tion is avoided. This factor bears particular significance with microorganisms that produce irregular defined zones, *i.e.*, many filamentous fungi. According to Cavillito (9), complete absence of visual growth is the most reproducible and satisfactory end point in an inhibition test.

The effectiveness of most diffusion methods is dependent on those factors which govern the ability of the agent to diffuse both horizontally and vertically throughout the medium. Cooper (10) indicates the importance of these factors in critical assay procedures. They are less important when applied to general screening procedures which are primarily designed to merely distinguish between active and nonactive agents. Through the use of small tubes and thin layers of media, the extent of horizontal and vertical diffusion has been limited, thus rendering diffusion factors negligible.

Those compounds which were highly water insoluble and tested as nonaqueous solutions tended to crystallize on the surface of the medium after evaporation of the solvent. The occurrence of these crystals had no apparent effect on the growth of the microorganisms or on the suspected activity of the antimicrobial agents. For example, penicillin V, having the properties of low water solubility, antibacterial but not antifungal activity, demonstrated in this experiment no effect on the growth of the fungi while it did inhibit growth of the bacteria. Furthermore, when griseofulvin was tested it was shown that the surface crystals had no effect on the growth of *C. albicans*, against which this agent is ineffective, but it did exhibit its usual inhibitory effect on *T. mentagrophytes* (11). These findings were also substantiated in the experiment where the compounds were uniformly dispersed and added to the tubes in a thin film of the respective medium. In this instance the results did not vary from those observed in the original study.

A slight antifungal effect was noted when alcohol was used as a solvent for those agents tested against *T. mentagrophytes*, but this was easily overcome by merely extending the incubation period slightly and providing adequate control tubes for comparison.

The method appears to distinguish between fungistatic and fungicidal activity. This may be accomplished by extending the incubation period to 7 days or longer but not beyond 10 days, since the medium begins to show signs of drying. It would also be a simple matter to attempt to reculture the microorganism to determine the nature of the activity of the agent being studied.

Additional advantages afforded by this method are a minimum of expenditure for materials or apparatus, and that a relatively small amount of working area is required to test a series of compounds when compared to the space needed if Petri dishes are employed.

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Inhibition of Replication of Lee Influenza Virus in Tissue Culture by Puromycin

By K. S. PILCHER and J. N. HOBBS

Puromycin, an inhibitor of protein synthesis with general growth inhibitory properties, was found to inhibit replication of influenza virus in tissue culture in low concentrations without destroying all metabolic activity of the tissue. The aminonucleoside of puromycin, reported to be as effective as the complete compound against trypanosomes, was inactive as an inhibitor of the virus. Evidence suggests the mechanism of the virus inhibition is probably interference with protein synthesis, and that the antitrypanosomal activity has a different mechanism.

PUROMYCIN (I) is an antibiotic whose structure, proved by total synthesis by Baker *et al.* (1), is that of a nucleoside bound to an unusual amino acid.

Puromycin has been found to inhibit the growth of a variety of cells, including bacteria (2), protozoa (3, 4), and animal tumors (5). This compound has been shown to inhibit protein synthesis in several biological systems, including a cell free rat liver extract (6), Ehrlich ascites tumor cells, and rabbit reticulocytes (7). It was effective in curing experimental infections of mice and rabbits with several species of trypanosomes (3, 8), and also in the therapy of human trypanosomiasis (9).

Puromycin's mechanism of action seemed to offer a possible new approach to the inhibition of virus protein formation. During the course of this study, the inhibition of poliovirus replication in tissue culture by puromycin was reported (10).

