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The Antiviral Activity of Caprochlorone. (32076)

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Although many different compounds have been shown to suppress viral replication *in vitro,* caprochlorone [levo-4-phenyl-4-(2-chlorobenzyl)-5-oxy-hexanoic acid] is one of the few which provides consistent activity against a viral infection in animals(1,2). A prophylactic treatment regimen produced an increase in survival of mice infected with a strain of influenza A/PR8. Mean survival time of mice was increased, and virus content of lungs was suppressed. Inhibition of virus HA titers was also demonstrated in embryonated and deembryonated eggs infected with the same strain of influenza; activity was ascribed to intracellular inhibition both early and late in the infectious process(2,3).

Paucity of information on the spectrum of activity of caprochlorone and its action mechanism prompted the present investigation using cell culture systems. Studies with antiviral compounds l-adamantanamine·HCl (4,5,6) and ammonium phosphate(7,8,9) were carried out in conjuction with those using caprochlorone to better elucidate the site of compound action.

Materials and methods. Viruses. Influenza strains A/WSN, A/PR8, and B/Maryland were used for action mechanism studies. The former was obtained from W. Henle, the latter 2 from the American Type Culture Collection. Viruses were propagated in embryonated eggs; virus in allantoic fluid was partially purified by 2 cycles of centrifugation with sedimentation at 26, 700 $\times$ *g* for 1 hour and resuspension in phosphate buffered saline (pH 7.6; PBS) containing 0.1% bovine serum albumin (PBS/BSA). Other viruses, used for antiviral spectrum studies, were grown in appropriate cell culture systems or embryonated eggs. All specimens were stored at —60°C prior to use.

Compounds. Caprochlorone, l-adamantanamine·HCl and dibasic ammonium phosphate were employed. Solutions were prepared in maintenance media; pH of media was adjusted to 7.6 by appropriate addition of NaOH or HCl.

Virus antisera. Antisera to influenza strains A/WSN, A/PR8, and B/Maryland were prepared in adult White Leghorn roosters. Ten ml of infected allantoic fluid was inoculated intravenously, and 2 ml intramuscularly, into each bird. After 7 days, birds were similarly reinjected. Ten days later serum was collected. Prior to use, serum was mixed with freshly trypsinized chick embryo (CE) cells (2×10^7 cells/ml of serum) for 2 hours at 37°C to adsorb non-viral antibody. The serum, clarified by centrifugation, was heated to 56°C for 30 minutes and then treated with potassium periodate(10) to remove non-specific inhibitors. Hemagglutination inhibition tests, using 4 units of corresponding virus, provided titers of 1024, 512, and 640 for A/WSN, A/PR8, and B/Maryland, respectively.

Media. Liquid medium for maintenance of CE cell cultures (MM) was composed of Earle's saline with 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 10% tryptose phosphate broth, 2mM L-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin·SO_4. Medium for plaque inhibition tests with human embryonic lung (HEL),

strain WI-26, has been previously described (11). The same medium was employed for HEp-2 cells.

Cell culture. Monolayer cultures of CE cells (11) and HEL cells(12) were established in 100 mm diameter plastic Petri dishes. Similar cultures of HEp-2 cells were prepared by the technic used for HEL cells.

Antiviral spectrum. Tests for inhibition of hemagglutinin production were carried out as described for Growth Curve Experiments (below). Compound in MM was added to cultures immediately after virus adsorption. Total content of virus in cultures was determined after a 12-hour period of incubation at 37°C in an atmosphere of 5% CO_2. The plaque techniques employed for rhinovirus (12) and for several different viruses in CE cells(11) have been described. The latter procedure was also employed for the remaining viruses in CE cell cultures using a 4-day period of incubation. Plaques were produced with herpes zoster(13) and respiratory syncytial viruses in HEL cells. A 1-hour interval was used for adsorption of infected cells or virus, respectively, to cell monolayers. Six ml of HEL maintenance medium was added with an additional 6 ml after incubation for 4 days. Plaques were visible after 11 days following staining with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride (INT, 14). Plaques with Coxsackie were obtained in HEp-a cells after incubation for 4 days using 6 ml HEp-2 maintenance medium and terminal INT staining. In all tests for plaque inhibition, cellulose discs (0.25 inches in diameter), impregnated with compound, were placed on the maintenance medium immediately after its solidification. Antiviral activity was denoted by a plaque-free zone surrounding the disc or zone of cytotoxicity.

