THE USE OF QUATERNARY AMMONIUM RESIN-TRIIODIDE COMPLEX TO INACTIVATE VIRUS AND SELECTED BACTERIA

by 6402

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INTRODUCTION

The need for a water disinfectant that would release antibacterial chemicals on demand without leaving any residue is being met. A strong base anion exchange resin that forms a very insoluble stable complex with the triiodide ion has been developed. This complex is a very efficient bactericide against Gram positive and Gram negative bacteria. However, there are many questions yet to be answered: (1) how effective is this resin complex on micro-organisms that are encapsulated or sporulated; (2) does it inactivate DNA and RNA virus? The work included in this thesis is an attempt to answer these questions.

LITERATURE REVIEW

It has been established that the bactericidal and viricidal properties of iodine are greatly reduced by high concentration of iodide ion (1,2,3,4,6,9). Although diatomic iodine has sporicidal activity, quantitative investigation showed that triiodide ion has a relatively negligible effect (18). Iodine in the form of iodate present in sea water was also found to be toxic to coliforms (10). Although research by the United States Army has shown that chlorine and iodine treated water could be used to reconstitute dehydrated foods, it was also found that there was some reduction in the quality of the flavor (13,14).

In the removal of virus from water by use of several kinds of sand filters and a flocculation-filtration process, surface phenomena, as well as the kind of sand and flow rate, were found to be important (12). Other studies have indicated that activated-sludge sewage processing can remove 90-95 per cent of the viruses present (7,8); while flocculation and settling remove 95-99 per cent (5). Chlorination can be used to kill animal virus under proper conditions

of temperature, concentration, pH, and exposure time (17).

Ion exchange resins are commonly employed to soften and deionize water but have not been used as disinfectants. Strongly basic anion exchange resin-triiodide complexes were found to kill five different Gram positive and Gram negative organisms. These were Staphylococcus aureus, Streptococcus faecalis, Salmonella typhimurium, Escherichia coli, and Pseudomonas aeruginosa (16). The organisms were not only filtered from the water but also inactivation was instantaneous and irreversible.

MATERIALS AND METHODS

Preparation of Column

Thirty grams of strong base quaternary ammonium ion exchange resin in the chloride form*, packed in a 20 mm pyrex glass tube, was saturated with triiodide solution prepared by adding solid iodine to a one molar aqueous solution of potassium iodide. The KI solution was previously heated to 80 C and the mixture cooled to room temperature (16). A molar ratio of potassium iodide to iodine of 3.5 to 1.0 was used. The triiodide ions replaced the chloride ions on the reactive sites of the resin forming an ion association compound. The column was cleaned by rinsing alternately with distilled water and 1 M aqueous potassium iodide, until no iodine was detected in the water rinse using the cadmium iodide-linear starch reagent (11). The potassium iodide converts any higher polyiodide forms to the triiodide. The distilled water rinse removes the uncombined iodine and excess iodide, triiodide, potassium, and chloride ions from the column. The amount of triiodide required was calculated by referring to the exchange capacity of the resin.

Action on Various Bacteria

Bactericidal action on capsulated and noncapsulated strains of Streptococcus bovis:

Strept. bovis was inoculated into trypticase soy broth containing 5 % sucrose to promote encapsulation and incubated for 24 to 48 hours at 37 C without shaking. To obtain noncapsulated Strept. bovis, the culture was inoculated into the same broth and, under the same conditions, without sucrose. Different dilutions of both were passed separately through a 30 gram column of Ionac A 540-I_3 complex at 28 ml per minute flow rate. Organisms were counted by the standard pour plate method (15) before and after passing through the column.

*Ionac A 540
(Matheson, Coleman & Bell)

Sporicidal effect on two spore forming bacteria:

A preliminary experiment was conducted using an unheated and heated culture of Bacillus cereus. The culture was grown in nutrient broth with continuous shaking for 27 hours at 37 C. Part of this culture was heated at 63 C for 30 minutes to kill vegetative forms. A 10^3 dilution of each was passed separately through a 30 gm resin-I_3 complex at 48 ml/min flow rate. Samples were taken before and after passing. The standard pour plate technique utilizing nutrient agar was used for counting.

