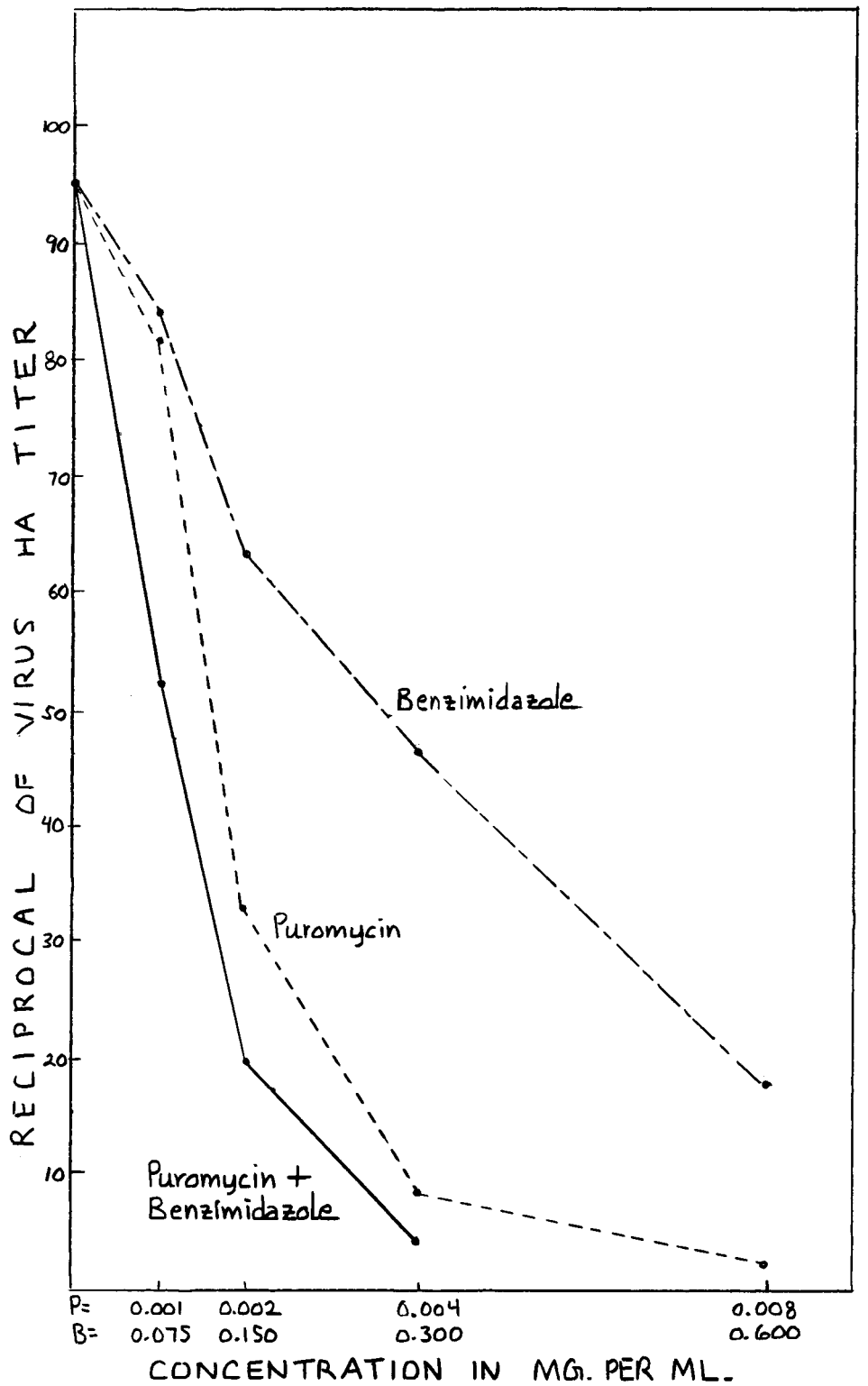
FIGURE 5

Combined effect of benzimidazole and puromycin on the development of Lee influenza virus in tissue culture

1. Hemagglutinin titers are expressed as the highest dilutions of tissue culture fluids giving complete agglutination of chicken erythrocytes.

2. Each point represents the geometric mean of the hemagglutinin titers of fifteen tissue cultures in the compound groups, and twenty-four tissue cultures in the control.