Virus inactivation. Virus at an approximate concentration of 10,000 EID_{50}/ml in MM was incubated at 37°C for 24 hours in the presence and absence of compound. Effect of compound on stability of the virus was then determined by comparative titration of samples in embryonated eggs.

Adsorption and elution. Compound mixed with 0.5% chicken rbc and 100 HA units of influenza A/WSN per ml in PBS was incubated at 37°C. At different intervals after mixing, from zero time to 24 hours, rbc were sedimented by centrifugation and virus in the supernate was titered by hemagglutination. Amount was compared with that obtained with an untreated control.

Action on viral neuraminidase. Neuraminidase obtained from influenza B/Lee (General Biochemicals) was assayed for activity against N-acetyl neuramin-lactose substrate in the presence of compound(11).

Growth curve experiments. One ml of virus diluted in PBS/BSA was added to each culture of CE cells to provide a ratio of approximately 2 EID_{50} of virus per cell. After 5 minutes at 25°C, unadsorbed virus was removed and cultures were washed quickly 3 times with 4 ml of PBS. At this time (considered zero time), 5 ml of MM with or without compounds was added; and cultures were incubated at 37°C. In experiments where sequential treatment with antiserum or acidification was employed, the initial incubation medium was removed from both treated and control cultures; and the cultures were washed 5 times with 8 ml of PBS. Antiserum treatment or acidification was then performed as described below. Five ml of fresh MM was subsequently added and cultures were reincubated. Duplicate cultures were employed in each series of each experiment and were pooled for assay. Extracellular, intracellular, or total virus content of cultures was determined by hemagglutination at different times after infection.

Extracellular virus content was provided by titration of culture medium. Intracellular virus was that released by disrupting the washed cells of a culture with 3 cycles of freezing and thawing (between −70°C and 37°C) into 5 ml of fresh MM. Total virus content of cultures was the amount obtained after similar disruption into the incubation medium.

Acidification. In growth curve experiments, infected cultures were acidified as follows. After removal of MM, 4 ml of PBS adjusted to pH 2.0 with HCl were added for 1 minute. The solution was then removed, the cultures washed with 8 ml of PBS, and MM replaced.

TABLE I. Antiviral Spectrum of Activity of Caprochlorone *in vitro*.

Virus	Cell*	Method†	Activity‡
Influ. A/PR8	CE	HA	+
" A/WSN	CE	HA, PI	+
" A/NWS	CE	HA, PI	+
" A1/FM1	CE	HA	+
" B/GL	CE	HA	+
" B/Md.	CE	HA	+
Newcastle disease, Roakin	CE	HA	+
" " , Victoria	CE	HA	+
Parainfluenza 1/Sendai	CE	HA	+
Influ. A/Swine	CE	HA	±
" A/WS	CE	HA	±
" A2/Japan 305	CE	HA	±
" B/Lee	CE	HA	±
Vaccinia, WH	CE	PI	±
Coxsackie B-1/Conn. 5	HEp-2	PI	—
Herpes simplex/HF	CE	PI	—
Herpes Zoster/EY	HEL	PI	—
Influ. A/Jpt	CE	PI	—
Pseudorabies/Aujeszky	CE	PI	—
Respir. Syncytial/Long	HEL	PI	—
Rhino/1059	HEL	PI	—
Semliki Forest/original	CE	PI	—
Vesicular stomatitis/Indiana	CE	PI	—

* CE = chick embryo; HEL = human embryonic lung, WI-26; HEp-2 = human epidermoid carcinoma.