Another experiment was done using a pure spore suspension of Bacillus megaterium QM. Spores were obtained by streaking plates of nutrient agar, containing 10 ppm of $MnCl_2$, with the culture and incubating four days at 37 C, and then two days at 10 C. The cells were loosened with distilled water and centrifuged down at 3000 rpm for 10 min. The process of washing and centrifuging at 6 C was repeated six times. The spores were then suspended in distilled water and refrigerated. A spore stain* was done to check for vegetative cells. A serial dilution of these pure spores in distilled water was passed through a 30 gm column at flow rate of 20 ml/min. Numbers of organisms were checked on samples taken before and after passing.

Viricidal Properties

Effect of resin on the DNA polyoma virus:

A 0.45 ml sample of the virus in Eagles** medium (control count 6 X 10^7 particles/ml) was passed through a 3.8 gm column of Ionac A 540-I_3 complex. The sample was first added to the column and allowed to flow into the resin bed. Distilled water was added in 0.5 ml portions to replace the 0.5 ml of the eluent collected every 10-20 seconds. Aliquots were received in test tubes each con-

*See appendix for procedure.

** Grand Island Biological Company, New York, p. 83.

taining 0.2 ml of Eagles medium. Ten such samples (0.7 ml total each) were obtained and tested for hemagglutination (HA), and plaque forming units (PFU). Mouse embryo tissue culture was used in the PFU experiment. The plaques were counted after 13 days incubation. (See appendix).

A second experiment was done but the virus (8×10^8 particles per ml) was diluted 10^3 times in distilled water and then passed through a 30 gram resin-I_3 column at 30 ml per min flow rate. The PFU and HA were done on samples taken before and after passing.

An experiment to determine if viruses are filtered from solution:

The *DNA of polyoma virus* was labeled with ^3H thymidine. A 0.45 ml sample of the radioactive virus (control count was 6×10^7 particles/ml) was passed through a 3.8 gram column of the resin-I_3 complex. Each of these samples were collected and tested for radioactivity using a scintillation counter. (Actually this and the first experiment were done simultaneously.)

Effect of time and different types of water treatment on the viability of Newcastle Disease virus:

Newcastle disease virus (NDV) was diluted ten fold in two types of liquid: distilled water and phosphate buffer saline (PBS). Plaquing on chick embryo tissue culture was made immediately on samples taken at 0, 10, 30, 60, and 120 minutes (two hours is the average time needed for passing any sample through the column). These samples were then frozen for 7 days and retested to determine if storage at 4 C was a factor. A control count was 1.5×10^6 particles per ml.

Effect of the resin-triiodide eluent on tissue culture:

Distilled water was passed through a 30 gram column at a flow rate of 30 ml/min. Serial dilution (routine procedure, see appendix) of the eluent in 1X

Eagle's medium were placed on a monolayer of mouse embryo tissue culture (see appendix). The plaquing procedure was followed.

A similar second experiment was done substituting rabbit kidney cells, 5X of 199 medium* and deionized water. This water was passed through the column as previously (30 ml/min.). The eluent, deionized water and 199 medium were mixed in the following proportions respectively: 0:4:1, 4:0:1, 3:1:1, 2:2:1, and 1:3:1. These mixtures were used to make rabbit kidney cell suspensions. These suspensions were seeded in bottles and incubated to determine any adverse effect on the growth of monolayers.

Effect of resin on an RNA virus:

Newcastle disease virus (NDV) suspended in Eagle's (control count was 1.6×10^6 cells per ml) was diluted one hundred times in distilled water. It was then passed through a 30 gm Ionac-I_3 column at a flow rate of 31 ml per min. A PFU determination was conducted on samples taken before and after passing through the column. Chick embryo tissue culture was used in this experiment. (See appendix).

* Grand Island Biological Company, New York, pp 82-83.

RESULTS

Action on Various Bacteria

Bactericidal action on capsulated and noncapsulated strains of Streptococcus bovis:

Ionac A 540-I_3 complex killed 8 X 10^5/ml of capsulated and 1 X 10^5/ml of noncapsulated Streptococcus bovis. The killing efficiency of the resin triiodide complex on the capsulated Strept. bovis was somewhat more than for the noncapsulated organisms (Table 1).

