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STUDIES ON VIRUS-INHIBITING SUBSTANCES OF BACTERIAL ORIGIN

II. INHIBITION OF ANIMAL VIRUS PLAQUE FORMATION BY CULTURE FILTRATES OF LACTIC ACID BACTERIA

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SYNOPSIS

Filtrates of the culture media of *Streptococcus faecalis, Lactobacillus acidophilus* and *Bifidobacterium bifidum* (of both rod and bifid forms) were shown to inhibit plaque formation *in vitro* by human-pathogenic viruses, e.g., herpes simplex type 1, chikungunya, influenza type A. Poliovirus type 1 was not affected by the same filtrates. The virus-inhibiting activity paralleled amounts of lactic and acetic acids which were produced by the bacteria and which were contained in the culture fluids. The drop of pH of fluids showed the same effect. It was believed, therefore, that the virus-inactivating action as observed was mainly, if not entirely, attributed to the production of organic acids and subsequent drop of pH. At the same time certain differences were noted between the virus-inhibitory effects of organic acids and of pH; residual amounts of the active viruses were detected in buffered solutions of any of the test pH's while the same viruses suspended in the organic acid-containing culture filtrates of the same pH's were more strongly affected. The significance of the data for the human viral infections is considered in this connection.

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INTRODUCTION

Virus-inhibiting capacities of bacterial culture filtrates have been recognized in the literature. The bacteria used for tests included Achromobacter, Pseudomonas, Proteus, Streptococcus, Staphylococcus, *Bacillus subtilis*, Corynebacterium, *Escherichia coli*, etc. Groupé *et al* [2,3] reported that a substance derived from a culture filtrate of *Pseudomonas viscosa* (termed 'viscosin' by them) inhibited infections of bronchitis virus in embryonated eggs and of influenza A virus in mice. The same authors moreover demonstrated that the plumonary lesions of influenza-infected mice were suppressed by acid-precipitable materials (APM) from a culture filtrate of Achromobacter sp. 134, which, however, failed to inactivate the virus particles directly. Recently Mahdy *et al*[8] described a substance derived from a *Proteus mirabilis* culture filtrate, which inhibited plaque formation of Sindbis and vesicular stomatitis viruses, without any direct inactivation effect on the virus particles themselves.

In the present study some so-called lactic acid bacteria were chosen as the subjects of tests and filtrates of culture media in which they were grown were examined for their actions of inhibiting viral plaque formation *in vitro*.

MATERIALS AND METHODS

Viruses.

Test viruses were: Herpes simplex virus type 1 (HSV-1) and poliovirus type 1 (polio-1) grown in HeLa-S_3 cell cultures; chikungunya virus (CHV) grown in BHK-21 cell cultures; and influenza virus A-2 (Infl-A2) grown in Vero cell cultures. The culture medium consisted of Eagle's minimum essential medium (MEM), 10% calf serum and 60 μg/ml kanamycin. Fluids harvested from infected cultures were used as viral inocula which, when needed, were diluted with Sörensen's phosphate buffered saline (PBS, pH 7.0) containing 0.02% bovine serum albumin as a virus-stabilizer.

Virus Titration.

Two-tenths ml of virus suspension was inoculated into each of the cell cultures in 3 oz. bottles and incubated at 37°C for 1 hr. to allow the complete cell-virus adsorption. After removing the excess fluid, 5 ml of overlay

medium was introduced, which consisted of Eagle's MEM, 1.2% methylcellulose (MC), 10% calf serum and 60 μg/ml kanamycin; in the case of polio-1, 1% Bacto-agar was used instead of the MC. After incubation at 37°C for 4 days, the cells were stained with 0.1% crystal violet in ethanol (except for the case of polio-1 in which the double overlay method using 0.004% neutral red solution was applied), and plaques which had formed were counted.

Bacteria and Bacterial Culture Filtrate.

Streptococcus faecalis (SF), *Lactobacillus acidophilus* (LA) and *Bifidobacterium bifidum* (BB) were used. Of the BB, the rod form (*r*BB) and bifid form (*b*BB) (Kojima *et al*[6]) were tested separately. The SF and *r*BB were cultivated in a "basic medium" consisting of VF bouillon, 1% glucose and 0.04% cystine. A medium consisting of the basic medium and 0.1% tween-80 was used for the LA, and the basic medium plus 1.4% NaCl for *b*BB. The SF and LA were cultured aerobically, and the BB anaerobically by the alkali-pyrogallol method. Temperature of incubation was 37°C throughout. Twenty hr. after the beginning of incubation the bacterial cells were removed by centrifugation (10,000 *g*, 15 min.) and the supernatant was passed through a membrane filter (Millipore Corporation, U.S.A., 0.45 μm pore size) for sterilization and used for antiviral tests.

RESULTS

Effect of Bacterial Culture Filtrate on Viral Plaque Formation.