† HA = hemagglutination production; PI = plaque inhibition.

‡ + = ≧ 25 mm diameter zone of PI or ≧ 4-fold inhibition of HA; ± = < 25 mm diameter zone of PI or 2-fold inhibition of HA; — = no activity. Forty μg/ml was used in HA tests; 100 μg/disc, for PI.

Antiserum treatment. Treatment with virus antiserum was accomplished by adding 3 ml of a dilution of MM which contained 100 HI units/ml to each infected culture following removal of MM; after 30 minutes cultures were washed 5 times with 8 ml of PBS. Five ml of MM were then added and cultures were reincubated.

Results. Toxicity. Caprochlorone was well tolerated for maintenance of CE cells in MM at 40 μg per ml over a 4-day period of incubation as determined by microscopic examination and protein content of cultures as compared with untreated controls. Eighty μg per ml produced slight cytopathic alterations and 25% diminution of protein content under similar conditions. Forty, but not 80, μg/ml permitted optimal growth of CE cells through 15 generations in growth medium (growth medium, 11).

Antiviral spectrum. As shown in Table I, 9 strains of virus were clearly sensitive to inhibition by caprochlorone; marginal activity was obtained against 5 additional strains, and 9 strains were not sensitive under the conditions employed. With the exception of vaccinia all those against which activity was obtained are myoxoviruses. Activity was demonstrated not only by inhibition of hemagglutinin production, but also by plaque inhibition. Although several myxoviruses are represented in the refractory group, the majority are not members of this taxonomic category.

Dose response. The relationship of caprochlorone concentration to antiviral activity was studied using CE cell cultures infected with influenza A/WSN as described under Growth Curve Experiments. Ten to 80 μg/ml of compound were added in 10 μg increments to cultures immediately after infection. After incubation for 12 hours, total hemagglutinin content was determined relative to that in untreated cultures. Inhibition of viral production was directly proportional to caprochlorone concentration from 5 μg/ml, the limit of activity as determined by extrapolation, to 80 μg/ml at which a 1.3 log inhibition was obtained.

Action mechanism. Virus inactivation. Eighty μg of caprochlorone per ml was not virucidal in that it did not enhance the rate of influenza A/WSN, A/PR8, or B/Maryland inactivation over a 24-hour period at 37°C.

Adsorption and elution. In the presence of 80 or 40 μg/ml of caprochlorone or unsupplemented media, 92% of influenza A/WSN was adsorbed to rbc within 10 minutes. At 37°C virus then gradually eluted from the cells at the same rate with or without compound in the medium. After 24 hours, added virus was essentially recovered in all series. These data indicate no effect of caprochlorone on interaction of virus with specific cell receptors or on viral neuraminidase.

Further study of viral neuraminidase. The enzymatic action of purified neuraminidase from influenza B/Lee on N-acetyl neuraminlactose was not inhibited by 80 μg of caprochlorone per ml of incubation mixture.