FIGURE 5


RECIPROCAL OF VIRUS HA TITER
100
90
80
70
60
50
40
30
20
10
Benzimidazole
Puromycin
Puromycin +
Benzimidazole
P= 0.001 0.002 0.004 0.008
B= 0.075 0.150 0.300 0.600
CONCENTRATION IN MG. PER ML.

concentrations of the two compounds were combined. It does appear from these results that the nature of the combined effect of these two compounds may be one of addition.

Attempts to Reverse the Inhibitory Activity of Puromycin in Tissue Culture

The ability of the compound to inhibit the formation of infectious virus as well as virus hemagglutinin in tissue cultures, and also, to inhibit the growth of new cells suggests that inhibition induced by this compound may be due to interference with protein synthesis as shown in certain other systems. Aside from this possibility the structural resemblance of the compound to amino-acyl ribonucleic acid and to natural occurring purine presented the possibility that the inhibitory activity of the compound might be the result of an antagonism to natural purines or pyrimidines or their nucleosides or nucleotides involved in the metabolism of viral nucleic acid. Also it has been shown that the puromycin inhibition of trypanosome infection in mice could be blocked or reversed by adenine and adenine derivative. To investigate this possibility purines and pyrimidines, nucleosides and nucleotides were added to tissue culture fluids containing minimal inhibitory concentrations of puromycin. The compounds were dissolved at maximum solubility in Hanks

balanced saline solution and were sterilized by filtration through ultrafine porosity sintered glass filters. In each case, the maximum concentration of the compound tested in tissue cultures in the first experiments was approximately 2500 fold greater than that of puromycin; or if impossible, the maximum soluble level was used. The purine or pyrimidine derivative and puromycin were tested both singly and in combination using the tissue culture-virus system described previously. Tables VII, VIII, IX, X and XI show the results of several experiments in which attempts were made to reverse the inhibition of virus development caused by puromycin. These compounds were observed in preliminary studies to exert a slight partial reversal effect on the inhibitory activity of puromycin and were further investigated in the tissue culture-virus system. The results of experiment I in Table VII seemed to indicate that cytidylic acid might have exerted some slight reversing effect but this was insignificant and was not confirmed by results of experiment II. Replicate experiments with cytidylic acid gave results similar to those observed with those of experiment II. The results shown in Table VIII seemed to indicate that guanylic acid might exert a partial reversal effect on the activity of puromycin. This effect observed with guanylic acid could

Table VII. Inhibition of Lee-R Influenza Virus Development by Puromycin: Attempted Reversal by Cytidylic Acid.

Experiment number	Hemagglutinin units per ml of tissue culture fluids inoculated with 4.7×10^6 EID_{50} of Lee-R influenza virus per ml			
	Control	Nutrient fluid plus 0.004 mg of puromycin per ml	Nutrient fluid containing 0.004 mg of puromycin plus the following concentration of cytidylic acid.	
			4.0*	0.016*
I	120	4	13	11
II	110	6	7	16

1. The number of hemagglutinin units of virus was calculated from the geometric means of 3 replicate cultures in the compound group, and 6 replicate cultures in the control group. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of fluid giving complete agglutination of chicken red blood cells.

* Milligram per ml.

not be confirmed however in additional experiments. The same situation is evident with uridine, uracil and uridylic acid. It was concluded that none of these compounds could significantly block the inhibitory activity of puromycin in this system. The results of these experiments are shown in Tables IX, X and XI. It appears from these results that the inhibitory activity of puromycin for influenza virus was not reversed, or reproducibly blocked, by any purines of the pyrimidines, or their nucleosides or nucleotides.

Table VIII. Inhibition of Lee-R influenza virus development by puromycin: attempted reversal by guanylic acid.

Experiment number	Hemagglutinin units per ml of tissue culture fluids inoculated with 4.7 x 10^6 EID_{50} of Lee-R influenza virus per ml				
	Control	Nutrient fluid plus 0.004 mg of puromycin per ml	Nutrient fluid containing 0.004 mg of puromycin per ml plus the following concentration of guanylic acid		
			0.120*	0.060*	0.024*
I	120	4	16	-	12
II	98	4	36	18	-

1. Hemagglutinin units of virus were calculated from the geometric means of 3 replicate cultures in the compound group and 6 replicate cultures in the control group. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of fluid giving complete agglutination of chicken red blood cells.

* Milligram per ml.