Sporicidal effect on two spore forming bacteria:

Bacillus cereus:

When a 10^3 dilution in distilled water was made from the original culture (count of 2 X 10^8 cells/ml), a count of 1.5 X 10^5 viable cells/ml (both vegetative and spores) was decreased, after passing through the column, to 4.5 X 10^2 cells per ml. This is about 99.9 % kill. Heating the original culture dropped the viable count to 3 X 10^6 cells/ml. This represents the spore or thermoresistant count in the culture. When a 10^3 dilution of this heated culture was made (viable heat resistant plus dead cells) and passed through the column, the count dropped from 2.5 X 10^3, to 3.5 X 10^2 cells/ml, or 90 % kill of the heat resistant forms. (Table 1).

Bacillus megaterium QM:

The resin-I_3 complex killed about 50 % of the pure spore suspension of Bacillus megaterium QM. (Table 1).

Viricidal Properties

Effect of resin-I_3 complex on DNA virus:

When a 0.45 ml sample with 6 X 10^7 PFU/ml of polyoma virus was passed through a 3.8 gram column, the PFU decreased to 33 X 10^5 and hemagglutination unit (HA) dropped from 288 to 160. When a sample with 3 X 10^5 PFU/ml of the polyoma virus was passed through a 30 gram column, it decreased to zero and the HA decreased from 20 to zero. (Table 2).

Effect of resin on an RNA virus:

The killing capability of the resin triiodide complex on Newcastle disease virus (NDV) was one hundred percent when starting at 2 X 10^4 particles /ml. (Table 2).

An experiment to determine if viruses are filtered from solution:

One hundred percent plus of ^3H thymidine labeled virus was recovered after passing through the column. (Table 3).

Effect of time and different types of water treatment on the viability of Newcastle disease virus:

Results showed that neither time nor distilled water killed the virus. (Table 4).

Effect of the column eluent on tissue culture:

Results showed that the column eluent has no harmful effect on the monolayer of mouse embryo tissue culture, and also has no deleterious effect on the development and growth of monolayers of rabbit kidney cells.

Table 1. Antibacterial capabilities of Ionac A 540-triiodide complex on capsulated and sporulated bacteria.

Organism*	Flow rate (ml per min.)	Viable count per ml**	
		Before passing	After passing
Streptococcus bovis			
a) encapsulated	28	8.0×10^5	0.0
b) noncapsulated	28	1.0×10^5	0.0
Bacillus cereus			
a) unheated	46	1.0×10^5	4.5×10^2
b) heated at 63 C	45	2.5×10^3	3.5×10^2
Bacillus megaterium***	20	3.2×10^6	1.5×10^6

* The organisms were suspended in deionized water and passed through 30 gm columns.

** All plate counts were run in duplicate, and results shown are: Strept. bovis and B. megaterium average counts of two experiments, B. cereus average counts of three experiments.

*** Pure spore suspension

Table 2. Antiviral properties of Ionac A 540-I_3 complex

Virus	Size of column*	Hemagglutination unit**		Plaque forming unit per ml.	
		Before passing	After passing	Before passing	After passing
Polyoma virus	3.8 gm	288	160	6×10^7	33×10^5
	30. gm	20	0	3×10^5	0
Newcastle disease virus	30. gm	---	---	2×10^4	0

* In cases of 30 gram columns, the flow rate was 30 ml/min. Flow rate for 3.8 gram column was approximately 0.5 ml every 10-20 seconds.

** This represents the titer

Table 3. Radioactive counts per minute, hemagglutination unit, and plaque forming units of ^{3}H-thymidine labeled polyoma virus before and after passing through a 3.8 gram resin-I_3 column.

Fraction No.	ml aliquote in each fraction collected	Observed * count per min.	** HA	*** PFU/0.7ml
#1	1.8	117	--	--------
#2	0.7	140	20	3.43×10^5
#3	0.7	175	80	7.00×10^5
#4	0.7	357	20	4.90×10^5
#5	0.7	210	10	1.89×10^5
#6	0.7	238	10	2.10×10^5
#7	0.7	203	10	3.5×10^5
#8	0.7	77	10	2.31×10^5
#9	0.7	98	--	--------
#10	0.7	133	--	--------
#11	0.7	105	--	--------
Totals	----	1853	---	2.513×10^6

* Starting with 1.787×10^3 per 0.45 ml-observed counts

** HA 640 initial titer

*** PFU 6×10^7 per ml. initial count

Table 4. Effect of distilled water and phosphate buffer saline on the viability of Newcastle disease virus. *

