PENETRATION OF PSEUDOMONAS AERUGINOSA INTO EGGS TREATED WITH COPPER VITRESAN

by

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INTRODUCTION

The use of embryonated eggs in the field of virology is a comparatively recent development. Eggs, especially chicken eggs, are used because:

(1) They are seldom contaminated by microorganisms.

(2) Few, if any, antibodies are present in embryonated eggs.

(3) Fertile eggs are readily available.

(4) Eggs are more easily handled and require less storage space than animals.

Since many viral antigens are now prepared commercially in embryonated eggs, companies engaged in their production and preparation desire eggs which are not contaminated by micro-organisms. One contaminated egg may render an entire lot of the antigenic material worthless. For this reason, these experiments were carried out to attempt to find a suitable compound which could be used as an antipenetrant to lessen the possibility of contamination by micro-organisms but still not affect the viability of the embryo.

HISTORICAL

Most authorities agree that eggs are not contaminated with micro-organisms when laid. Contamination occurs after laying by improper care of the eggs.

Rettger (1), Mallmann and Davidson (2) and others who have made extensive investigations all conclude the egg is sterile unless laid by a diseased bird.

It is evident then, that contamination occurs after laying, a view held by the egg industry. For this reason, anti-penetration compounds have been studied in hopes that some compound could be found which would prevent penetration of micro-organisms into the egg during periods of adverse storage thus giving the trade a better product and protecting it against the large losses which it now has.

The shell of the egg is porous. Haines and Moran (3) reports pore sizes as high as 15 microns in diameter, which is large enough to admit some of the largest bacteria, to say nothing of mold hyphae.

Wilm (4) was apparently the first to show that bacteria could penetrate an egg. Using a culture of Vibrio comma and immersing the egg in the culture, the organism was able to penetrate the shell in 15 to 16 hours. Other workers have shown the same thing using various organisms.

Egg albumin and possibly the shell membranes possess bactericidal properties. Wurtz (5) was apparently the first to note this property in eggs. Meyer, Palmer, Thompson and Khonazo (6) were able to isolate this substance called lysozyme. Sharp and Whitaker (7) found that the apparent discrepancies in the bactericidal action of eggs was due to the hydrogen ion concentration which decreased rapidly during the first few days after laying due to the diffusion of carbon dioxide from the egg. They pointed out that older eggs have

less bactericidal growth due to the high pH and not necessarily to the action of the lysozyme.

Despite the conclusions reached by investigators about how the egg becomes infected and the subsequent proliferation of the organism in the egg, the fact remains that once an organism does gain entrance, the possibility of the egg deteriorating is still most important.

For this reason, proper care of the eggs is important from the time they are laid until they reach the consumer. Since small producers rely on the large storage centers for their market, their equipment and methods of handling the eggs for shipment to these centers are often open to question. With this in mind, many investigators have attempted to find compounds which could be used to treat the eggs to prevent contamination by micro-organisms during periods of adverse handling.

A survey from the producers point of view of the problem and recommendations concerning handling of eggs are given by Mallmann and Davidson (8) and a more complete picture of all phases of the egg industry is given in the U. S. Department of Agriculture Circular 588, 1941.

The problem of evaluating a compound for antipenetration properties is quite complex. It can be approached from several different viewpoints: (1) eggs used for consumption, (2) eggs used for virus work, and (3) eggs used for other purposes.

In the first case, the problem of off odors and flavors is most important, while in the other two cases, effect on the embryo or other portion of the egg to be used must be considered.

Since this study was largely conceived with the second viewpoint in mind, no attention was paid to off odors and tastes.

Fertile eggs are used in virus work. They must be of the best quality in all respects. Beveridge and Burnet (9) briefly review the criteria for eggs used in this work. They advise the use of clean, unwashed, white-shelled eggs, not stored over ten days before incubation is begun. They state that the temperature of storage should be between 4.5° and 20.0° C. and incubation temperature should be between 37.3° and 37.8° C. If proper care has been exercised, the egg should be sterile at the time of use, however, such is not always the case. Some of the larger biological preparation houses engaged in virus production find their eggs contaminated, thus increasing production costs. As pointed out before, if the producer could use an effective antipenetration compound, then a much better product would result.

The evaluation of the compound largely resolves itself into the following factors: (1) temperature at which the eggs are stored, (2) relative humidity, (3) concentration of the compound used, and (4) storage conditions and length of storage. As can be seen, all of these factors are intimately related.

In virus work, eggs should be stored at 4.5° to 20.0° C. after collection if they are not immediately used. Relative humidity is not important during the first twelve days of incubation. During incubation the temperature should range between 37.3° and 37.8° C. During the storage period the eggs should not be allowed to sweat, for it is under these conditions that the bloom is destroyed and the egg becomes much more susceptible to bacterial invasion.

Circular 583, 1941 of the U. S. Department of Agriculture covers briefly the factors involved in eggs produced for the consumer market. At present, methods used in preserving eggs for this market are not applicable to those used in virus work.

Jones and DuBois (10) classify egg preservation into four groups: (1) low temperature storage, (2) airtight packing, (3) sealing with various agents, and (4) immersing in preserving solutions. Of these classifications, the latter is the least used. None of the other classifications are entirely satisfactory, but of late, a combination of sealing the pores, usually with oil, controlling the carbon dioxide concentration in the atmosphere, and storing at low temperatures has proven quite satisfactory. Spoilage is still very high pointing to contamination before eggs reach the storage center.

Funk (11), Mallmann and Davidson (8), and others have described various methods for preserving eggs. The method of oil preservation gives excellent results in that the egg

loses much less weight than with other treatments. Funk claims excellent results with 1.0 per cent NaOH, other compounds tested giving poorer results. The oil wash cannot be applied to fertile eggs for it interfers with the respiration of the embryo. To date, no work using fertile eggs has been reported using various chemical agents as possible antipenetration compounds. For this reason it was decided to attempt an evaluation of chemical agents as possible antipenetration compounds and their subsequent use in the field of eggs produced for virus work.