Mouse Test

Puromycin was prepared as a sterile solution containing 2.5 mg/ml in physiological saline. A single dose, containing 1.0 mg, was administered to the animals in a volume of 0.40 ml per injection. This dose of the compound was tested for inhibitory activity against the development of Lee virus in the mouse lung. Six mice, weighing 25 to 28 grams each, were given one intraperitoneal injection of the above dose and inoculated intranasally with a 0.05 ml volume containing 10 LD_{50} of mouse adapted Lee virus, after one hour. The mice were injected with the same dose

Table IX. Inhibition of Lee-R influenza virus development by puromycin: attempted reversal by uridine.

Experiment number	Hemagglutinin units per ml of tissue culture fluids inoculated with 4.7 x 10^6 EID_{50} of Lee-R influenza virus per ml		
	Control	Nutrient fluid plus 0.004 mg of puromycin per ml	Nutrient fluid containing 0.004 mg of puromycin per ml plus the following concentration of uridine
			0.40*
I	123	7	21
II	82	12	16

1. Hemagglutinin units of virus were calculated from the geometric means of 3 replicate cultures in the compound group and 6 replicate cultures in the control group. Hemagglutinin titers are expressed as the reciprocals of the highest dilution of fluid giving complete agglutination of chicken erythrocytes.

* Milligram per ml.

twice daily for a three day period. Six control mice received injections of sterile physiological saline and were inoculated with the same virus inoculum. Approximately 12 to 15 hours after the last injection, the treated and control mice were sacrificed. The lungs of each group were removed, washed in physiological saline, scored for pulmonary consolidation, and weighed. Physiological saline was added to each lung pool to give a dilution of 1:20 and the suspension was ground in the VirTis homogenizer. The ground lung suspension was treated to remove lung tissue inhibitors as described by Bowen and Pilcher

Table X. Inhibition of Lee-R influenza virus development by puromycin: attempted reversal by uracil

Experiment number	Hemagglutinin units per ml of tissue culture fluids inoculated with 4.7 x 10^6 EID_{50} of Lee-R influenza virus per ml				
	Control	Nutrient fluid plus 0.004 mg of puromycin per ml	Nutrient fluid containing 0.004 mg of puromycin per ml plus the following concentrations of uracil		
			2.0*	1.0*	0.20*
I	115	5	-	15	12
II	118	3	10	5	-

1. Hemagglutinin units of virus were calculated from the geometric means of 3 replicate cultures in compound group and 6 replicate cultures in the control group. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of fluid giving complete agglutination of chicken erythrocytes.

* Milligram per ml.

(4, pp. 458-459) and then titrated for virus hemagglutinin. The hemagglutinin titer of the lung suspension from the puromycin treated group infected with Lee virus was 320; that of the control group was 160. This two fold difference between titers is not significant with the number of animals used in the experiment. Puromycin produced no measurable inhibition of virus development in the lung, even though it was effective in curing trypanosome infection in mice.

Table XI. Inhibition of Lee-R influenza virus development by puromycin: attempted reversal by uridylic acid

Experiment number	Hemagglutinin units per ml of tissue culture fluids inoculated with 4.7 x 10^6 EID_{50} of Lee-R influenza virus per ml		
	Control	Nutrient fluid plus 0.004 mg of puromycin per ml	Nutrient fluid containing 0.004 mg of puromycin per ml plus the following concentration of uridylic acid
			2.0*
I	124	2	10
II	72	1	4

1. Hemagglutinin units of virus was calculated from the geometric mean of replicate cultures in the compound group and 6 replicate cultures in the control group. Hemagglutinin titers are expressed as the reciprocals of the highest dilutions of fluid giving complete agglutination of chicken red blood cells.

* Milligram per ml.

A Comparison of the Activity of Puromycin and Puromycin Amino Nucleoside in Tissue Culture

The final phase of this investigation involved the comparison of the inhibitory activity of puromycin with that of the amino nucleoside for virus development in tissue cultures. The puromycin amino nucleoside as described by Baker *et al.* (1, p. 2838) has been found to have a more powerful effect upon trypanosome infections in mice and rabbits than puromycin itself (13, pp. 1222-1227). It has not been found active against bacteria. The amino nucleoside was tested using the tissue culture-

virus system described previously. The results obtained in the preliminary study indicate that this compound, at concentrations equivalent to those of puromycin, in tissue culture fluid was stimulatory for the development of Lee influenza virus. It appears from the results of these experiments that the amino nucleoside gave a significant augmentation of virus yield, and as a result, further experiments were undertaken to determine the degree of this stimulation.