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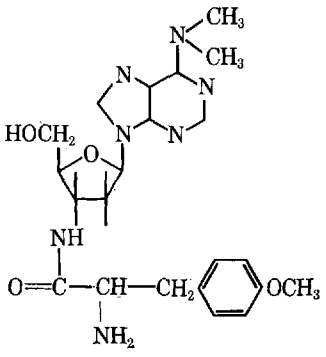
TABLE I.— INHIBITION OF REPLICATION OF LEE INFLUENZA VIRUS IN TISSUE CULTURE BY PUROMYCIN

	Concn. of Puromycin in Culture Fluid or Diluent, mcg./ml. 8.0	4.0	2.0	0
HA[a] units of virus/ml.[b,c] of tissue culture fluid	2.0	14.8	61.4	102.9
EID_{50} of virus/ml.[b,d] of tissue culture fluid	NT[e]	$10^{6.4}$	$10^{8.0}$	$10^{8.5}$
Infectivity of virus[d] exposed to puromycin 24 hr. at 35° in buffer at pH 8.0, EID_{50}/ml.	NT	$10^{7.4}$	NT	$10^{7.0}$
Effect of puromycin on outgrowth of chorio-allantoic cells in vitro	No growth	No growth	Definite cell growth	Definite cell growth

[a] HA indicates hemagglutinating units. [b] Virus hemagglutinin and infectivity measured after 44–48 hr. incubation on shaker at 35°. Initial virus concentration from inoculum about 5×10^5 EID_{50}/ml. [c] Each value is a geometric mean of 30–32 cultures from 5 experiments. [d] Values from a single representative experiment. [e] NT indicates not tested.



Puromycin (I)

MATERIALS AND METHODS

The Lee strain of influenza virus was employed in these experiments and was maintained as frozen chick embryo allantoic fluid stored at −60°. Puromycin and its aminonucleoside were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio.

The tissue culture system has been previously described (13). Each culture consisted of about 4 cm.² of chorio-allantoic tissue from 10-day chick embryos. The tissue was suspended in 2.0 ml. of Hanks' balanced saline (BSS) in 25 × 150 mm. Pyrex culture tubes.

The method of measuring virus concentration in culture fluids by hemagglutinin titration, and the method of determining toxicity for tissue cells have also been described (13). For the measurement of infectious virus, a series of decimal dilutions of the culture fluid was made and each dilution inoculated into a group of five 10-day chick embryos. After 48-hr. incubation, the number of infected embryos in each group was determined, and the number of 50% infectious doses (EID_{50}) per milliliter of the undiluted virus calculated.

RESULTS AND DISCUSSION

The combined results of 5 experiments designed to determine the effects of puromycin on virus replication are presented in the first row of data in Table I. Puromycin was incorporated in the fluids of the tissue cultures which had been inoculated with the Lee strain of influenza virus, and the concentration of the latter after incubation was determined by hemagglutination. It is evident that a concentration of 4 mcg./ml. reduced the virus yield markedly, and even 2 mcg./ml. produced a slight reduction, which was significant in view of the relatively large number of replicate cultures. In some experiments virus concentration was measured by infectivity titrations in 10-day chick embryos. Results of a representative experiment of this type are shown in the second row of Table I. In the presence of 4 mcg. of puromycin per milliliter the titer of infectious virus was 2 log units lower than in control cultures. Relatively few virus inhibitors are active at such low concentrations.

In vitro experiments in which the virus was exposed to a puromycin concentration of 4 mcg./ml. in a phosphate–glycine buffer[1] of pH 8.0 for 24 hr. at 35° yielded results which indicated that the titer of infectious virus was essentially the same as in control preparations similarly treated, but without puromycin. Data from such an experiment are shown in the third row of Table I. Thus, the compound seemed to have no direct effect on the virus itself. It was also found to cause no interference with the hemagglutination reaction, or with adsorption of the virus to the chick chorio-allantoic tissue. These observations pointed to an effect of the compound on the infected cell as the basis for inhibition of virus replication.

It was noted that fragments of chorio-allantoic tissues exposed to a puromycin concentration of 4 mcg./ml. for 44–48 hr. appeared very similar to those in control cultures, and some metabolic processes were still active, as indicated by continuing acid production in such cultures. Thus, gross or destructive tissue toxicity was not apparent. However, employing a more sensitive criterion, it was found that outgrowth of new cells from tissue fragments in roller tube cultures containing BSS, did not occur in the above concentration of the antibiotic. Cell growth did occur, however, in a concentration of 2 mcg./ml. It is of interest to note that the concentration of 4 mcg./ml. is close to that which Yarmolinsky and de la Haba (6) found necessary for marked inhibition of leucine-^{14}C incorporation into protein in a rat liver extract.