EXPERIMENTAL WORK

A. METHODS DEVELOPED FOR OPENING EGGS

The first portion of the experimental work was devoted to developing a method for opening eggs for sampling giving as little contamination as possible to the egg contents. Several techniques were tried: (1) blowing off the shell over the air sac portion of the egg with a small Bunsen Burner, (2) disinfecting the egg and puncturing the air sac end with a sterile pipette, (3) disinfecting the egg, filing a small triangular portion of the shell over the air sac, removing this portion exposing the shell membrane, then inserting the sterile pipette.

To hold the egg, a 50 ml. beaker was found to work for normal size eggs, while for smaller eggs, a 30 ml. beaker was used. The beaker was cleaned carefully by washing, then a 10 per cent solution of Roccal was used to disinfect it prior to setting the egg in place.

The first method for opening the eggs was discarded due to the variable results obtained. Fresh eggs very seldom broke clean and in most cases excessive heating caused some coagulation of the albumin making sampling difficult. Older eggs gave better results due to the larger air cell they contained, which on heating caused the air to expand thus exploding the shell.

The second method was also discarded because of the large amount of cracking caused by plunging the pipette through the unbroken shell. Subsequent treatment of the shell was almost impossible due to the large number of cracks.

The third method proved to be the best. Not only was the shell removed, but sterility of the contents maintained due to the intact membrane which could be punctured by a sterile pipette when the sample was to be taken.

The egg was disinfected by swabbing it with a 10 per cent solution of Roccal and allowing it to dry. A triangular file was sterilized in the same manner then a triangular portion of the shell, large enough to admit a 2 ml. pipette, was filed to the shell membrane. This portion of the shell was then removed exposing the shell membrane. The pipette was inserted when the sample was to be taken. As far as could be found during the subsequent use of this method, little if any, contamination resulted from outside sources.

B. PENETRATION OF VARIOUS ORGANISMS INTO THE EGG.

After a suitable technique has been developed for opening the eggs, penetration tests using various bacteria were made to find the approximate penetration time of the various organisms under optimum conditions, namely 37° C. and a relative humidity of 100 per cent. Under these conditions, motile organisms exhibit the maximum motility and the 100 per cent humidity gives a moist environment at all times which is necessary to the life of the organism as well as a microscopic film of moisture which would serve as the motility medium.

The eggs used in this series of experiments were from two sources, M.S.C. College and the Hamilton Farm Bureau. Storage eggs were received from the latter source. Bacteriological examinations were made on some of the eggs to insure the absence of the test organisms. Both sets of eggs were found to be free of the test organisms. The eggs received from the Hamilton Farm Bureau were found to be grossly contaminated in most instances with a Gram positive micrococci which was not identified. The use of these eggs was discontinued because of this. Most of the preliminary penetration tests were made using eggs from this source. M.S.C. College eggs were used in the final penetration tests.

The test organisms used were all stock cultures of Bacillus subtilis, Escherichia coli, Micrococcus pyogenes, var. aureus, and Pseudomonas aeruginosa. These organisms were carried on TGE agar slants. A twenty-four hour F.D.A. broth culture of the organism was used for penetration

The test eggs were washed with tap water and wiped with a clean cloth, the control eggs were used as received from the poultry department. The culture was placed on the shell in two ways: (1) swabbing, using a clean piece of cotton, and (2) pooling several tubes of culture and immersing the egg for about a minute or two. After treatment with the culture, the eggs were stored in one-half gallon wide mouthed Ball jars in which has been placed a small piece of absorbent cotton thoroughly wetted with water. The jars were sealed and incubated at 37° C. and sampled at appropriate intervals.

Only the white of the egg was sampled. Transfers were made to a tube of broth and a dilution agar plate. In case of doubt, physiological and microscopic tests were made for identification. The results of this work are given in Table 1.

It was found that organisms varied in their ability to penetrate the shell of the egg but that the majority of organisms penetrated in about 14 to 18 hours in each type of treatment used.

C. PHYSICAL CHARACTERISTICS AND BACTERIOLOGICAL EXAMINATION OF EGGS.

The next series of tests were concerned with the physical characteristics and the degree of bacterial contamination of the eggs. This information was necessary for evaluation of the results attained in subsequent experiments.

TABLE 1

Penetration of Various Organisms into Eggs

Organism	No. of Eggs Sampled**	Time of Sampling in Hours	Treatment*	
			Washed	Untreated
B. subtilis	6	4	0	0
	6	8	0	0
	6	12	3	1
	12	14	5	2
	12	16	10	8
	12	18	11	10
	6	20	6	5
E. coli	6	4	0	0
	6	8	0	0
	6	12	2	0
	12	14	8	3
	12	16	10	7
	12	18	10	11
	6	20	6	6
M. pyogenes var. aureus	6	4	0	0
	6	8	1	0
	6	12	1	2
	12	14	4	2
	12	16	11	10
	12	18	9	11
	6	20	6	5
Ps. aeruginosa	6	4	1	0
	6	8	0	0
	6	12	4	3
	12	14	6	7
	12	16	10	9
	12	18	11	12
	6	20	6	4

*Incubation at 37° C. and 100% humidity.

**Number of eggs sampled for each treatment.

The examination was divided into four parts: (1) determination of contaminating organisms, (no attempt was made to identify the organisms found), (2) loss of weight of the eggs under incubation conditions, (3) shell thickness, and (4) viability of the embryo in the case of fertile eggs.

The eggs were divided into two groups, fertile and infertile. They were set in a 600 egg Jamesway gravity type circulation incubator. Humidity could not be controlled as desired, but a maximum humidity of about 90 - 93 per cent could be obtained with all vents closed. Two dozen eggs of each group were set in the middle tray where the temperature was maintained at 37° C. Eight eggs were sampled at random from each group in the sixth, fifteenth, and nineteenth days of incubation. The eggs were untreated and weighed to the nearest 0.5 gram before setting. They were re-weighed when removed from the incubator. The shell thickness was measured by means of a micrometer measuring in ten thousandths of an inch. The results of these investigations are given in Table 2.