The maximum stimulatory concentration of the amino nucleoside was determined by preparing groups of tissue cultures containing varying concentrations of the amino nucleoside in Hanks' balanced saline solution and inoculating them with an appropriate dilution of virus. After incubation for 44 to 46 hours at 35°C., the culture fluids were titrated for virus hemagglutinin. The results of this investigation are shown in Table XII. The amino nucleoside at a concentration of 0.20 mg/ml in culture fluid increased the virus hemagglutinin titer to 165, compared to a control value of 91, a significant enhancement of virus yield. Slightly lower virus yields were observed in tissue culture fluid containing 0.40 mg and 0.10 mg per ml of amino nucleoside, 136 and 137 hemagglutinin units per ml, respectively. Statistical analysis

by the Student's "t" test (8, pp. 153-157) reveals that based on the 15 replicate cultures in each experimental group the mean hemagglutinin titers were highly significant.

Fisher's Table of "t", "n" (degrees of freedom) gives:

P (probability)	0.05
"t"	2.045

The value of "t" calculated from the data is:

Concentration	"t"
0.40	3.263
0.20	3.819

The value of "t" in each experimental group exceeds the value of "t" given in Fisher's table for the 0.05 probability level.

If it is a true increase in the virus yield this suggests that the amino nucleoside may in some way augment the transfer of amino acids into proteins thus stimulating protein synthesis for both cell and virus protein, or it may become associated in the function of transfer ribonucleic acid in the attachment of the amino acids to ribosome. The results also clearly indicate that the amino nucleoside does not interfere with the function of amino acyl-ribonucleic acid as is thought to be the case with puromycin. Its lack of inhibitory effect on influenza virus seems to resemble that on bacteria instead of its action on trypanosomes.

Table XII. Relation of concentrations of amino nucleoside (puromycin) to the degree of stimulation of Lee-R influenza virus in tissue cultures.

EID_{50} of virus per ml in tissue cultures	Culture number	Virus hemagglutinin titers of tissue culture fluids containing the following concentrations of amino nucleoside.			
		0.4*	0.2*	0.1*	Control
4.7×10^6	1	160	80	160	60
	2	160	320	240	80
	3	160	240	80	160
	4	160	240	160	80
	5	240	160	160	80
	6	120	240	160	160
	7	120	240	80	80
	8	80	160	160	80
	9	120	240	60	80
	10	120	240	160	40
	11	160	120	160	160
	12	120	160	120	120
	13	80	120	160	80
	14	160	80	160	120
	15	160	80	160	80
	Geo. Mean	136	164	137	91

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

* Milligram per ml.

Stimulation by Cytidylic Acid

The stimulatory activity of cytidylic acid (2'3' mixed isomers) for Lee influenza virus was noted in reversal studies in tissue cultures and was further studied in this system to determine the degree of stimulation.

A range of stimulatory concentrations of cytidylic

acid was similarly determined, using the tissue culture-virus system described previously. The results of these experiments are shown in Table XIII. Cytidylic acid at a concentration of 4.0 mg/ml in culture fluid increased the mean virus hemagglutinin titer of 20 cultures to 242, compared to a control value of 117, a significant enhancement of virus concentration in culture fluid. Slightly lower virus yields were observed in tissue culture fluid containing 3.2 mg, 1.6 mg, 0.8 mg, 0.4 mg, 0.2 mg and 0.1 mg of cytidylic acid per ml. The lowest concentrations gave the least stimulation. Statistical analysis by the Student's "t" test (8, pp. 153-157) using the logs of the hemagglutinin values, reveals that based on the 20 replicate cultures in each experimental group, the increase in the mean hemagglutinin titers was highly significant for the 4.0 mg/ml concentration and significant even at the 0.1 mg/ml level.

Fisher's Table of "t", "n" (degrees of freedom) gives:

P (probability)	0.05
"t"	2.042

The value of "t" calculated from the data is:

Concentration	"t"
4.0 mg/ml	6.078
0.1 mg/ml	2.335

The value of "t" in each experimental group exceeds the value of "t" given in Fisher's table for the 0.05 probability level.

The effect of this mixed nucleotide is obviously a real one, but it is not known whether the overall yield of virus has been increased, or only the amount released into the culture fluid. Neither has the maximum active concentration been determined. This observation needs further study, but these preliminary results are included here because the compound is a nucleotide and thus related in structure to puromycin, but apparently has just the opposite effect on the virus concentration in the culture fluid.