Time of action. The foregoing observations

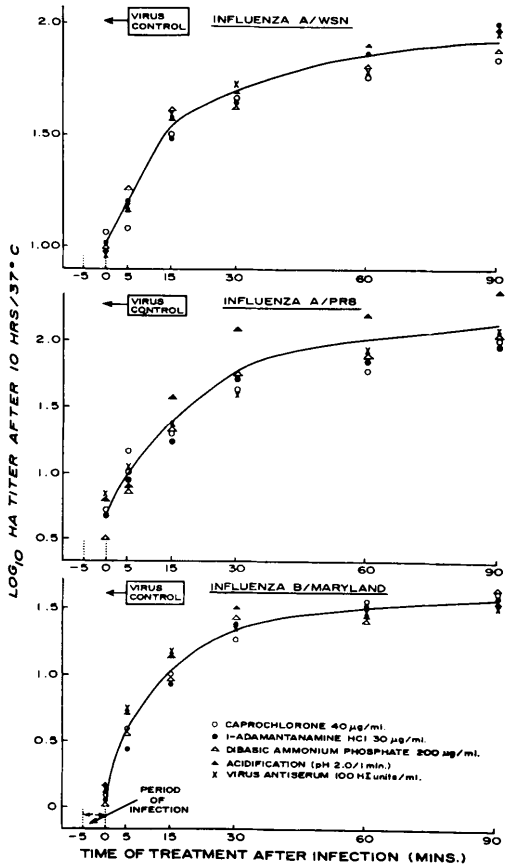

FIG. 1. Time of action of compounds, virus antiserum, and acidification.

suggested action of caprochlorone on an event subsequent to viral adsorption. Activity with respect to time of addition after virus adsorption was therefore examined in growth curve experiments to provide information on the stage of viral replication subject to inhibition. CE cell cultures were infected with influenza A/WSN, A/PR8, or B/Maryland. Caprochlorone at 40 µg/ml was added to the culture medium at different intervals after virus adsorption. After incubation for 10 hours (1 cycle of virus replication) total virus content of cultures was determined by hemagglutination. As shown in Fig. 1, inhibition was greatest when compound was added immediately after infection. Activity rapidly diminished with delay of compound addition during the initial 30-minute interval after infection. The observed rapid loss of activity was demonstrated with all 3 viruses. Parallel studies were carried out with l-adamantanamine·HCl, ammonium phosphate, virus antiserum, and acidification. l-Adamantanamine·HCl was used at 30 and ammonium phosphate at 200 µg/ml; these represented maximum tolerated concentrations for maintenance of CE cells over a 4-day period of incubation. With all 3 viruses, time of action for all compounds, for specific virus antiserum, and for acidification was similar. With influenza A/PR8 only, later acidification consistently produced a slightly higher yield of virus.

Sequential treatment. Inhibition of A/WSN, A/PR8, and B/Maryland infections was examined when initial 2-hour incubation with compounds was followed by compound removal and subsequent treatment with viral antiserum or acidification (Table II). Parallel controls indicated that inhibition was always obtained when compounds, added to cultures at zero time, were retained in the medium for the entire 12-hour incubation period. Little or no inhibition was obtained when added 2 hours after infection. When added at zero time, removed after 2 hours, and replaced with MM, inhibition was reversed except with B/Maryland infected cultures which had been treated with caprochlorone or l-adamantanamine·HCl. The effect of sequential treatment on the latter cultures could not, therefore, be studied. In all other instances, specific viral antiserum added after 2 hours of compound treatment retained inhibition whereas no inhibition was obtained when added 2 hours after incubation of infected cultures in MM. Similar results were obtained when acidification was employed in lieu of antiserum treatment for A/WSN infected cultures, but not for cultures infected with A/PR8 or B Maryland. In these instances no effect was shown following 2 hours treatment with any of the compounds.

To account for the apparent lack of reversal of inhibition of B/Maryland-infected cultures with caprochlorone and l-adamantanamine·HCl, it was postulated that compound may have caused virus to become more loosely attached to the cell, resulting in loss of virus in wash fluids during subsequent manipulations. However, assay of fluids removed or

TABLE II. Sequential Treatment of Cultures Infected with Influenza A/WSN, A/PR8, or B/Maryland with Compounds and Antiserum or Acidification.