The biological activity of the aminonucleoside of puromycin or that portion of the molecule remaining after splitting off the amino acid, has been studied by several workers. In the experimental infection of mice with *Trypanosoma equiperdum*, both puromycin and its aminonucleoside were found

[1] Concentrations of buffer components in final mixture with virus were: glycine, 0.025 *M*; NaCl, 0.025 *M*; and Na_2HPO_4, 0.0167 *M*.

TABLE II.—REPLICATION OF LEE INFLUENZA VIRUS IN TISSUE CULTURES CONTAINING PUROMYCIN AMINONUCLEOSIDE

	Concn. of Aminonucleoside in Tissue Culture Fluid, mcg./ml.			
	400	200	100	0
HA units of virus/ml.[a,b] of tissue culture fluid	136	164	137	91

[a] Virus concentration measured as hemagglutinating (HA) units after 44–48 hr. incubation on shaker at 35.° Each value is a geometric mean of 15 cultures from 3 experiments. [b] Initial virus concentration from inoculum about 5×10^5 EID_{50}/ml.

about equally effective on a molar basis in curing the disease (11), and the *in vivo* activity of each was reversed by administration of adenine.

The aminonucleoside was compared with puromycin for its ability to inhibit virus replication in tissue culture. It was found that the former compound had no inhibitory activity in concentrations comparable on a molar basis to the effective levels of puromycin shown in Table I. Much higher concentrations resulted consistently in somewhat higher yields of virus in the culture fluid than those in control cultures. The results obtained are presented in Table II. In the experiments represented, tissue culture fluids containing 200 mcg. of the aminonucleoside per milliter were found to yield virus titers nearly twice that of controls. These experiments showed conclusively that the amino acid moiety of the puromycin molecule was essential for inhibition of influenza virus formation. They also indicated that the mechanism of action of puromycin against trypanosomes is apparently different from that responsible for the virus inhibition. A further difference was revealed by experiments in which attempts were made to block or reverse the virus inhibition by means of adenine, guanine, cytosine, uracil, and the corresponding nucleosides and nucleotides added to the tissue culture medium. In no case could any significant or reproducible reversal be demonstrated.

Preliminary experiments were performed to determine the activity of puromycin in mice infected with minimal doses of Lee influenza virus. These animals were given intraperitoneal injections of 1 mg. at 12–14-hr. intervals for 3 days. This treatment had no effect on the virus concentration in the lungs at the end of that period. Larger doses and more frequent injections should be tried, as well as administration by aerosol inhalation.

Yarmolinsky and de la Haba found that while puromycin inhibited protein synthesis in their rat liver extract system, the aminonucleoside showed only slight activity in equivalent molar concentrations (6). Rabinovitz and Fisher found the complete antibiotic molecule a potent inhibitor of protein synthesis in Ehrlich ascites tumor cells and in rabbit reticulocytes, while the aminonucleoside was inactive (7).

Several characteristics of the influenza virus inhibition by puromycin suggest that the mechanism probably depends on inhibition of protein synthesis. The concentrations required for the virus inhibition are very similar to those found necessary by the above investigators to inhibit incorporation of amino acids into protein. The aminonucleoside has been found to have little or no inhibitory activity for either virus replication or protein synthesis. In the virus tissue culture system described, when puromycin was present in inhibitory concentrations, multiplication of the host cells was also inhibited, without destruction of metabolic activity as measured by acid production. The amino acid moiety of puromycin, which was essential for virus inhibition, is *p*-methyoxyphenylalanine. *p*-Fluorophenylalanine is an antimetabolite which has also been found to interfere with protein metabolism (12). When combinations of puromycin and the latter compound were studied in the influenza virus system as previously reported for other inhibitor combinations (13), it was found that the degree of virus inhibition was no greater than that produced by either agent alone. This suggested a similar site of action for the 2 inhibitors.

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