Time in minutes	Plaque forming units per ml	
	Distilled water	Phosphate buffer saline
0	5×10^5	7×10^5
30	11×10^5	9×10^5
60	6×10^5	6×10^5
120	6×10^5	6×10^5
7 days	1×10^5	2.3×10^4

* At room temperature (25-30 C) except for 7 day storage at 0 C

DISCUSSION

Strongly basic resin-triiodide complexes were previously reported to have antibacterial capabilities against five different Gram positive and Gram negative organisms. It was felt that it would be necessary to try to determine its effect on encapsulated and sporulated bacteria, as well as on DNA and RNA viruses. This study showed that the Ionac A 540-triiodide complex has great antibacterial capabilities on encapsulated and noncapsulated bacteria. Unfortunately, sporicidal effects were not as clean cut. Kills were significant but not satisfactory. The killing capabilities varied from 50% with pure spores of Bacillus megaterium QM, to 90-99.9% of Bacillus cereus.

The viricidal properties of the resin-I_3 complex were quite conclusive with two types of viruses: a DNA polyoma virus and an RNA Newcastle disease virus (NDV). As many as 2.0×10^4 particles per ml of the RNA virus were killed. With the DNA virus, the kill was 95% when a 3.8 gram column and concentrated virus (6×10^7/ml) suspended in Eagle's were used. In a second experiment the virus (8×10^8/ml) was diluted 10^3 times in distilled water and passed through a 30 gram column. The kill, 3×10^5 particles/ml, was complete. The results obtained with the DNA virus varied when two different sizes of column were used. This difference could be due to the very concentated virus when the 3.8 gram column was used, or to the presence of Eagle's medium rich in organic material which may interfer with the iodine present in the resin-I_3 complex.

The use of ^{3}H-thymidine labeled polyoma virus showed that the virus was not filtered out by the column, but passed through and emerged in non viable form. There was no harmful effect produced by the eluent on tissue cultures. In addition, the amount of iodide ion in the eluent released from the column is 0.2 to 0.5 PPM.* This is well below the taste threshold.

* Unpublished data of research now in progress for Ph. D. by the author.

The exact mechanism of antibacterial and antiviral action of the column as well as the amount of iodine necessary to kill one bacterium are not known, but are being investigated.

SUMMARY

A strong base quaternary ammonium ion exchange resin in the chloride form saturated with triiodide was prepared. This resin-I_3 complex killed 8 X 10^5 cell/ml. of an encapsulated strain of Streptococcus bovis and 1.0 X 10^5 cell/ml of the noncapsulated strain. Its killing effect on sporulated bacteria was not conclusive; however, it appeared that it killed 50% of a pure spore suspension of Bacillus megaterium and from 90-99.9% of spore forming Bacillus cereus. The antiviral capabilities of the resin-I_3 complex was conclusive. It killed 2.0 X 10^4/ml of the RNA virus (NDV) and 3 X 10^5/ml of the DNA virus (polyoma virus).

By using ^{3}H-thymidine labeled polyoma virus, it was shown that there was no filtering action by the column, and the virus emerged in a nonviable form. The resin-I_3 complex eluent was found to have no harmful effect on the tissue culture. The amount of iodide released from the column was far less than that needed to surpass the taste threshold.

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APPENDIX

Preparation of mouse embryo tissue culture:

Primary mouse embryo cells are obtained by aseptically removing embryos from 18-20 day pregnant mice. The embryos are washed in PBS, and the extemities removed. Minced tissue is washed twice in PBS with the aid of a magnetic stirrer to remove excess red blood cells. The tissue is trypsinized (0.25% trypsin in PBS, pH 7.2) several times at 15 to 20 min. intervals until all the tissue has been digested. The trypsinized suspension of cells is then centrifuged ten min. at 1000 rpm, resuspended in Eagle's medium plus 10% fetal calf serum and seeded in 60 mm dishes at a concentration of 1.3×10^6 cells/ml. These dishes are incubated at 37 C in a CO_2 incubator. This forms monolayers in 3-4 days.

Chick embryo tissue culture:

The same procedure is followed as in the mouse tissue culture preparation, except that chick embryos are used. To obtain the embryos, fertilized eggs are incubated at 37 C for 9-13 days, and then examined.