Treatment after infection		Increase in virus titer† (log_{10} HA units/ml)		
Initial (0-2 hr)	Final* (2-12 hr)	A/WSN	A/PR8	B/Maryland
—	—	2.00	1.85	2.10
Caprochlorone	Caprochlorone	.90	.90	.60
—	"	1.95	2.15	1.95
Caprochlorone	—	1.90	1.85	.60
"	Antiserum	1.00	1.00	—
"	Acidification	.90	1.90	—
—	—	2.10	2.80	2.20
$(NH_4)_2HPO_4$	$(NH_4)_2HPO_4$	1.00	1.20	.30
—	"	2.00	2.30	2.00
$(NH_4)_2HPO_4$	—	2.00	2.80	2.10
"	Antiserum	1.00	1.15	.30
"	Acidification	.95	2.50	1.80
—	—	2.20	2.30	2.10
l-Adamantanamine • HCl	l-Adamantanamine • HCl	1.15	1.00	.75
—	"	1.95	2.25	1.95
l-Adamantanamine • HCl	—	2.10	2.50	.70
"	Antiserum	1.15	1.10	—
"	Acidification	1.05	2.40	—
—	Acidification	2.10	2.40	2.05
—	Antiserum	2.05	2.35	2.00

* Viral antiserum: 300 HI units for 30 min followed by MM. Acidification: PBS at pH 2.0 for 1 min followed by MM.

† Mean values from 5-12 experiments.

used in washing the cultures disproved this hypothesis: pooled wash fluids (3 × 4 ml) from cultures incubated for 2 hours after infection with caprochlorone or MM provided titers (EID_{50}) of $10^{3.5}$ and $10^{4.5}$/.2 ml, respectively. Indeed, data suggest that compound may even enhance the binding of virus to the cell surface.

Virus release. A compound with surface-active properties providing inhibition of cell penetration by virus might likewise inhibit release of newly formed virus. To investigate this possibility with caprochlorone, compound at 40 μg/ml was added to CE cell cultures 2 hours after infection with influenza A/WSN. Virus content of incubation medium and washed cells in fresh medium was subsequently determined after different intervals of incubation (Fig. 2). A marked inhibition in rate of release of cell-associated virus was observed with respect to untreated controls. Similar experiments with l-adamantanamine • HCl and ammonium phosphate showed no such effect on release of virus from the cell.

Discussion. The *in vitro* activity of caprochlorone differs from that of l-adamantanamine • HCl (6) although both compounds are predominantly active against myxoviruses. Several different myxoviruses are also susceptible to the action of ammonium ion (7,8,9) although its spectrum of activity has not been extensively documented.

Two stages of infection have been shown susceptible to inhibition by caprochlorone: penetration of the infectious unit into the cell, and later, release of newly formed virus.

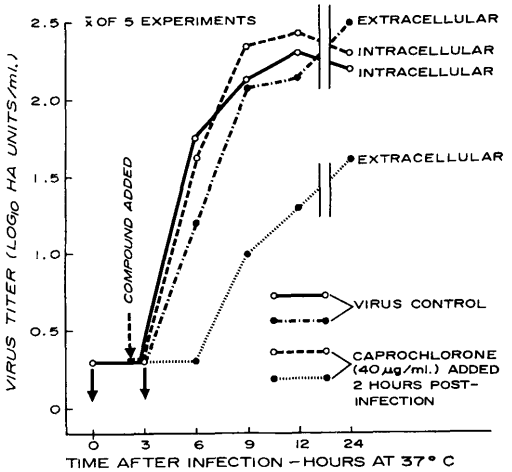

FIG. 2. Kinetics of virus production in presence of caprochlorone-compound added 2 hr post infection.

Sensitivity of the penetration step to inhibition by caprochlorone was indicated by ability of compound to inhibit virus replication at a locus permitting virus inactivation by antiserum. Similar results obtained with l-adamantanamine · HCl and ammonium ion confirm previous findings for these compounds (4,5,8) not only with similar but also with additional viruses to those employed. Use of antiserum as a reference for penetration has been justified by observations that sensitivity of cell-adsorbed virus to antiserum is rapidly lost by a temperature-dependent step occuring at the cell membrane(15,16,17).

The mechanism of influenza entry into the cell is not clear. Previous studies suggest that the initial temperature-dependent step leading to infection is either engulfment of the intact virus particle(18) or uncoating of the virus prior to penetration of the component nucleic acid(19). Further clarification of compound action must await a better understanding of the early stages of infection with influenza.