Fertile eggs were found to be about as contaminated as infertile eggs, but the extent of contamination was relatively slight. The viability of the embryos was found to be about 50 per cent in each lot sampled. No correlation could be found to exist between the initial weight and the final weight, this probably being due to the fluctuation in the humidity of the incubator. Shell thickness seemed to

TABLE 2

Contamination, Loss of Weight, Shell Thickness, and Viability of Embryos*

Egg No.	Sampled Days	Contamination [1]	Initial Weight [2]	Loss of Weight [2]	Shell Thickness [3]	Viability of Embryo
INFERTILE						
1	6	-	63.5	3.0	0.0125	
2		-	52.5	2.5	0.0137	
3		-	56.0	1.0	0.0128	
4		-	63.5	2.5	0.0144	
5		✓	51.0	2.0	0.0130	
6		-	62.0	2.0	0.0128	
7		-	54.5	1.5	0.0136	
8		-	59.0	2.0	0.0132	
9	15	-	65.0	4.5	0.0129	
10		-	63.5	4.0	0.0136	
11		✓	60.5	3.5	0.0142	
12		-	59.5	6.0	0.0120	
13		-	56.0	4.5	0.0139	
14		✓	54.5	4.0	0.0128	
15		-	54.5	4.0	0.0122	
16		-	54.0	6.0	0.0135	
17	19	-	62.5	5.5	0.0132	
18		-	55.5	4.0	0.0124	
19		-	59.5	4.5	0.0129	
20		-	62.5	4.5	0.0135	
21		✓	62.0	5.5	0.0141	
22		-	58.5	5.0	0.0130	
23		-	61.0	6.0	0.0127	
24		✓	64.0	5.5	0.0125	
FERTILE						
1	6	-	63.0	2.0	0.0141	✓
2		-	47.5	2.0	0.0135	✓
3		✓	55.0	2.5	0.0128	✓
4		-	52.5	3.5	0.0130	-
5		-	51.0	3.0	0.0127	✓
6		-	49.5	2.0	0.0139	✓
7		-	60.5	2.5	0.0121	-
8		-	54.5	2.5	0.0126	-
9	15	-	64.0	4.5	0.0134	✓
10		✓	63.5	4.0	0.0121	✓
11		-	61.5	5.0	0.0130	-
12		✓	56.5	5.0	0.0121	✓
13		✓	52.0	5.5	0.0152	-
14		-	50.0	4.5	0.0133	-
15		-	46.5	3.0	0.0123	-
16		-	46.0	4.5	0.0124	-
17	19	✓	59.5	4.5	0.0128	-
18		-	55.0	5.5	0.0145	✓
19		-	61.5	4.5	0.0132	✓
20		✓	65.0	6.0	0.0151	-
21		-	63.5	5.0	0.0146	✓
22		-	60.5	4.5	0.0125	-
23		✓	51.5	5.5	0.0133	-
24		-	49.0	5.0	0.0141	-

TABLE 2 LEGEND

1 M.S.C. College eggs incubated at 37° C. in a relative humidity of 40 to 80 percent.

2 Albumin only sampled.

3 Weighed to the nearest 0.5 gram.

4 Thickness measured by a micrometer reading to ten thousandths of an inch.

have no influence on weight loss or penetration. It is possible that porosity might play the more important role.

D. TESTS ON COPPER VITRESAN AS A POSSIBLE ANTIPENETRATION COMPOUND

The viability of the embryo must be considered when selecting a compound to be used as an antipenetrant for eggs used in virology work. Any compound which causes the death of the embryo could not be used. For this reason, the usual methods for preserving eggs cannot be used with eggs in virology work.

A compound to be used as an antipenetrant should have the following attributes:

(1) Easily handled and placed on the eggs.

(2) Will not cause embryo mortality either directly or indirectly.

(3) Prevent the entrance of microorganisms in the egg.

The compound need not be germicidal but can be bacteriostatic, so long as penetration is prevented. Realizing the limits placed upon the choice of a compound, it was decided to try the salts of the heavy metals as possible antipenetrants.

The majority of the heavy metals and their salts exert oligodynamic action, copper being perhaps the most toxic to bacteria.

Several copper compounds as well as one silver compound were used in these tests to determine which compound would be chosen for more extensive tests.

Copper sulfate, Copper Vitresan, and Silver Vitresan were the compounds examined in the preliminary trials.

In the preliminary tests with these compounds it was found that both copper sulfate and Silver Vitresan had distinct disadvantages. The copper sulfate was highly soluble and washed off the egg shell during subsequent treatment with the culture of the organism. Silver Vitresan would precipitate whenever any chloride ion was present. When the egg was exposed to light, the silver chloride would turn brown and render the egg aesthetically objectionable. These two compounds were discarded and more extensive tests made on Copper Vitresan which had none of these disadvantages.

Copper Vitresan is one of a large group of compounds, which, because of their glassy or vitreous nature and their usefulness as sanitizing agents, have been named the vitresans. This group of compounds is produced by the Economics Laboratory, Inc., of St. Paul, Minnesota. The series produced by this company include the heavy metal phosphates of copper through uranium. At present, most of these compounds are produced for experimental use only. Copper Vitresan was produced in the course of a more general program which was directed principally to the production of Silver Vitresan.

The compound is prepared by fusing copper nitrate, or some other salt of copper with a volatilizable anion, with sodium hydroxide and phosphoric acid. The ratios of the quantities of the constituents and its subsequent method of cooling determine the type compound produced. After the

mixture has been fused at 600° to 700° C., the liquid is quenched by pouring on a water cooled stainless steel plate. The resulting compound is a water soluble phosphate glass containing a trace of copper.

Tests by the Economics Laboratory show that these water soluble phosphate glasses containing a trace of the heavy metal are much more bactericidal than the salts of the heavy metal alone. Why this is true is not known at present.

In addition to being very soluble in water, the heavy metal apparently forms a very stable complex ion with the phosphates. The intensity of the color of the various solutions of Copper Vitresan varies as the concentration of copper present, the greater the percentage of copper, the more intense and deeper the color.

Copper Vitresan is highly soluble, but goes into solution rather slowly, leaving a rust-like deposit in the container. This rust-like stain disappears upon standing, the length of time depending upon the concentration of copper present.

The glassy sodium phosphates in low concentrations act as sequestering agents in hard water and form complex ions with calcium and magnesium, thus acting as a water softener. The Vitresans react in the same way, except with the presence of the heavy metal ion, the compounds have enhanced germicidal properties, or exert oligodynamic action.

