Antiviral Activity of a Fraction of Abalone Juice. (27259)

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In the search for antimicrobial agents, major attention has been focused on microbial products. More recently it was reported that whole abalone juice possessed antimicrobial activity(1,2). Of greater interest, perhaps, was the observation that mice were fed canned abalone juice displayed some resistance to experimental poliomyelitis(1). Thus, the rates of paralysis and death were 20 to 50% lower in treated animals than in controls and incubation periods were, in general, several days longer in the treated mice. Subsequently, the chromatographic separation of abalone juice yielded a fraction which was found to inhibit growth of a number of bacteria *in vitro*(3).

The present report extends the earlier observations of fractionation of abalone juice (3) and demonstrates that one fraction is rich in antibacterial material and another in antiviral substance.

Materials and Methods. Abalone juice and its fractionation. Fresh, whole abalones without shells were shipped from California in a frozen condition to our laboratory and stored at −20°C. Upon thawing, 12 whole abalones yielded about a liter of juice. The juice was dialyzed and applied to an anion-exchanger diethylamino-ethylcellulose (Cellex-D) column. Elution of the material was carried out with a series of Tris-H_3PO_4 buffers of varying ionic strength and pH. Details of these procedures have been described(3).

Tissue cultures. Two tissue culture systems were used in assaying antiviral activity of the abalone fractions. These were primary monolayer mouse embryo tissue cultures (METC)‡ in roller tubes maintained in Eagle's medium with 2% calf serum and primary monolayer monkey kidney tissue cultures (MKTC)§ grown in 199 medium with 2% calf serum and maintained in the same medium without serum.

Virus strains. 1. *Polyoma.* The strain of polyoma virus employed in the present work, designated as 5019ME9, was isolated and identified in this laboratory(4). 2. *Influenza.* Influenza type A virus, Japanese 305 strain, was used.

Assay for antiviral activity against polyoma virus. In assaying the activity of abalone juice fractions against polyoma virus, the fractions were diluted in maintenance medium to the desired concentration and the solution was passed through a very fine fritted glass filter. In some cases the fractions were diluted in saline and heated to 75°C for 30 to 45 minutes before being incorporated into the medium. In performing an assay, each of 6 METC tubes was treated either for 24 hours or for 5 days with 1 ml of medium containing 50 mg% of the test abalone fraction, after which the test fluid was removed and replaced with 0.9 ml of fresh test medium. Then, to each of the 6 tubes, was added 0.1 ml of a suspension containing either 250 or 2,500 $TCID_{50}$ of polyoma virus. Infected and treated tubes were incubated for 2 or 3 weeks; medium was replaced every 3 or 4 days. Tubes were examined periodically for CPE and for viral hemagglutinin. Hemagglutination tests were performed on days 7, 13 and 21, using tissue culture fluid from individual tubes or pooled from 6 tubes. Viral content was measured by inoculating serial 10-fold dilutions of the test fluid into METC tubes, using 6 tubes per dilution. The $TCID_{50}$ was calculated by the method of Reed and Muench(5).

The same number of infected control tubes was handled in the same way except that the abalone fractions were omitted from all media.

Assay for antiviral activity against influ-

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‡ Purchased from Microbiological Associates, Inc., Bethesda, Md.

§ Kindly supplied by Section on Tissue Culture, Laboratory of Viral Immunology, DBS.

TABLE I. Antimicrobial Activity of Various Fractions from Abalone Juice.*

Fraction No.	Pool from tubes	Eluent Tris-H_3PO_4 buffer		Yield, mg	Inhibition of growth by 50 mg % abalone juice		
		Conc. molar	pH		*Staph. aureus*	Polyoma virus†	Influenza virus‡
1	1- 8	.01	7.6	680	+	0	0
2	9- 17	.01	7.6	300	+	0	0
3	18- 30	.01	7.5	1,540	±	0	0
4	31- 48	.01	7.2	1,320	0	0	0
5	49- 64	.1	7.3	1,370	0	0	0
6	65- 83	.1	7.3	1,270	0	0	±
7	84- 95	.1	7.2	1,800	0	±	+
8	96-144	.1	7.3	5,200	0	+	+
9	145-170	.1	7.2	2,320	0	+	+
10	M NaCl wash			14,000	0	+	+

* Abalone fractions were tested for activity against microbial agents at a concentration of 50 mg %.

† Test dose of polyoma virus ± 250 to 2500 $TCID_{50}$.

‡ Test dose of influenza virus = 50 to 100 $TCID_{50}$.

+ = Suppression of viral or bacterial growth.