Acid lability, like antiserum sensitivity, is presumed to be characteristic of certain viruses in an extra- but not intracellular state (20,21). Therefore, acidification was used as an alternate treatment to that with antiserum to study the mechanism of compound action. Results obtained were comparable to those with antiserum in A/WSN-infected cultures. In contrast, however, acidification had no effect on A/PR8 infection following 2-hour treatment with any of the 3 compounds. This difference was also observed following ammonium phosphate treatment of cultures infected with B/Maryland. The reason for discrepancy in action of antiserum and acidification following compound application in these instances remains obscure. With influenza A/PR8 and B/Maryland but not A/WSN, the interaction of compound, cell, and virus in some manner stabilizes the infectious unit to acid pH inactivation.

The slight, but consistent, stimulation of influenza A/PR8 production following acidification at later periods of time probably resulted from a physiolgic modification of the culture.

Contrary to experience with cultures infected with influenza A/WSN or A/PR8 the inhibitory action of caprochlorone and l-adamantanamine · HCl but not that of ammonium ion, was retained by B/Maryland-infected cultures after washing. This effect did not result from a compound-mediated release of virus from the cell with subsequent loss during washing manipulations. The differences observed in reversal of compound inhibitory action, as with acid pH effect following compound treatment, point to differences in the nature of the complex formed by virus, cell, and compound which are not now evident.

Although caprochlorone, l-adamantanamine · HCl and ammonium ion produce similar inhibition of virus penetration, only caprochlorone delays release of virus from the cell. In this respect, the activity of caprochlorone, at least superficially, resembles that of α-amino-p-phenylmethane-sulfonic acid which inhibits an early stage of infection with influenza and also release of newly synthesized virus(22).

Summary. Caprochlorone demonstrates clear-cut or suggestive activity in cell culture systems against 15 myxoviruses and vaccinia, while 9 other virus strains are insensitive. The action mechanism of caprochlorone against influenza A/WSN, A/PR8, and B/Maryland was compared with that of l-adamantanamine · HCl, ammonium phosphate, viral antiserum, and acidification. The time at which viral replication could be inhibited, up to 15 minutes after addition of virus, was similar in all cases. This inhibition resulted from failure of virus to penetrate the cell membrane as indicated by sensitivity of virus to neutralization by antiserum even 2 hours after addition of virus and inhibitor. Reversal of inhibition of A/WSN and A/PR8-infected cultures treated for 2 hours is accomplished by removing the compounds and replacing with medium; at this time, acidification inactivates A/WSN, whereas A/PR8 is refractory. Inhibition of B/Maryland-infected cultures with ammonium phosphate is reversed by removal of compound after 2 hours, but resistance to acidification is shown. By contrast, caprochlorone or l-adamantanamine · HCl inhibition of B/Maryland-infected cultures could not be reversed. In addition to in-

hibition of cell penetration by virus, caprochlorone delayed release of newly formed virus from the cell, whereas l-adamantanamine · HCl and ammonium phosphate did not.

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Histopathology of Guinea Pigs with Cholesterol-Induced Anemia.* (32077)

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Hemolytic anemia, induced in guinea pigs by dietary cholesterol, was accompanied by enlargement of liver and spleen(1) and by hyperplasia of the bone marrow(2). Other organs did not show any gross changes, although the lipid composition of the liver, spleen(3), bone marrow(2), and adrenal(4), as well as that of lung, kidney and heart (unpublished), was greatly altered. We have now subjected these organs to a thorough microscopic examination in an attempt to determine the mechanism of the anemia.

Materials and methods. Male guinea pigs, weighing about 200 g, were fed a semisynthetic diet(2) with or without the addition of 1% cholesterol. Development of the anemia was monitored by red blood counts. The time required to produce the anemia varied from 10-14 weeks. When the red cell count had fallen to about 3.5 mil/mm^3, the animal was sacrificed and the liver, spleen, lung, kidney, adrenal, heart, pancreas, and testis were fixed in 10% buffered neutral formalin. The ends of

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