The pH of a solution of the Vitresans depends upon the ratio of sodium hydroxide and phosphoric acid used in preparing the product. The usual range is about pH 7.3 to 8.1 for these compounds.

The selection of the concentration of Copper Vitresan to be used was based upon tests using eggs treated with varying strengths of the compound and penetration tests run on these eggs using Ps. aeruginosa as the test organism. The appearance of the egg was also taken into consideration when the selection of the final concentration was made. The eggs were prepared in the same manner as in the penetration tests. A twenty-four hour broth culture of the organism was employed and fresh M.S.C. College eggs not over twenty-four hours old were employed.

The eggs were washed to remove any dirt and bloom, wiped with a clean cheesecloth, immersed in the concentration of the compound employed, allowed to air dry and then swabbed with the culture of the organism.

The results of this work are presented in Table 3.

It was found that a 2.5 per cent concentration of Copper Vitresan gave the best results, both from an antipenetration standpoint and appearance, higher concentrations gave the egg a blue color. The stock Copper Vitresan solutions were prepared from the commercial compound which contained 10 per cent copper.

E. ANTIPENETRATION TESTS USING 2.5 PER CENT COPPER VITRESAN

After the concentration of Copper Vitresan to be used was determined, more extensive tests for its effectiveness as an antipenetration compound were made.

In all of these tests, clean, fresh, infertile M.S.C. College eggs were used.

Control eggs were used as received. The test eggs were washed with water by wiping with a piece of clean cheese cloth moistened with tap water similar to methods used by many small producers in preparing their eggs for market. These eggs were then immersed in the 2.5 per cent Copper Vitresan solution and then allowed to air-dry.

The broth culture of Ps. aeruginosa was then swabbed on the cleaned eggs. A different strain of Ps. aeruginosa was employed in these tests. This new culture was chosen because of its remarkable pyocyanin and flourescein production. This particular culture was isolated from a diseased chicken and fulfilled all of the characteristics of Ps. aeruginosa given in Bergey's Manual of Determinative Bacteriology, 6th Edition.

The eggs, after the treatment with the broth culture of the organism, were then allowed to dry slightly and stored under the following conditions: (1) 100 per cent relative humidity, incubated at 37° C., (2) 100 per cent relative humidity, incubated at 20 - 25° C., (3) 35 - 53 per cent relative humidity, incubated at 20 - 25° C., (4) 100 per cent relative humidity, incubated at 7° - 10° C.

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TABLE 3

SELECTION OF THE CONCENTRATION OF COPPER VITRESAN TO BE TESTED*

Number of Eggs Sampled for each Concentration	Time of Sampling in Hours	Concentration of Copper Vitresan				
		10%	5%	2.5%	1%	0.5%
		Number of Infected Eggs				
6	14	0	1	1	1	2
6	20	1	0	2	3	4
6	24	1	1	2	2	4

*Ps. aeruginosa, test organism: Eggs incubated at 37° C. in a relative humidity of 100 percent.

The eggs were sampled at appropriate intervals using the following procedure:

(1) The egg was opened as previously described.

(2) A sterile pipette was inserted and 1.0 ml. of white withdrawn and diluted in 99.0 ml. of sterile saline, mixed well and 1.0 ml. of this mixture plated.

(3) The hole was then enlarged using sterile forceps and the contents of the shell emptied into a Petri plate. The contents was examined under black-light for presence of the test organism.

(4) The contents of the egg were allowed to drain from the shell which was then reinverted in the holder. Ten ml. of sterile saline were then run into the empty shell and with the pipette, the membrane was scraped until portions of it were removed from the shell. One ml. of the mixture was then plated and another 1.0 ml. mixed with broth for culturing.

(5) The remainder of the saline was then removed by inverting the shell and the interior of the shell swabbed by means of a sterile swab. The swab was passed over a TGE agar slant, then cultured in broth.

TGE agar was used in preparing the plates and FDA broth for liquid cultures. These media were used because the organism grew well on them and good pigment production was obtained. Only the white was sampled, because only the initial contamination was observed. Plates and tubes were incubated at 37° C. for 48 hours.

The white was diluted to facilitate plate counts. It was found that 1.0 ml. of the undiluted white did not disperse sufficiently when preparing the plates. Black light examinations were made of the plates and tubes to detect sparse growths.

With the incubation temperatures used, it was found that pyocyanin production was diminished slightly. The best method of detecting the organism was by checking for flourescein.

The results of these tests are given in Tables 4 to 7 inclusive.

F. INTERPRETATION OF TESTS MADE ON COPPER VITRESAN

As can be seen from Table 4, when eggs treated with Copper Vitresan, were incubated at 37° C. and 100 per cent relative humidity, the compound showed some value as an antipenetrant. The eggs in this test were exposed to the most favorable conditions for penetration, i.e. a moist atmosphere and a temperature at which the organisms have maximum motility.

The eggs treated with Copper Vitresan show a much lower percentage of infectivity than those washed with water, while the control eggs show a slightly higher percentage of infectivity than those treated with the compound. It is interesting to note that the eggs treated with Copper Vitresan have a slightly lower infectivity at 72 hours than have the control eggs.

TABLE 4

THE PENETRATION OF EGGS BY PS. AERUGINOSA, STORED AT 37° C. IN A RELATIVE HUMIDITY OF 100 PER CENT

Hour of Sampling	Number of Eggs Sampled	Inner Shell		Inner Shell Swab		Albumin		Average Plate Count per ml.	
		No. Infected	%	No. Infected	%	No. Infected	%	Albumin	Shell
Eggs Treated with Copper Vitresan									
0	18	0	0	1	6	0	0	0	0
3	18	0	0	0	0	0	0	0	0
6	18	0	0	0	0	0	0	0	0
12	18	0	0	0	0	0	0	0	0
24	18	1	6	1	6	0	0	0	70
48	18	5	28	6	33	1	6	2600	320
72	18	9	50	9	50	3	17	1200	300
Control Eggs (Washed with Water)									
0	18	3	17	3	17	0	0	0	30
3	18	2	11	1	6	0	0	0	50
6	18	3	17	3	17	0	0	0	110
12	18	6	33	7	26	1	6	200	1520
24	18	9	50	12	67	3	17	700	2300
48	18	15	83	15	83	8	44	3200	1080
72	18	16	89	17	94	10	56	Over 5000	860
Control Eggs (Untreated)									
0	18	0	0	0	0	0	0	0	0
3	18	0	0	1	6	0	0	0	0
6	18	0	0	0	0	0	0	0	0
12	18	0	0	1	6	0	0	0	0
24	18	1	6	7	39	1	6	2000	280
48	18	6	33	11	61	3	17	Over 5000	1760
72	18	11	61	12	67	6	33	Over 5000	780

