Inhibitory Effect of Simple Aliphatic Amines on Influenza Virus in Tissue Culture. (28076)

E. M. JENSEN* AND O. C. LIU†
Smith Kline and French Laboratories, Research and Development Division, Philadelphia, Pa.

Ammonium ions in very low concentrations have been shown to inhibit completely the cytopathic effect (CPE) of influenza virus in tissue culture(1,2). This inhibition was accompanied by a very marked suppression of viral replication as measured by both infectivity and hemagglutination titrations. Subsequent studies(3) showed that this effect can be demonstrated in a number of cell systems, but that it appears to be specific for the influenza group of viruses. Since publication of our papers(1,3) we have become aware of an earlier report on this phenomenon. Higo, Kaneko, Kobayashi, Nakajima and Sasaki(4) described the effect of ammonium salts on influenza virus in suspended chorioallantoic membrane cultures. Although viral inhibition was measured only by hemagglutination titrations, the results obtained were very similar to ours, and those of Eaton(2). In view of these observations, it was of interest to determine whether or not organic derivatives of ammonia, *i.e.*, the lower aliphatic amines, might possess a similar activity. This paper presents the results observed with several primary, secondary and tertiary amines against influenza virus in tissue culture.

Materials and methods. The dog kidney cell culture employed in this study has been described(1) and was originally obtained from A. H. Fieldsteel, University of California School of Medicine. At the time of these studies, the cultures had undergone approximately 90 subpassages. As in the previous studies, the growth medium consisted of Eagle's minimum essential medium(5) supplemented with 10% calf serum. Maintenance medium consisted of the same medium without glutamine and only 2.5% calf serum.

Influenza A, strain PR8, virus was employed throughout this study, and stock cultures were prepared in embryonated eggs inoculated by the allantoic route. Virus dilutions were prepared in maintenance medium described above and all hemagglutination (HA) titrations were conducted by the method of Salk(6).

The compounds tested for antiviral activity were all used as the hydrochloride salts, and dilutions were prepared in maintenance medium. Whenever necessary, the pH of each compound solution was adjusted to approximately 7.0 before addition to the tissue cultures.

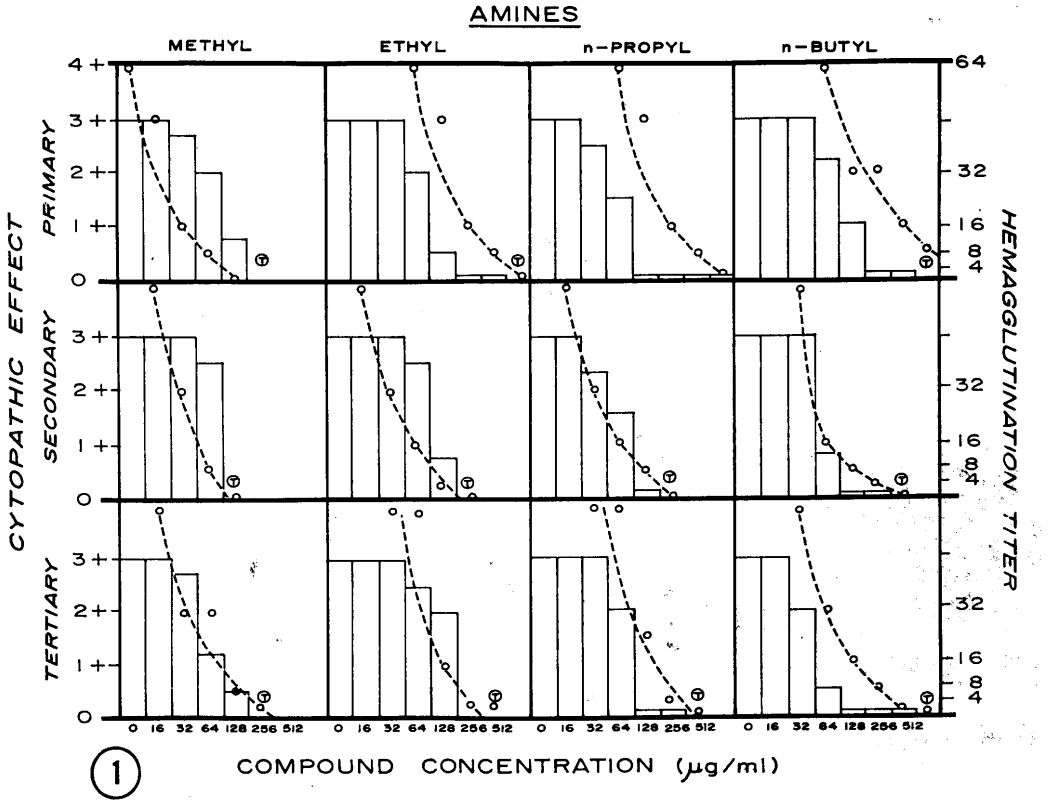
Virus inhibition studies were conducted by a tube dilution method which has been described(1), and virus replication was recorded both as CPE and HA titers. In all studies, a one hour period of virus adsorption was allowed after which the medium was changed and, where necessary, compound was readded. CPE was generally observed at 72 hours post infection and recorded on a scale of 0 for no cellular degeneration to 4+ for complete destruction of cells. Compounds are recorded as toxic at the lowest concentration which shows any effect upon the normal structure of the cells.