Table XIII. Relation of concentrations of cytidylic acid to the concentration of Lee-R influenza virus hemagglutinin in tissue culture fluid

EID_{50} of virus per ml in tissue cultures	Culture number	Virus hemagglutinin titers of tissue culture fluids containing the following concentrations of cytidylic acid							
		4.0*	3.2*	1.6*	0.8*	0.4*	0.2*	0.1*	Control
	1	240	150	120	80	80	80	120	80
	2	240	160	120	320	320	160	160	40
	3	160	160	160	160	160	120	80	40
	4	160	80	80	240	80	160	120	80
	5	320	480	320	160	240	160	240	160
	6	320	480	320	160	240	240	160	160
	7	320	640	320	160	240	320	160	160
	8	320	160	320	320	240	240	160	160
	9	320	160	240	320	160	160	240	120
4.7×10^6	10	240	320	320	160	80	160	120	80
	11	160	320	240	120	320	160	120	160
	12	320	240	160	320	160	160	240	160
	13	320	120	160	160	80	160	160	120
	14	160	320	160	240	160	160	160	160
	15	160	240	320	160	240	240	160	120
	16	320	320	240	240	320	240	240	120
	17	240	240	160	320	160	160	80	160
	18	160	160	160	320	160	320	240	160
	19	240	160	160	160	240	160	160	120
	20	320	240	240	160	320	160	160	160
	Geo. Mean	242	227	201	199	178	177	156	117

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

* Milligram per ml.

DISCUSSION

Results obtained in tissue culture experiments indicate that puromycin caused a significant inhibition of the development of influenza virus at very low concentrations. Few virus inhibitors are active in the microgram range. The findings suggest that the compound probably interferes with some phase of the intracellular development of the virus. Results of toxicity tests showed that the compound prevented growth of the chick embryo cells at the 0.004 mg per ml concentration, but did not block all metabolic activity of the cells. As it is known to interfere with protein synthesis in some animal cells, this may well explain its effect on these chick embryo cells. At the 0.002 mg per ml concentration cell multiplication could occur, and a detectable degree of interference with virus multiplication was still noted. Thus it seems possible that inhibition of virus development may well be due to the same underlying effect of this compound, interference with protein synthesis.

Results of the <u>in vitro</u> test for virucidal activity of puromycin indicated that the compound caused no significant inactivation of virus infectivity of hemagglutinin after 24 hours exposure to an inhibitory concentration. This means that the activity of puromycin was not directed

against the virus particle itself. The results obtained from tests with the hemagglutination reaction *in vitro* showed the compound did not interfere with the agglutination of chicken erythrocytes caused by the virus nor did it cause such agglutination. The results of the adsorption experiments indicated that puromycin did not interfere with the adsorption of the virus by the tissue, at least not within a short exposure period. Thus a direct effect on the virus or its adsorption by host cells, or upon the hemagglutination reaction, were eliminated as possible mechanisms for the observed virus inhibition.

The inhibitory activity of puromycin was not reversed in tissue cultures by adenine, adenosine, adenylic acid, guanine, guanosine, guanylic acid, cytosine, cytidylic acid cytidine, uracil, uridine, and uridylic acid under the test condition. The reversal by purines and their nucleotides of growth inhibition due to the action of puromycin has been demonstrated in certain other systems (5, pp. 146-159; 2, pp. 770-776; 13, pp. 1222-1227; 14, pp. 177-191). The negative results in this tissue culture virus system thus shed little light on the mechanism of virus inhibition.

Results obtained in combined tissue culture experiments revealed that combinations of puromycin and certain amino acid analogs resulted in a significant enhancement

of virus inhibition. The combination of L-canavanine plus puromycin was synergistic. Ribonuclease plus puromycin, benzimidazole plus puromycin and m-tyrosine plus puromycin showed marked combined effects which were essentially additive. In the case of p-fluorophenylalanine, however, combination with puromycin did not result in an additive degree of inhibition. The mechanism by which each of these amino acid analogues produces virus inhibition has been partly elucidated. In each case the effect has been shown to be blocked, in some cases, competitively by the analogous amino acid. Benzimidazole is thought to interfere in some phase of nucleic acid metabolism and ribonuclease enzymatically attacks cellular ribonucleic acid. Thus all of them may interfere directly or indirectly with protein synthesis. Puromycin is also known to interfere with protein synthesis in some animal cells. The puromycin inhibition of influenza virus development may be due to this same interference. The combined effects found with puromycin and the other inhibitors are compatible with this hypothesis. It is thought that puromycin interferes with protein synthesis at the stage where transfer ribonucleic acid functions to permit attachment of amino acids to ribosomal ribonucleic acid. Because of its structural similarity to the amino acid-bearing end of transfer ribonucleic acid it is proposed that it may