TABLE 4

THE PENETRATION OF EGGS BY PS. AERUGINOSA, STORED AT 37° C.
IN A RELATIVE HUMIDITY OF 100 PER CENT

Hour of Sampling	Number of Eggs Sampled	Inner Shell		Inner Shell Swab		Albumin		Average Plate Count per ml.	
		No. Infected	%	No. Infected	%	No. Infected	%	Albumin	Shell
Eggs Treated with Copper Vitresan									
0	18	0	0	1	6	0	0	0	0
3	18	0	0	0	0	0	0	0	0
6	18	0	0	0	0	0	0	0	0
12	18	0	0	0	0	0	0	0	0
24	18	1	6	1	6	0	0	0	70
48	18	5	28	6	33	1	6	2600	320
72	18	9	50	9	50	3	17	1200	300
Control Eggs (Washed with Water)									
0	18	3	17	3	17	0	0	0	30
3	18	2	11	1	6	0	0	0	50
6	18	3	17	3	17	0	0	0	110
12	18	6	33	7	26	1	6	200	1520
24	18	9	50	12	67	3	17	700	2300
48	18	15	83	15	83	8	44	3200	1080
72	18	16	89	17	94	10	56	Over 5000	860
Control Eggs (Untreated)									
0	18	0	0	0	0	0	0	0	0
3	18	0	0	1	6	0	0	0	0
6	18	0	0	0	0	0	0	0	0
12	18	0	0	1	6	0	0	0	0
24	18	1	6	7	39	1	6	2000	280
48	18	6	33	11	61	3	17	Over 5000	1760
72	18	11	61	12	67	6	33	Over 5000	780

TABLE 5

THE PENETRATION OF EGGS BY PS. AERUGINOSA, STORED AT 20° - 25° C.
IN A RELATIVE HUMIDITY OF 100 PER CENT

Hour of Sampling	No. of Eggs Sampled	Inner Shell		Inner Shell Swab		Albumin		Average Plate Count per ml.	
		No. Infected	%	No. Infected	%	No. Infected	%	Albumin	Shell
Eggs Treated with Copper Vitresan									
6	10	0	0	0	0	0	0	0	0
12	10	0	0	0	0	0	0	0	0
24	10	0	0	1	10	0	0	0	0
48	10	1	10	1	10	0	0	0	60
72	10	2	20	3	30	1	10	300	110
96	10	2	20	2	20	1	10	1200	580
Control Eggs (washed with water)									
6	10	0	0	0	0	0	0	0	0
12	10	0	0	0	0	0	0	0	0
24	10	2	20	3	30	0	0	0	50
48	10	3	30	4	40	2	20	2300	870
72	10	5	50	6	60	2	20	1100	Over 5000
96	10	8	80	10	100	6	60	Over 5000	over 5000
Control Eggs (Untreated)									
6	10	0	0	0	0	0	0	0	0
12	10	1	10	1	10	0	0	0	20
24	10	2	20	3	30	0	0	0	380
48	10	2	20	3	30	1	10	500	1210
72	10	3	30	5	50	1	10	1200	4030
96	10	5	50	4	40	3	30	Over 5000	Over 5000

TABLE 6

THE PENETRATION OF EGGS BY PS. AERUGINOSA, STORED AT 20° TO 25° IN A RELATIVE HUMIDITY OF 35 TO 55 PER CENT

Hour of Sampling	No. of Eggs Sampled	Inner Shell		Inner Shell Swab		Albumin		Average Plate Count per ml.	
		No. Infected	%	No. Infected	%	No. Infected	%	Albumin	Shell
Eggs Treated with Copper Vitresan									
0	10	2	20	2	20	1	10	300	150
6	10	0	0	0	0	0	0	0	0
12	10	0	0	0	0	0	0	0	0
24	10	1	10	1	10	1	10	500	1100
48	10	2	20	3	30	2	20	Over 5000	Over 5000
72	10	1	10	1	10	1	10	2500	250
Control Eggs (Washed with water)									
0	10	0	0	1	10	0	0	0	0
6	10	2	20	2	20	1	10	500	710
12	10	0	0	1	10	0	0	0	0
24	10	2	20	4	40	0	0	0	50
48	10	4	40	7	70	2	20	1500	Over 5000
72	10	5	50	7	70	4	40	Over 5000	Over 5000
Control Eggs (Untreated)									
0	10	0	0	0	0	0	0	0	0
6	10	1	10	2	20	0	0	0	200
12	10	3	30	6	60	2	20	1500	1220
24	10	3	30	4	40	3	30	800	Over 5000
48	10	3	30	6	60	1	10	200	300
72	10	2	20	5	50	2	20	Over 5000	Over 5000

TABLE 7

PENETRATION OF EGGS BY PS. AERUGINOSA, STORED AT 8° TO 10° C. IN A RELATIVE HUMIDITY OF 100 PER CENT

Time of Sampling	Number of Eggs Sampled	Inner Shell		Inner Shell Swab		Albumin		Average Plate Count per ml.	
		No. Infected	%	No. Infedted	%	No. Infected	%	Albumin	Shell
Eggs Treated with Copper Vitresan									
26 days	10	0	0	0	0	0	0	0	0
30 Days	10	0	0	0	0	0	0	0	0
35 Days	10	0	0	0	0	0	0	0	0
40 Days	10	2	20	4	40	2	20	Over 5000	Over 5000
Control Eggs (Washed with Water)									
26 Days	10	0	0	0	0	0	0	0	0
30 Days	10	1	10	2	20	1	10	Over 5000	Over 5000
35 Days	10	3	30	3	30	3	30	Over 5000	Over 5000
40 Days	10	4	40	4	40	3	30	Over 5000	Over 5000
Control Eggs (Untreated)									
26 Days	10	0	0	0	0	0	0	0	0
30 Days	10	0	0	1	10	0	0	0	0
35 Days	10	2	20	4	40	0	0	0	Over 5000
40 Days	10	3	30	3	30	3	30	Over 5000	Over 5000

The results revealed by this table would indicate that if the eggs are washed to remove dirt, then the use of an antipenetration compound is warranted. If, however, the eggs are not washed and not exposed to adverse storage conditions which would destroy the bloom, the extent of penetration would be slight.