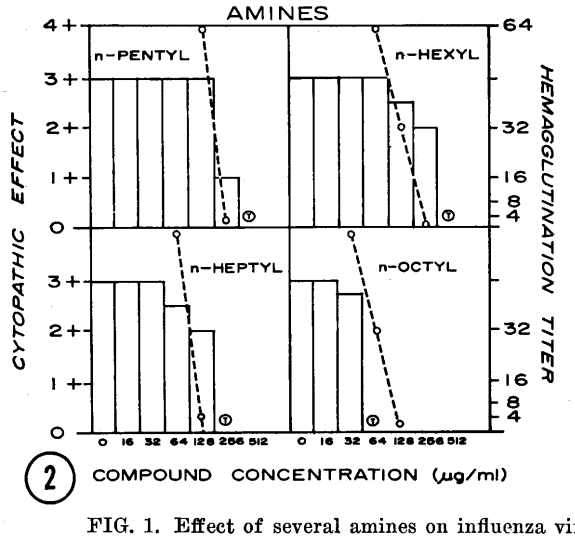
Results. To determine the presence or absence of a pattern of antiviral activity in the lower amines, the primary, secondary and tertiary forms of methyl-, ethyl-, n-propyl- and n-butylamines were used. Compounds with longer hydrocarbon chains were available only in the primary forms and were tested as high as the 8-carbon chain; and for simplicity, only the normal chain forms were used. The inhibitory effects of the lower amines on influenza virus are shown in Fig. 1. CPE is shown by the bar graphs; HA titers, by the broken lines; and compound concentrations are indicated as μg/ml of the hydrochloride salt. Effective levels of compounds are arbitrarily selected as the lowest level which reduced the CPE to 1+ or less.

Taking the primary amines as a group, it is

* Present address: John L. Smith Memorial for Cancer Research, Maywood, N. J.

† Present address: National Drug Co., Germantown, Pa.





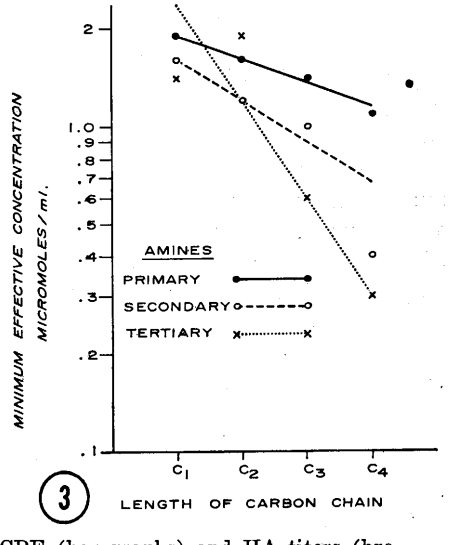


FIG. 1. Effect of several amines on influenza virus CPE (bar graphs) and HA titers (broken lines). Minimum toxic level indicated by encircled T.