Typical examples of the results obtained are summarized in Table 1 and Fig. 1. The HSV-1, CHV and Infl-A2 were inactivated by being mixed with the culture filtrates. The inactivation was practically complete within 60 min. at 37°C. Under the conditions studied, the action of LA filtrate was strongest, and that of BB comparatively weak; however, no difference was noted between *r*BB and *b*BB. The poliovirus resisted the action of culture filtrates.

Table 1 Virus-inactivating activity of culture filtrates of lactic acid bacteria.

Virus	Bacterium	Infective titer of virus-culture filtrate mixture (PFU/0.2ml)	
		Before incubation	After incubation at 37°C for 60 min.
HSV-1	*S.faecalis*	1.2×10^4	0
	L.acidophilus	1.2×10^4	0
	B.bifidum (*r*)*	1.2×10^4	0
	(*b*)	1.2×10^4	0
CHV	*S.faecalis*	1.1×10^4	0
	L.acidophilus	1.1×10^4	0
	B.bifidum (*m*)	1.1×10^4	3+
Infl-A2	*S.faecalis*	4.3×10^4	0
	L.acidophilus	4.3×10^4	0
	B.bifidum (*m*)	4.3×10^4	0
Polio-1	*S.faecalis*	7.8×10^2	6.3×10^2
	L.acidophilus	7.8×10^2	7.9×10^2
	B.bifidum (*m*)	7.8×10^2	11.0×10^2

One part of virus suspension and nine parts of culture filtrate were mixed and incubated at 37°C. After 60 min., portions of the mixture were taken and assayed for viral infectivity by the plaque counting method described in text. Control tests were done in the same way by the use of PBS in place of the culture filtrate.

**r*, rod form; *b*, bifid form; *m*, mixed population of the rod and bifid forms.

+ In other experiments carried out in the same way, the residual viruses were similarly demonstrated. However, actual numbers of plaques were less than 10 in all cases.

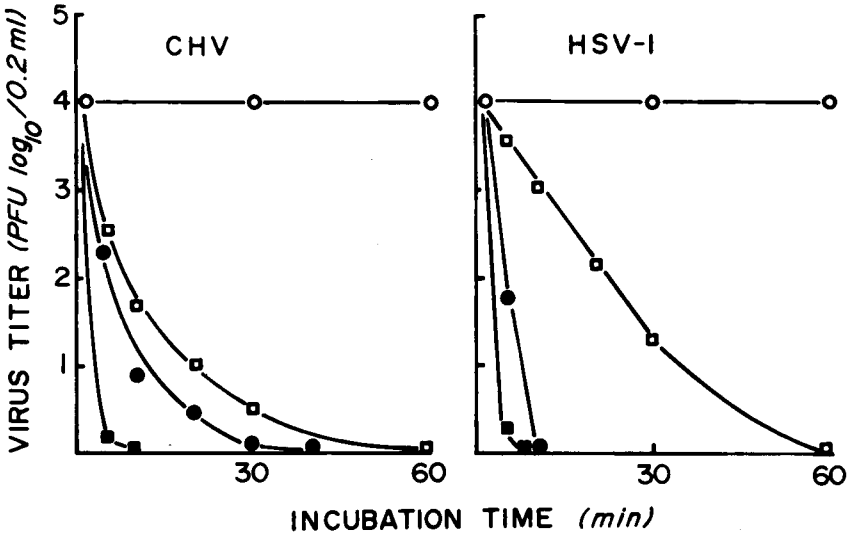

Fig. 1 Inactivation of CHV and HSV-1 by bacterial culture filtrates.
Experimental procedures were the same as those stated in Table 1. Ordinate indicates virus titers (PFU $\log_{10}$/0.2 ml) and abscissa indicates incubation time in min.
●, SF filtrate; ■, LA filtrate; □, BB filtrate; and ○, control fluid (filtrate of medium not inoculated with virus).

Virus-Inactivating Effect of Lactic Acid and Acetic Acid.

Since the test bacteria produce organic acids, especially lactic and acetic acids,* the effects of these acids on viral infectivity were examined. The results obtained (Fig. 2) indicated that the HSV-1, CHV and Infl-A2 were rapidly inactivated by the organic acids, and that the grades of inactivation were proportional to the concentrations of acids. The poliovirus was completely refractory to both acids at a concentration of 10 mg/ml.

* In preliminary experiments it was indicated that amounts (mg per ml culture filtrate) of the lactic acid (determined by the method of Barker and Summerson [1]) and acetic acid (determined by the method of Keenan [4]) produced by the test organisms were: SF (5.7 and 2.6), LA (7.3 and 1.6), *r*BB (2.4 and 1.2), and *b*BB (2.0 and 1.1), respectively.