Table 5 would indicate that when eggs are stored at room temperatures and 100 per cent relative humidity, Copper Vitresan would offer considerable protection against the penetration of organisms. Washed eggs, however, exhibit a little more infectivity than eggs of the other two groups. In unwashed eggs the bloom of the egg appears to be almost as good a protection against penetration as treatment with the compound.

Table 6 shows, that under the conditions of storage, i.e. a temperature of 20° - 25° C. and a relative humidity of 35-55 per cent, Copper Vitresan offers a definite protection against the invasion of the egg by micro-organisms. Both the washed and the untreated eggs had much higher percentages of infectivity than the treated group. This is probably due to ionization of the Copper Vitresan as increased humidities would offer more moisture which is necessary for its ionization.

Table 7 gives the results obtained using eggs stored at low temperatures and 100 per cent relative humidity. As in the preceding cases, eggs which have been washed show the

greatest penetration of the test organism, while in the other two groups penetration is not as great. The eggs treated with Copper Vitresan were better protected than were the untreated eggs.

G. BLACK LIGHT EXAMINATION OF EGGS

The use of black light was begun during the course of study of Copper Vitresan. It was originally thought that it could be used to check plate counts and growth in tubes, but it was found that it was even more effective in detecting contaminated eggs after they had been broken from the shell.

A General Electric Black Light Lamp was used for these examinations. Shell, white, and yolk were examined under the lamp as well as all plates and culture tubes. By using the lamp as a check, more exact plate counts could be obtained because very small colonies which had just begun to develop showed up equally as well as the more fully developed colonies due to the diffusion of the pigments of Ps. aeruginosa in the medium adjacent to each colony.

Ps. aeruginosa produces fluorescein, pyocyanin, and pyrorubrin. Of these, pyocyanin is uaually associated with the organism because of the greenish-blue color which it imparts to the medium on which the organism is grown. The organism loses the power to produce pyocyanin at low temperatures and at higher temperatures this pigment producing power is also diminished. The optimum temperature for pigment

production is about 20° to 25° C. Fluorescein production seems to have a wider range. It was found that this pigment was produced at temperatures as low as 7° - 10° C. and as high as 37° - 40° C.

The detection of this pigment was by means of black light. Under this light the medium turned a brilliant yellow color. If pyocyanin was also produced, then the medium appeared a bright yellowish-blue color. Since small amounts of the pigment is visible under black light, many small colonies which would have remained uncounted on plates were detected. Fluorescein production started before pyocyanin production in all cultures ~~was~~ observed.

After samples were made of the eggs, the contents were examined under black light. Albumin has some natural fluorescence, but if organisms were present in sufficient number fluorescence was enhanced and the albumin appeared a distinct yellowish-blue to a yellowish-green color. If the egg had recently become infected with the organism, then the fluorescence was confined to several foci in the albumin. If the egg had been contaminated for some time and the organisms spread throughout the entire albumin, then the entire mass fluoresced under the lamp. Pyocyanin usually appeared at this stage and the albumin assumed a yellowish-green or yellowish-blue color, usually visible to the eye under ordinary light.

Infection of the yolk could not always be determined by this method. The natural yellow color of the yolk masked the yellow color of the fluorescein.

The shell surface was examined by means of black light to determine if penetration of the organism could be predicted. Under black light, a fresh normal egg has a deep scarlet hue, older eggs have a more purplish color, and a very old egg has a definite purple color characteristic of the light. Brown shelled eggs gave a deeper scarlet color than white eggs of comparable ages.

Close examination of the shell revealed many fluorescent pin-point spots over the entire area. It was found that these eggs usually were infected when measured bacteriologically. It was further noted that when these eggs were examined closely under ordinary light, these spots appeared in the pores of the egg indicating that the organisms had proliferated enough in the pores to produce the pigment which was observed under the lamp. These flourescent pin-point spots did not appear until about six hours had elapsed after treatment of the eggs with the culture.

Tables 8 to 10 inclusive give the comparison between the black light examinations and the bacteriological examinations. As can be seen from these, black light examinations usually showed a higher percentage infectivity than the bacteriological examinations. They may be due to several factors. First, natural fluorescence of the white

may have been mistaken for contamination, but this is doubtful due to the characteristic color of fluorescein under black light. Secondly, in the case of newly infected eggs, the organisms may not have spread through the entire albumin and were missed in the sampling procedure, or if they were few in numbers they may have been diluted sufficiently that they failed to grow in the medium used, or they were again missed when the dilution was plated.

Considering these factors, there is a remarkable correlation between the examinations of the eggs made bacteriologically and examinations of the eggs made with black light.

TABLE 8

COMPARISON OF BLACK LIGHT EXAMINATIONS WITH BACTERIOLOGICAL EXAMINATIONS INCUBATED AT 37° C. IN A RELATIVE HUMIDITY OF 100 PER CENT

Hour of Sampling	Black Light Examinations					Bacteriological Examinations			
	Number of Shell Exterior			Albumin		Inner Shell		Albumin	
	Eggs Sampled	No. Infected	%	No. Infected	%	No. Infected	%	No. Infected	%
Eggs Treated with Copper Vitresan									
0	18	0	0	0	0	0	0	0	0
3	18	0	0	0	0	0	0	0	0
6	18	0	0	0	0	0	0	0	0
12	18	1	6	0	0	0	0	0	0
24	18	3	17	1	6	1	6	0	0
48	18	9	50	2	11	5	28	1	6
72	18	10	56	5	28	9	50	3	17
Control Eggs (Washed with Water)									
0	18	0	0	0	0	3	17	0	0
3	18	0	0	0	0	2	11	0	0
6	18	0	0	0	0	3	17	0	0
12	18	2	11	0	0	6	33	1	6
24	18	5	28	4	22	9	50	3	17
48	18	16	89	11	61	15	83	8	44
72	18	18	100	13	72	16	89	10	56
Control Eggs (Untreated)									
0	18	0	0	0	0	0	0	0	0
3	18	0	0	0	0	0	0	0	0
6	18	0	0	0	0	0	0	0	0
12	18	2	11	1	6	0	0	0	0
24	18	5	28	4	22	1	6	1	6
48	18	7	39	4	22	6	33	3	17
72	18	12	67	7	39	11	61	6	33