FIG. 2. Effect of higher primary amines on influenza virus CPE (bar graphs) and HA titers (broken lines). Minimum toxic level indicated by encircled T.

FIG. 3. Relationship between carbon chain length and minimum effective level (μmoles) of each series of amines.

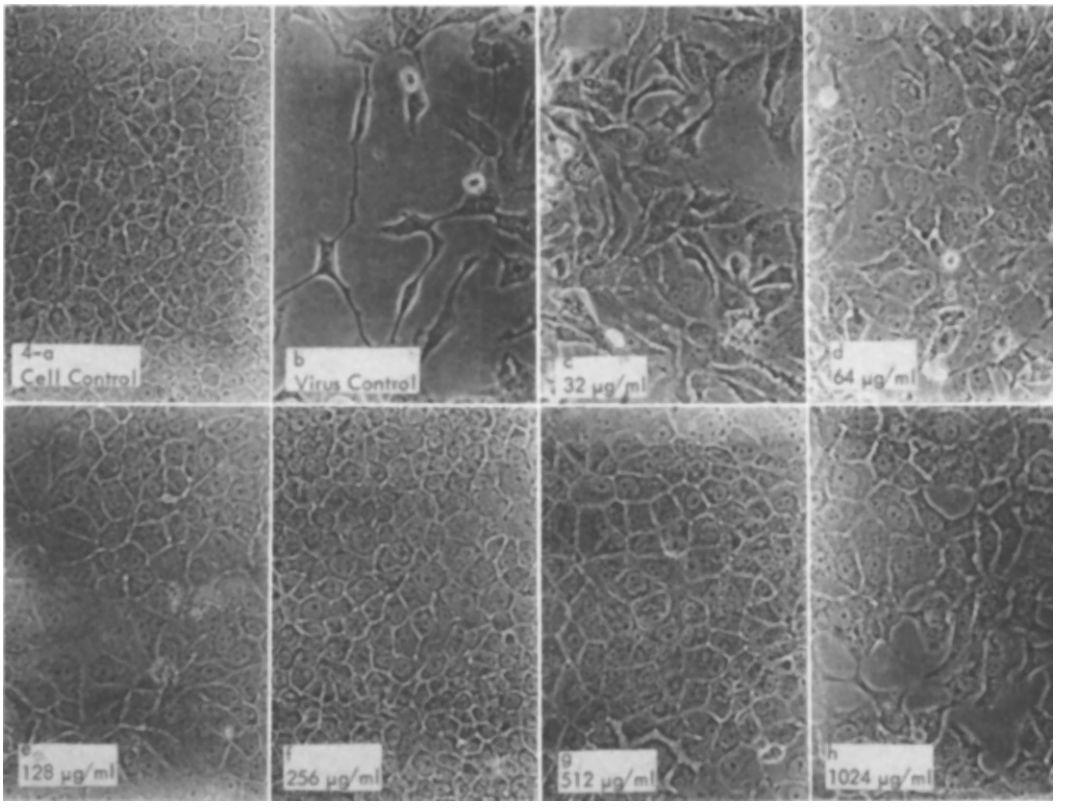


FIG. 4. Phase contrast photomicrographs illustrating protective effect of n-propylamine against influenza virus CPE in dog kidney cells. Fig. c through h show treated cultures. (Magnif. 177×.)

apparent that lengthening of the carbon chain increases the therapeutic index up to n-propylamines. This increase in therapeutic index appears to be due to an increase of minimum toxic concentration (MTC) rather than a lowering of the effective level. The MTC of n-propylamine is 2048 μg/ml, whereas n-butylamine is slightly more toxic and consequently less effective. With the higher primary amines, as shown in Fig. 2, the toxicity increases markedly with increased chain length with a consequent loss of antiviral activity. The secondary and tertiary amines studied (Fig. 1), also show a similar pattern, *i.e.*, increasing therapeutic index with increasing chain length.