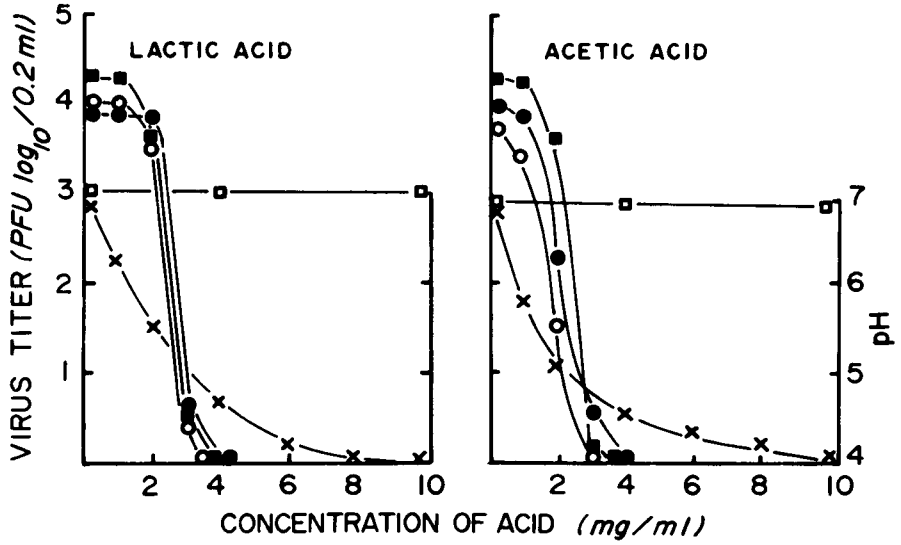

Fig. 2 Effect of lactic acid and acetic acid on viral infectivity. One part of the virus suspension was mixed with nine parts of fresh bacterial culture medium containing lactic or acetic acid (amounts indicated on abscissa). Following incubation at 37°C for 60 min., the viral infectivity was assayed by the plaque method.
● , HSV-1; ○ , CHV; ■, Infl-A2; □, Polio-1; and × , pH value

Effect of pH on Viruses.

As the increase of acid concentrations paralelled the decrease of pH (Fig. 2), the effect of pH on viruses was examined. As shown in Fig. 3, the test viruses, except for polio-1, were rapidly inactivated by lowering the pH. Other tendencies noted were that the pH sensitivity was different from one test virus to another; the CHV was most labile, Infl-A2 comparatively stable, and the HSV-1 intermediate. Still residual amounts of the active viruses were detected at pH 4.0. The poliovirus was completely stable within the pH range tested.

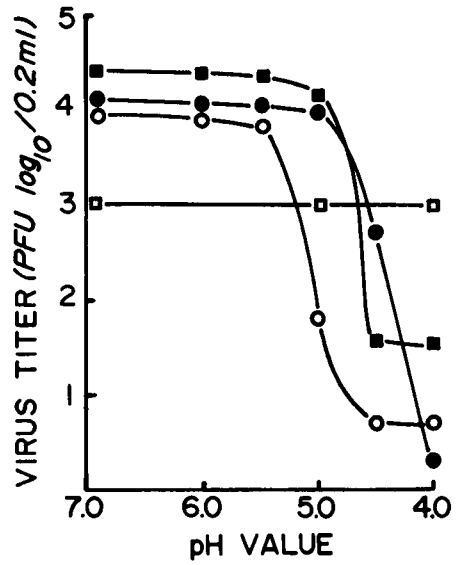

Fig. 3 pH inactivation of viruses.
One part of virus suspension was mixed with nine parts of buffered solutions (0.05 M phosphate buffer, for pH range 5.8 – 7.0; and 0.05 M acetate buffer, for pH range 4.0 – 5.8). Following incubation at 37°C for 60 min., the infectivity of virus was assayed by the plaque method. ●, HSV-1; ○, CHV; ■, Infl-A2; and □, polio-1.

DISCUSSION

It was shown in the present study that culture filtrates of *Streptococcus faecalis, Lactobacillus acidophilus* and *Bifidobacterium bifidum,* which are popular members of the lactic acid bacteria, inhibited the plaque formation of HSV-1, CHV and Infl-A2, but not of polio-1. The virus-inhibiting activity was mainly attributed to organic acids (lactic acid and acetic acid) produced by the bacteria. All of the three viruses inhibited have the envelope which the poliovirus lacks. A possibility is strongly suggested, therefore, that the organic acids alter the structure of the viral envelope, thus causing the loss of infectivity. Mak *et al*[9] reported that the pH inactivation of mengo encephalitis virus resulted in the dissociation of viral capsids.

The action of organic acids well paralleled the effect of lowering the pH of the media. Certain discrepancies, however, were noted between the virus-inactivating kinetics by the organic acids contained in culture filtrates (Fig. 2) and by the buffered solutions of the same pH's (Fig. 3). A possible explanation of the data may be that some ingredients of the culture media have the additional influence(s) of stabilizing the viruses. An alternative possibility is that the organic acids may posseses certain specific characteristic(s) for which pH cannot be substituted. In either case the poliovirus is completely refractory to both the organic acids and pH. The data are well compatible with those by Loring and Schwerdt [7] and Ketler *et al*[5] who demonstrated the stability of polio and related picornaviruses in wide ranges of pH.

It is to be added that the bacteria tested in the present study are among common species of the intestinal flora of man. The interaction between these bacteria and viruses is perhaps an important factor influencing the pathogenesis of the infections by viruses under study.

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