TABLE 9

COMPARISON OF BLACK LIGHT EXAMINATIONS WITH BACTERIOLOGICAL EXAMINATIONS INCUBATED AT 20° TO 25° C. IN A RELATIVE HUMIDITY OF 100 PER CENT

Hour of Sampling	No. of Eggs Sampled	Black Light Examinations				Bacteriological Examinations			
		Shell Exterior		Albumin		Inner Shell		Albumin	
		No. Infected	%	No. Infected	%	No. Infected	%	No. Infected	%
Eggs Treated with Copper Vitresan									
6	10	0	0	0	0	0	0	0	0
12	10	1	10	0	0	0	0	0	0
24	10	0	0	0	0	0	0	0	0
48	10	2	20	1	10	1	10	0	0
72	10	3	30	1	10	2	20	1	10
96	10	3	30	3	30	2	20	1	10
Control Eggs (Washed with Water)									
6	10	0	0	0	0	0	0	0	0
12	10	1	10	0	0	0	0	0	0
24	10	4	40	2	20	2	20	0	0
48	10	3	30	3	30	3	30	1	10
72	10	7	70	5	50	5	50	1	10
96	10	9	90	8	80	8	80	3	30
Control Eggs (Untreated)									
6	10	0	0	0	0	0	0	0	0
12	10	2	20	0	0	1	10	0	0
24	10	4	40	1	10	2	20	0	0
48	10	5	50	3	30	2	20	1	10
72	10	4	40	3	30	3	30	1	10
96	10	7	70	4	40	5	50	3	30

TABLE 10

COMPARISON OF BLACK LIGHT EXAMINATIONS WITH BACTERIOLOGICAL EXAMINATIONS INCUBATED AT 20° TO 25° C. IN A RELATIVE HUMIDITY OF 35 - 55 PER CENT

Hour of Sampling	No. of Eggs Sampled	Black Light Examinations				Bacteriological Examinations			
		Shell Exterior		Albumin		Inner Shell		Albumin	
		No. Infected	%	No. Infected	%	No. Infected	%	No. Infected	%
Eggs Treated with Copper Vitresan									
0	10	0	0	0	0	2	20	1	10
6	10	0	0	0	0	0	0	0	0
12	10	0	0	1	10	0	0	0	0
24	10	1	10	2	20	1	10	1	10
48	10	3	30	3	30	2	20	2	20
72	10	3	30	2	20	1	10	1	10
Control Eggs Washed with Water									
0	10	0	0	0	0	0	0	0	0
6	10	0	0	0	0	2	20	1	10
12	10	2	20	0	0	0	0	0	0
24	10	3	30	3	30	2	20	0	0
48	10	5	50	2	20	4	40	2	20
72	10	8	80	3	30	5	50	4	40
Control Eggs (Untreated)									
0	10	0	0	0	0	0	0	0	0
6	10	0	0	0	0	1	10	0	0
12	10	2	20	0	0	3	30	2	20
24	10	5	50	3	30	3	30	3	30
48	10	4	40	2	20	3	30	1	10
72	10	6	60	3	30	2	20	2	20

TABLE 10

COMPARISON OF BLACK LIGHT EXAMINATIONS WITH BACTERIOLOGICAL EXAMINATIONS INCUBATED AT 20° TO 25° C. IN A RELATIVE HUMIDITY OF 35 - 55 PER CENT

Hour of Sampling	No. of Eggs Sampled	Black Light Examinations				Bacteriological Examinations			
		Shell Exterior		Albumin		Inner Shell		Albumin	
		No. Infected	%	No. Infected	%	No. Infected	%	No. Infected	%
Eggs Treated with Copper Vitresan									
0	10	0	0	0	0	2	20	1	10
6	10	0	0	0	0	0	0	0	0
12	10	0	0	1	10	0	0	0	0
24	10	1	10	2	20	1	10	1	10
48	10	3	30	3	30	2	20	2	20
72	10	3	30	2	20	1	10	1	10
Control Eggs Washed with Water									
0	10	0	0	0	0	0	0	0	0
6	10	0	0	0	0	2	20	1	10
12	10	2	20	0	0	0	0	0	0
24	10	3	30	3	30	2	20	0	0
48	10	5	50	2	20	4	40	2	20
72	10	8	80	3	30	5	50	4	40
Control Eggs (Untreated)									
0	10	0	0	0	0	0	0	0	0
6	10	0	0	0	0	1	10	0	0
12	10	2	20	0	0	3	30	2	20
24	10	5	50	3	30	3	30	3	30
48	10	4	40	2	20	3	30	1	10
72	10	6	60	3	30	2	20	2	20

TABLE 10

COMPARISON OF BLACK LIGHT EXAMINATIONS WITH BACTERIOLOGICAL EXAMINATIONS INCUBATED AT 20° TO 25° C. IN A RELATIVE HUMIDITY OF 35 - 55 PER CENT

Hour of Sampling	No. of Eggs Sampled	Black Light Examinations				Bacteriological Examinations			
		Shell Exterior		Albumin		Inner Shell		Albumin	
		No. Infected	%	No. Infected	%	No. Infected	%	No. Infected	%
Eggs Treated with Copper Vitresan									
0	10	0	0	0	0	2	20	1	10
6	10	0	0	0	0	0	0	0	0
12	10	0	0	1	10	0	0	0	0
24	10	1	10	2	20	1	10	1	10
48	10	3	30	3	30	2	20	2	20
72	10	3	30	2	20	1	10	1	10
Control Eggs Washed with Water									
0	10	0	0	0	0	0	0	0	0
6	10	0	0	0	0	2	20	1	10
12	10	2	20	0	0	0	0	0	0
24	10	3	30	3	30	2	20	0	0
48	10	5	50	2	20	4	40	2	20
72	10	8	80	3	30	5	50	4	40
Control Eggs (Untreated)									
0	10	0	0	0	0	0	0	0	0
6	10	0	0	0	0	1	10	0	0
12	10	2	20	0	0	3	30	2	20
24	10	5	50	3	30	3	30	3	30
48	10	4	40	2	20	3	30	1	10
72	10	6	60	3	30	2	20	2	20