In all cases, the suppression of viral CPE was accompanied by a corresponding drop in HA titer. In Fig. 1 and 2, HA titers are plotted on an arithmetic scale to correspond with the CPE scale. With those compounds showing a significant therapeutic index, the HA titers indicate a slight degree of viral synthesis at the levels which completely suppress CPE.

Although with increasing molecular weight, the hydrochloride portion of the salt becomes less significant, the effectiveness of each series can be realistically compared only on a molar basis. Fig. 3 shows the effective level of each compound in micromoles per milliliter when plotted against carbon chain length. In each series, the concentration required for antiviral activity decreases with increased chain length.

The degree of protection from the virus by n-propylamine is illustrated in Fig. 4a through 4h. These phase contrast photomicrographs of dog kidney epithelial cells show that as little as 32 μg/ml affords a slight but definite protective effect which increases with concentration. Although a slight toxic effect (vacuolation) is observed at 512 μg/ml, cell destruction appears only at

TABLE I. Period of Sensitivity of Influenza Virus to n-Propylamine.

Time of treatment with 256 μg/ml of n-propylamine HCl	Cytopathic effect	HA titer
Virus control	3-4+	256
1 hr before infection	0+	<2
Zero time	0+	<2
1 hr after infection	2-3+	128
2 " " "	3+	128
3 " " "	3-4+	256

1024 μg/ml; and even at this concentration, the majority of the cells appear intact. The degrees of CPE shown in these figures do not match exactly those shown in Fig. 1 since the latter graphs represent average results from several observations made with tube cultures and with the less sensitive bright field optical system.

Eaton and Scala(7) have shown that the period of sensitivity to inhibition of influenza virus by ammonia is less than one hour after infection. This appears also to be true of the lower amines as shown by the following study with n-propylamine. Plastic T-30 flask cultures of dog kidney cells were treated with 256 μg/ml of propylamine hydrochloride one hour before infection, at zero time and at 1, 2 and 3 hours post infection. One hour after the last treatment, all cultures were washed free of unadsorbed virus and compound, and refed with new maintenance medium. Compound was readded to those cultures which had been treated. Table I shows the results obtained after 72 hours. Both CPE and HA titers indicate that pretreatment or simultaneous treatment is necessary for protection from the virus. A delay in treatment of only one hour results in no significant protection.

Discussion. Since the lower amines are organic derivatives of ammonia, it was of interest to determine whether or not compounds in this series might possess virus inhibitory properties similar to those of ammonia. These studies show that most amines with aliphatic chains up to 4 carbons in length do inhibit influenza virus synthesis, although in some cases the activity appears to be masked by the toxic effects.

The means by which ammonia and lower amines inhibit viral synthesis have not been determined, but from this study it would appear that similar, if not identical, mechanisms are involved. Both ammonia(1,7) and the amines must be administered before or at the time of infection for inhibition; a delay of one hour or more results in no significant inhibition. When expressed on a molar basis, as shown in this and the previous(1) studies, approximately equal concentrations, 0.3 to 2.0 micromoles/ml, are required for inhibition. In view of the activity of the organic amines, it appears unlikely that any mechanism involving the transfer of ammonia groups of glutamine, such as in purine synthesis, is involved. Since ammonia and the amines studied have similar basic qualities with approximately equal dissociation constants, the inhibition may be due to interference with some ionic mechanism in the early stages of virus absorption or replication.

Summary. Several primary, secondary and tertiary amines have been shown to inhibit influenza virus in tissue culture. Compounds with the greatest therapeutic index are n-propylamine and tertiary butylamine. The virus inhibitory effect of these amines is similar to that produced by ammonia and it is postulated that the mechanisms of inhibition may be the same for these compounds.

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