0 = Absence of inhibiting effect against test agents.

enza virus. Procedures used for assay of activity of abalone fractions against influenza virus were essentially those employed for assay of activity against polyoma virus except that MKTC in 199 medium without calf serum was used and infected culture fluids were harvested for hemagglutination tests and for determination of infectious virus at 48 hours. A somewhat different procedure was employed in studying the time after infection with influenza virus at which abalone fractions were effective in inhibiting viral growth. Fifty $TCID_{50}$ of influenza virus were inoculated into each of 30 MKTC tubes distributed into 5 groups. At 0, 2, 6, 18 and 24 hours, respectively, infected tubes in a given group were washed 3 times and 2 ml of 199 medium containing 50 mg% of abalone fraction were added to each tube. The tubes of each group were further incubated for another 48 hours and then the culture fluids were harvested, pooled and titered for virus activity. The same number of MKTC tubes in 199 medium without abalone fraction was employed for controls and was treated in the same way.

Growth curves for influenza virus were determined in MKTC which had been treated for 24 hours with medium containing 50 mg% of abalone fraction. Five $TCID_{50}$ of virus were added to each tube. At 16, 24, 32, 48, 72 and 96 hours, respectively, 6 tubes were removed and their fluids pooled for virus titration. Control tubes without abalone fraction were treated in the same way.

The *in vitro* virucidal effect of abalone fractions was tested by mixing 2 ml of 20 mg% solution of test fraction with 2 ml of fluid containing 100 $TCID_{50}$ of influenza virus. The resultant mixture was incubated at room temperature for 1 hour after which 0.2 ml of the mixture was inoculated into each of 10 MKTC tubes.

Hemagglutination. In the hemagglutination tests for quantitative estimation of polyoma and of influenza virus, serial 2-fold dilutions of the culture fluids were made in pH 7.0 phosphate buffered saline in 0.5 ml volumes and equal amounts of a 0.25% suspension of guinea pig red cells were added to each dilution. The mixtures were incubated at 4°C overnight after which readings were made. In tests for polyoma virus, the virus material was inactivated at 56°C before titration; influenza virus was used unheated.

Methods for assay of antibacterial activity. These have been previously described(1-3).

Results. Separation of antiviral and antibacterial activity. Some of the fractions obtained during the process of passing abalone material through an ion exchange column yielded materials active in inhibiting growth of *Staphylococcus aureus* but inactive against polyoma and influenza viruses. Other fractions behaved in a reverse fashion. Results of a typical experiment are presented in Table I. The activity of each fraction against polyoma and influenza viruses was determined in METC and MKTC, respectively.

TABLE II. Hemagglutination Titer* of Polyoma Virus in METC Cells Treated† with 50 mg % of Abalone Fraction 10.

	HA titers in cultures inoculated with			
	2,500 $TCID_{50}$		250 $TCID_{50}$	
Test No.	Treated	Control	Treated	Control
1	N.D.	64	0	32
2	4	256	0	64
3	8	128	0	32
4	32	128	0	64
5	16	64	0	64
6	8	16	0	32

* Test carried out with tissue culture fluid harvested from each of 6 mouse embryo tissue culture tubes on 7th day after virus inoculation.

† Primary mouse embryo cells treated with abalone fraction 10 for 5 days before virus inoculation.

N.D. = Not done.

Those fractions inhibiting growth of virus, as indicated by failure of polyoma or influenza hemagglutinin to appear in the treated cultures, were recorded as active and listed as positive in Table I. It is evident from the data that fractions 1 and 2 contained the antibacterial activity, while fractions 7 or 8 to 10 contained the antiviral substance. These observations extend the earlier ones(3) and clearly indicate that the antibacterial and antiviral activities of untreated abalone juice are readily separable by ion exchange chromatography.

Subsequent studies were limited almost entirely to those employing fraction 10.

Activity of fraction 10 against polyoma virus. Illustrative information on the results obtained in an experiment using fraction 10 and 2 different concentrations of polyoma virus are presented in Table II. It is apparent from the tabular data that cultures treated with medium containing 50 mg% of fraction 10 failed to produce polyoma hemagglutinins following inoculation with 250 $TCID_{50}$ of virus. On the other hand, when the inoculum contained 2,500 $TCID_{50}$, each of the treated tubes at the end of 7 days contained some demonstrable hemagglutinin. However, the amount of virus measured by this technic was appreciably less in the treated tubes than in the control. It is unlikely that these small amounts of HA in the treated tubes represented residual virus from the inoculum since the culture fluids were changed 3 or 4 days after virus inoculation.

The inhibitory effect of several concentrations of fraction 10 on development of polyoma virus hemagglutinin in METC is illustrated in Table III. It is apparent from the tabular data that with each of the 4 concentrations employed there was a considerable reduction in amount of viral hemagglutinin produced during the first 13 days of incubation. This inhibitory effect was less apparent in those tissue culture fluids tested on the 21st day of incubation.