TABLE 11

COMPARISON OF BLACK LIGHT EXAMINATIONS WITH BACTERIOLOGICAL EXAMINATIONS INCUBATED AT 8° TO 10° C. IN A RELATIVE HUMIDITY OF 100 PER CENT

Time of Sampling	No. of Eggs Sampled	Black Light Examinations				Bacteriological Examinations			
		Shell Exterior		Albumin		Inner Shell		Albumin	
		No. Infected	%	No. Infected	%	No. Infected	%	No. Infected	%
Eggs Treated with Copper Vitresan									
26 Days	10	1	10	0	0	0	0	0	0
30 Days	10	3	30	0	0	0	0	0	0
35 Days	10	5	50	2	20	0	0	0	0
40 Days	10	9	90	5	50	2	20	2	20
Control Eggs (Washed with Water)									
26 Days	10	2	20	0	0	0	0	0	0
30 Days	10	3	30	2	20	1	10	1	10
35 Days	10	3	30	3	30	3	30	3	30
40 Days	10	5	50	4	40	4	40	3	30
Control Eggs (Untreated)									
26 Days	10	1	10	0	0	0	0	0	0
30 Days	10	4	40	1	10	0	0	0	0
35 Days	10	4	40	2	20	2	20	0	0
40 Days	10	5	50	4	40	3	30	3	30

DISCUSSION

The data presented show that Copper Vitresan has value as an antipenetration compound, however, the conditions of storage greatly influence the value of it as an antipenetrant.

Variations in the results obtained may be attributed to several very closely related factors:

(1) Temperatures at which the eggs are stored.

(2) Relative humidity of the storage chamber.

(3) Concentration of the test compound employed.

(4) Inherent structure of the egg.

Low storage temperatures are used to prevent rapid decomposition of the eggs and in the case of eggs used in virology, to prevent embryonic development.

A relatively high humidity is used when storing market eggs. This prevents loss of weight due to the evaporation of water from the egg. In virology, humidity is not too important and is usually controlled to give either a small air cell or large air cell, depending upon the type of inoculation to be made.

The concentration of the test compound can be varied. In the case of Copper Vitresan, the higher the concentration the less infectivity occurred but with the higher concentrations the eggs assume a bluish hue and are rendered aesthetically objectionable.

The inherent structure of the egg plays an important role in penetration of the egg by microorganisms which is difficult to evaluate.

Checks, small cracks, and possibly large pores are conducive to penetration, while the bloom and the resistance of the shell membranes tend to lessen penetration. Since these factors cannot be evaluated without destroying the egg no attempt was made to consider them to any extent in this study.

Evaluation of the compound was based upon the swab sample of the inner shell. This method was used because it was a more certain measure of penetration than were the other methods. If the organism has penetrated the shell, then it will be a relatively short time until the albumin will become infected.

The other sampling procedures used, i.e., albumin sample and membrane sample, often failed to reveal the presence of the organism while the swab sample of the inner shell was positive.

Plate counts were made from both the albumin and saline samples. The counts were found to be erratic, possibly due to the sampling procedure. They offered no means of evaluating the compound, but only give an indication of the average number of organisms which have infected the eggs at a given sampling time.

Of all the factors concerned with penetration of microorganisms into eggs, temperature and relative humidity are the two most important.

The activity of microorganisms is directly dependent upon temperature. The higher the temperature, the faster the rate of reproduction and the greater the motility, if the organism is motile, until such a temperature is reached that destruction of either the organism or the substrate occurs. Conversely, the lower the temperature, the less the activity of the organism.

Activity of the organism is also directly related to the amount of moisture present. Reproduction and motility occur only in a moist state. If the relative humidity of the environment is high moisture is apt to condense on the egg shell and provide an excellent environment for reproduction of the organism.

Eggs removed from low storage temperatures to room temperatures usually have water of condensation on the shell in a short time. If the relative humidity is high, then substantial activity by the organism occurs and the eggs may become contaminated at this time. If the eggs are wiped dry, any organisms on the shell have a tendency to be pushed into the shell thus hastening contamination. If moist eggs are placed in a cooler temperature, contraction of air in the air cell draws the water and organisms through the shell contaminating the egg contents.

Ideally then, the most favorable conditions for preventing penetration of the shell by microorganisms is a low tem-

Since the average producer does not have the facilities to store his eggs properly, contamination is quite frequent, especially if he has cleaned them before sending them to market. If such a producer had some compound which would prevent penetration of microorganisms, then the large losses now experienced by the industry would be reduced considerably.

Copper Vitresan in a 2.5 per cent solution was found to act as a suitable antipenetrant. The use of this compound, or any compound as an antipenetrant, would be in no way a substitute for the proper handling and storage of eggs by the producer for once the egg is contaminated by microorganisms, an antipenetrant is of no value.

SUMMARY

(1) A method for opening eggs which gave very little contamination is presented.

(2) Sampling procedures are presented for testing compounds for antipenetrating properties.

(3) Penetration studies were made on eggs using as test organisms B. subtilis, E. coli, M. pyogenes var. aureus, and Ps. aeruginosa. It was found that the average time of penetration of these organisms was about 14 to 18 hours.

(4) Copper Vitresan in a 2.5 per cent concentration was found to act as an acceptable antipenetrant.

(5) Black light was found to be a good method for checking the penetration of eggs by Ps. aeruginosa.

CONCLUSIONS

Washed eggs were more susceptible to bacterial invasion than were unwashed eggs. Copper Vitresan in a 2.5 per cent solution when applied to washed eggs lessened bacterial invasion. A method of testing penetration of eggs by Ps. aeruginosa with black light is presented.

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