Hemagglutination test:

A quinea pig is bled from the heart and the blood stored in Alsever's solution until used. The blood is washed three times in PBS, in a 10 ml screw cap tube, by centrifuging for exactly 10 min. (middle setting in the small table centrifuge). The supernatant from the last wash is aspirated off and 0.4 ml, of the packed cells, is added to 100 ml of PBS. This gives a final concentration of 0.75% rbc in the suspension.

Serial two-fold dilutions of the sample are made in PBS (0.01 ml sample added to 1.0 ml PBS in the first well) in multiwelled plastic trays. An equal

volume (0.5 ml) of 0.75% rbc suspension is added to each well, mixed and allowed to react for 3 hr. at 4 C. Then the patterns were read. The end point or titer is the last well showing positive agglutination.

The Plaque Assay:

NDV:

The plaque assay is performed on monolayers of chick embryo cells. Media (Eagle's medium plus 10% fetal calf serum) is removed from the dishes and monolayers washed once with PBS. Serial ten-fold dilutions of NDV samples, are made in 1X Eagle's medium. Duplicate dishes are inoculated with 0.5 ml/dish of the diluted sample. The virus is allowed to adsorb for 30 min. at 37 C. Solid agar overlay (consisting of 50% agar and 50% of the following: adeno 91.5%, serum 7.5%, and glutamine 1%) is added to the dishes (8 ml/dish). After 3 days incubation at 37 C in a CO_2 incubator, a solid agar overlay containing 1% neutral red is added (2-3 ml/dish) and plaques counted 12 hrs. after neutral red addition. This plaquing technique allows viruses to be counted with about the same accuracy as is achieved with bacterial plate counts. Results are reported in terms of plaque-forming units per milliliter (PFU/ml).

Polyoma:

The same procedure is used as in the NDV plaquing procedure except mouse embryo tissue culture is used and another agar overlay (7 ml/dish) is added 8 days after the first addition, then 3-4 days later a solid agar overlay containing 1% neutral red is added (2-3 ml/dish). Plaques are counted 12 hrs. after the addition of neutral red.

Rabbit kidney cell monolayers:

Rabbit kidney cells are obtained by aseptically removing the kidney from

10-15 day old rabbits. The kidney is minced and washed twice with PBS for 5-10 min. and once with 0.25% trypsin for 2-3 min. with the aid of a magnetic stirrer. The same procedure is followed as per the chick and mouse embryo experiments from here on. In this preparation 1X of 199 medium is used and the cell suspension is seeded in bottles at a concentration of 21 X 10^6 cells/ml (12 ml per 8 oz bottle).

Spore stain:

A wet mount is stained with saturated, aqueous malachite green for 7 min. over boiling water, washed with tap water, and counterstained with 0.25% aqueous safranin (spores stain green, and vegetative cells stain red).

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It had been shown in previous work that strong base anion exchange resins form very stable insoluble complexes with triiodide. It was also reported that this resin-I_3 complex has great antibacterial capabilities against five different Gram positive and Gram negative organisms. It was then thought desirable to investigate the effect of this resin-I_3 complex on capsulated and sporulated bacteria, also DNA and RNA viruses.

The results showed that this resin-I_3 complex has the capability to kill 8×10^5 cell/ml. of an encapsulated Streptococcus bovis and 1.0×10^5 cell/ml. of a noncapsulated strain. The results obtained with the sporulated bacteria were not conclusive. The complex killed 50% of the pure spore suspension of Bacillus megaterium QM and 90-99.9% of spore forming Bacillus cereus.

The antiviral capabilities of the resin-I_3 complex was quite conclusive. As much as 2.0×10^4 particles/ml. of the RNA virus (Newcastle disease virus, NDV) were killed. With the DNA virus (polyoma virus) the kill was 95% when a concentrated virus in Eagle's medium (6×10^7/ml) was passed through a 3.8 gm. column. When the virus (8×10^8 particles/ml.) was diluted 10^3 times in a second experiment and passed through a 30 gm. column, 3×10^5 particles/ml. were killed.

The use of ^{3}H-thymidine labeled polyoma virus showed that the virus was not filtered out by the column, but passed through, and emerged in nonviable form. It was also found that the column eluent has no harmful effect on the monolayer of mouse embryo tissue culture or the growth of monolayers of rabbit kidney cells.