Activity of fractions 1 and 10 against influenza virus. Detailed information on an illustrative experiment in which fraction 10 was shown to have marked activity against influenza virus while fraction 1 was without an inhibitory effect against this virus is presented in Table IV. Here, the multiplication of virus in cell culture was followed by 3 different procedures, namely, infective titer, hemagglutination and cytopathic change. It is apparent that with this particular system the untreated control tubes all developed cytopathic changes within 48 hours after infection, and the fluids contained influenza hemagglutinins at a titer of 1:64. Furthermore, the infectivity of the pooled fluids was $10^{4.5}$. In contrast, the tubes treated with fraction 10 developed no CPE or HA and infective virus was barely detectable. Fraction 1 had no significant effect on influenza virus.

When similar studies with influenza virus were extended over a longer period of time, the inhibitory effect of fraction 10 was appreciably diminished (Fig. 1). In this particu-

TABLE III. Inhibition of Polyoma Virus by Fraction 10 at Various Concentrations.

	HA titers in cultures inoc. with			
	2,500 $TCID_{50}$ on day		250 $TCID_{50}$ on day	
Fraction 10 conc. as mg %	13	21	13	21
50	64	128	8	64
25	64	128	4	128
12.5	64	256	8	128
6.2	128	256	16	128
0 (control)	1024	512	128	512

Primary mouse embryo cells were treated with abalone fraction 10 at indicated concentration for 24 hr prior to virus inoculation, using 6 tubes for each concentration of fraction 10. Each hemagglutination titer indicated a test with tissue culture fluids harvested and pooled from 6 mouse embryo tissue culture tubes on 13th and 21st days after virus inoculation.

TABLE IV. Effect of Fractions 1 and 10 on Influenza Virus.

Fraction 1			Fraction 10			Control		
CPE	HA	$TCID_{50}$	CPE	HA	$TCID_{50}$	CPE	HA	$TCID_{50}$
+	64		0	0		+	64	
+	128		0	0		+	64	
+	16	$10^{5.5}$	0	0	$10^{0.5}$	+	64	$10^{1.5}$
+	16		0	0		+	64	
+	16		0	0		+	64	
+	64		0	0		+	128	

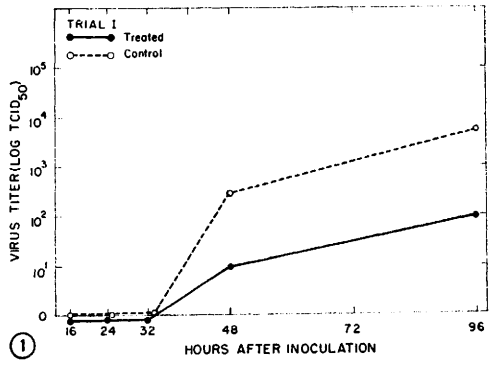
Three sets of primary monkey kidney tissue cultures, 6 tubes in each set, were treated prior to infection for 24 hr with fraction 1 or fraction 10 in a concentration of 50 mg %. Fifty $TCID_{50}$ of influenza virus was inoculated into each tube. The tubes were incubated for another 48 hr (see text).

CPE = Cytopathic effect HA = Hemagglutination

lar test, the original inoculum of 5 $TCID_{50}$ of virus was not detectable even in the controls at 16 hours, and infective virus was first demonstrated at 48 hours. The titer subsequently rose over the next 2 days to $10^{3.6}$ $TCID_{50}$. During this same period, from 32-96 hours, virus appeared in the treated culture but remained at an appreciably lower level than in the untreated control tubes. This seems to be in agreement with some other investigators'(6,7) work, showing that agents inhibiting influenza virus showed some, but not complete, inhibition.

It may be reemphasized that in each of the previously described experiments, the tissue culture cells were treated with the abalone material prior to infection with polyoma or influenza virus. The experiment summarized in Fig. 2 was designed to determine the latest time at which one might effectively treat cells infected with influenza and still demonstrate inhibition of growth of the virus. The data show that addition of abalone fraction 10, two hours after infection with influenza virus, was as effective in inhibiting growth as when the antimicrobial substance and virus were added to the culture at the same time. (This finding indicated that inhibition of virus multiplication was probably not due to prevention of adsorption or penetration into the cells by the virus.) However, when fraction 10 was added 6-18 hours after infection, only slight inhibition of viral growth resulted. When treatment was delayed for 24 hours, no antimicrobial effect could be demonstrated in the cultures which were examined 2 days later. It should be pointed out here that fraction 10 displayed no direct virucidal action *in vitro, i.e.,* 20 mg% did not inactivate 100 $TCID_{50}$ of virus (see Materials and Methods for procedures employed in experimental tests).