induce formation of an abnormal type of transfer ribonucleic acid-amino acid that cannot become attached to the ribosomal ribonucleic acid; or once attached the amino acid may not be released from the rest of the molecule. Another possibility is that an abnormal amino acid may be introduced into the polypeptide chain of the protein with the formation of a protein that is non functional. Whatever the details of the mechanism may be, the formation of virus protein could be blocked in a similar manner.

Two observations made in the present study are closely related to the mechanism by which the virus is inhibited. First is the finding that the amino nucleoside of puromycin is not inhibitory, but apparently slightly stimulating. This shows clearly that the amino acid moiety is essential for virus inhibition, and seems to support the hypothesis that puromycin interferes with formation of virus protein by one of the methods mentioned above.

The other observation is the fact that if puromycin is combined with p-fluorophenylalanine, another potent inhibitor of influenza virus, the resulting inhibition is no more than that of one of the compounds alone. This suggests that the mechanism of inhibition in each case may be similar and that when a certain effect has been achieved with one agent, the second cannot increase the

effect further. Such a possibility seems the more likely in view of the fact that the amino acid in puromycin is p-methoxyphenylalanine.

The intraperitoneal injection of 1.0 mg of puromycin twice daily for a period of three days failed to inhibit the development of influenza virus in mouse lung. The failure of puromycin to inhibit virus development in mouse lung was not too surprising. Most compounds which have inhibitory activity for virus development in tissue cultures have failed to show similar activity in mice or other laboratory animals.

The results obtained in tissue culture experiments indicated that the amino nucleoside of puromycin caused a significant enhancement of virus concentration in the culture fluids. No attempts were made to determine whether this was a real increase in virus yield or an increase in the release of virus from the tissue. Similar observations were made in tissue culture experiments with cytidylic acid. This compound also caused a significant augmentation of virus concentration in culture fluid.

SUMMARY

1. Puromycin, a specific inhibitor of protein synthesis, has been shown to inhibit the development of Lee influenza virus in tissue cultures.
2. The minimum concentration necessary to cause complete inhibition of influenza virus development in tissue cultures was 8.0 micrograms per ml. A concentration of 4.0 micrograms per ml caused marked, but not complete, inhibition. A concentration of 2.0 micrograms per ml was found to be sufficient to cause significant virus inhibition.
3. The compound was found to inhibit the formation of infectious virus in tissue cultures as well as virus hemagglutinin.
4. A concentration of 4.0 micrograms of puromycin per ml caused inhibition of cell growth, but did not block all metabolic activity. However, the degree of selectivity of the inhibitory effect on influenza virus was very slight.
5. At a concentration of 4.0 micrograms per ml, puromycin had no virucidal effect upon Lee influenza virus <u>in vitro</u>. This concentration did not influence the end point of the hemagglutination reaction.
6. A concentration of 4.0 micrograms of puromycin per ml had no effect upon the adsorption of virus by membrane

tissue *in vitro*.

7. The combination of canavanine plus puromycin in tissue culture showed a synergistic effect against virus multiplication. Combinations of ribonuclease plus puromycin, benzimidazole plus puromycin, and m-tyrosine plus puromycin in tissue culture showed additive effects, while a mixture of Dl-p-fluorophenylalanine plus puromycin showed no combined effect. A possible interpretation of these findings is proposed.
8. The inhibitory activity of puromycin for Lee influenza virus in tissue cultures was not reversed by any of a group of purines, pyrimidines or their nucleosides or nucleotides.
9. A dose of 1.0 milligram of puromycin administered twice daily for three days failed to inhibit the development of influenza virus in mouse lung.
10. The amino nucleoside of puromycin at a concentration of 0.20 mg per ml in tissue culture gave a significant enhancement of virus yield. Concentrations of 0.40 mg and 0.10 mg of the amino nucleoside per ml in culture fluid significantly increased the virus concentration.
11. The fact that the amino nucleoside did not inhibit multiplication of the virus proves that the amino acid moiety of the molecule is essential for this activity.

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