Discussion. There are many reports in the literature on the inhibitory effect of amino acid analogs, or other chemical compounds, on multiplication of influenza virus(8). Some of the inhibitors, such as methoxinine, affect



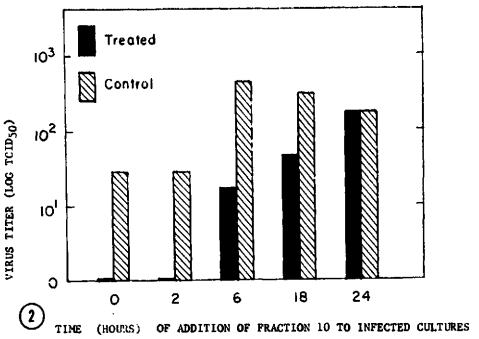


FIG. 1. Growth of influenza virus alone and in presence of 50 mg % of Fraction 10.

FIG. 2. Behavior of influenza virus in MKTC treated with 50 mg % of Fraction 10 at several time intervals after infection. Each group of cultures was assayed 48 hr after addition of Fraction 10.

only a particular phase in the latent period of viral development(9). Although our experiments illustrated in Fig. 2 suggest that abalone fraction 10 may also act on some specific phase of influenza virus replication, it is too early to discuss, at present, the mechanism of inhibition by abalone material. However, it appears unlikely that the inhibition is dependent on failure of virus to attach to or penetrate the host cell.

Recently, Jensen *et al.*(10) reported that inhibition of influenza virus in tissue culture systems exhibited by chemical compounds could be due to trace amounts of ammonium ion. In our study, the amount of ammonium ion in 2 aliquots of MKTC fluids at the 4th day of incubation—one in 199 medium with 50 mg% abalone fraction 10 and the other without it—was determined‖ and practically no difference was found. Therefore, inhibition of influenza virus in our case apparently was not due to ammonium ion.

Conclusion. The present experiments indicate that the antibacterial and antiviral activities of untreated abalone juice are readily separable by ion exchange chromatography.

‖ Determination of ammonium ion was kindly performed by Mr. H. G. McCann in the Laboratory of Chemistry, Nat. Inst. of Arthritis and Metab. Dis., N.I.H.

1. Li, C. P., PROC. SOC. BIOL. AND MED., 1960, v103, 522.
2. ———, *ibid,* 1960, v104, 366.
3. Prescott, B., Li, C. P., *ibid,* 1960, v105, 498.
4. Li, C. P., Jahnes, W. G., *Virology,* 1959, v9, 489.
5. Reed, R. J., Muench, H., *Am. J. Hyg.,* 1938, v27, 493.
6. Lum, G. S., Smith, P. K., *J. Pharm. and Exp. Therap.,* 1957, v119, 284.
7. Dickson, L., Thompson, M. J., *Brit. J. Pharmacol. and Chemotherap.,* 1957, v12, 66.
8. Tamm, I., 1959, In *Viral and Rickettsial Infections of Man,* Rivers, T. M. and Horsfall, F. L. (ed.), J. B. Lippincott Co., Philadelphia, p156.
9. Ackermann, W. W., Maassab, H. F., *J. Exp. Med.,* 1954, v100, 329; 1955, v102, 393.
10. Jensen, E. M., Force, E. E., Unger, J. B., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 447.

Received October 9, 1961. P.S.E.B.M., 1962, 109.

Depletion of Splenic Norepinephrine in Rat by Celiac Ganglionectomy. (27260)

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It has been shown that surgical denervation of the spleen in the sheep(1), cat(2), and dog(3) depletes tissue norepinephrine. This report describes a technic for celiac ganglionectomy which results in depletion of splenic norepinephrine in the rat. The ability of tissues to store norepinephrine has been related to functioning sympathetic innervation(4). How the storage and metabolism of norepinephrine are dependent on innervation is not clear, nor is it certain that all tissues in which norepinephrine can be measured are directly innervated(5). In attempting to study these problems, particularly with isotopic tracer technics, such factors as size of the animal and the ease with which a specific tissue may be studied became important considerations. The spleen of the rat seems suitable in these respects.

Methods. Albino male rats of the Osborne-Mendel strain, weighing 200-250 g, were used. Surgical denervation of the spleen was carried out in 14 animals; a sham operation was performed in 11 animals. Anesthesia consisted of ether or intraperitoneal sodium pentobarbital (25 mg/kg). Spleens from 22 unoperated animals served as normal controls. At the time of study the animals were killed by decapitation, asphyxia or with an overdose of sodium pentobarbital. The methods of sacrifice did not significantly influence the results. Rats subjected to denervation or sham procedures were sacrificed on the fourth postoperative day. Tissue nor-