PURIFICATION OF SUSPENSIONS OF THE VIRUS OF VACCINIA BY MEANS OF CARBON DIOXIDE[1]

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Introduction

The vaccine used to protect against smallpox, though very efficient, contains not only the virus of vaccinia but also a great deal of foreign matter.

The purpose of this work was to remove as much of this extraneous material and obtain the virus in as pure a form as possible. In order to accomplish this we contemplated precipitating the tissue at its iso-electric point, and thus we hoped to procure clear virulent suspensions of the virus.

Preliminary experiments revealed the fact that inorganic acids such as sulphuric or hydrochloric could not be used to obtain the proper hydrogen-ion concentration necessary to bring the tissue to its iso-electric point, because of their harmful effect on the virus. Organic acids such as acetic, carbonic, citric, lactic, succinic, and tartaric when used in such small amounts as are required to obtain the iso-electric point of the tissue were practically harmless in their effect upon the virus. Since carbonic acid is the weakest of these organic acids, it was hoped that the desired results could be obtained by bubbling carbon dioxide gas through tissue emulsions until the proper hydrogen-ion concentration was reached by the formation of carbonic acid.

Another seeming advantage of using carbonic acid as a precipitating agent was the fact that it may be easily displaced, and consequently its action stopped, by bubbling an inert gas through the suspension.

Historical

Many attempts in the past to separate the virus of vaccinia from the extraneous material have met with varying success. Among these are the cataphoresis experiments carried out by Douglas and Smith, and Lepine. Ward and Tang, Ward, and Yaoi and Kasai filtered the virus. Bland filtered and centrifuged it, Tang centrifuged it, and Yaoi and Kasai carried out diffusion and adsorption experiments on it.

Behrens and Morgan have previously obtained water clear preparations "free from tissue cells and extraneous matter, giving only a slight biuret reaction and carrying a high percentage of the virus." Two methods were used in obtaining these water clear preparations: one in which aluminum gel (Brewer-Kraybill) was used as the precipitating agent, and the other in which the iso-electric point of the tissue was brought about. The "Aluminum Gel Method" requires the use of an agent of varying concentration and composition; also it seems applicable only to the purification of dilute vaccines. For these reasons its value commercially is questionable.

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We felt that the "Iso-electric Point Method" could be developed to a greater degree, and this paper is devoted to that process.

Materials

Dermovirus.—The virus used in this work was prepared from the pulp obtained from a calf inoculated with a mixture of calf and rabbit dermovirus.

Carbon dioxide.—A tank of the compressed gas used as a source of the carbon dioxide.

Distilled water.—Single-distilled water, ranging from pH 5.5 to pH 5.8, was used.

Preparation of the Virus for Experiments

The pulp was weighed and placed in a mortar, or a large pyrex test tube, and ground with pyrex ground-glass, which had been passed through a number 80 sieve, until the tissue was completely disintegrated. Distilled water of a sufficient quantity was added slowly to the required concentration of the emulsion. A fine, homogeneous emulsion resulted.

Theoretical Considerations in Purification

The virus of vaccinia resembles colloidal particles in the nature of its behavior to certain chemicals. Douglas and Smith, working on the electrical charge of the virus of vaccinia, have pointed out that the virus carries a negative charge at pH 5.5 to pH 8.4 . Yaoi and Kasai, working on the adsorption of the virus of vaccinia on kaolin, show that the virus has a negative charge at pH 4.7 and can exist in the presence of kaolin at this low pH for at least 30 minutes. In our work we have recovered virus from the supernatant liquid of an emulsion treated with acid in which the pH went as low as 4.4.

We found that the iso-electric point of the tissue emulsion was pH 4.8. The iso-electric point of the virus has not been determined, since it shows signs of inactivation when remaining at lower pH values than 5.5 for any length of time (Douglas and Smith). We have demonstrated, by typical reactions on rabbits, the presence of the virus in solutions having had lower pH values than 4.8; but the solutions were not allowed to remain at these low pH values for any length of time for they were neutralized before injecting. Therefore, it would seem possible to free the virus from the tissue by bubbling carbon dioxide through the tissue emulsion until pH 4.8 was reached. To preserve the virulence of the virus, after centrifugating out the clumped tissue, the carbonic acid may be displaced by nitrogen or neutralized with sodium carbonate.

Experiments in Purification

The vaccinal activity of the purified virus suspension was tested by intradermal inoculations of 0.2 cc. of various dilutions into normal rabbits. The percentage of the virus in the purified vaccine was determined by its titre. The strength of the reactions and the number of days after inoculation required for the reactions to appear were used as an index to the virulence of the virus. The reactions produced by the

original emulsion were the standards for comparison. Both the emulsion and the purified vaccine were injected into the same rabbit.

Preliminary Experiments

Experiment 1: Carbon dioxide as a purifying agent.

This experiment was carried out to determine the possibility of using carbon dioxide as a purifying agent.

A one per cent emulsion was prepared and placed in an ice bath. Carbon dioxide was passed through a capillary tube into the emulsion and the bubbling allowed to proceed at a slow rate for 20 minutes. Clumping of the emulsion particles was then noticeable. The clumped emulsion (or mixture, as we shall identify it hereafter) was centrifuged for 10 minutes at high speed, and the supernatant fluid was neutralized by passing nitrogen through it for one hour. A transparent preparation resulted which gave a negative biuret test. Various dilutions of this preparation were made up, and 0.2 cc. of each dilution was injected intradermally into a normal rabbit. The results of the inoculations with dilutions from the original emulsion and the purified preparation are shown in Table 1.

From the results of this experiment we conclude that it was possible to separate the virus from the tissue. The long exposure of the virus to the acid decreased its virulence; no strong reactions were noted.

TABLE 1. The Effect of Bubbling Carbon Dioxide through the dermo Emulsion for 20 Minutes at 0° C.

Rabbit number	Prepared virus	Reaction with various Dilutions*								"Days"
		5	10	15	20	25	30	35	40	
1	Original emulsion	+	+	+	+	+	+	+	—	5
		++	++	++	++	++	++	++	+	7
1	Purified	+	+	—	—	—	—	—	—	7
		+	+	+	+	+	+	—	—	11
2	Original emulsion	+	+	+	+	+	+	+	+	5
		++	++	++	++	++	++	++	+	7
2	Purified	—	—	—	—	—	—	—	—	7
		+	+	+	+	+	+	—	—	11

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution).
+Indicates reddening of the skin.
++Indicates a strong reaction.
—Indicates no reaction.
"Days" is the number of days after injection.

Since it is not necessary to bring the pH of the emulsion below the iso-electric point of the tissue to obtain clarification, the following experiments were conducted in search of a method to control the pH.

Experiment 2: The pH of carbonic acid solutions.

The object of this experiment was to determine the pH obtained by bubbling carbon dioxide through distilled water, physiological solution of sodium chloride, and physiological solution of sodium chloride

adjusted to pH 7.4 with sodium carbonate. The time required to reach that pH was an additional element in this problem.

Each solution was placed in an ice bath, and carbon dioxide was passed through a bubbling tube[2] into each at a constant slow rate. Samples were removed at the end of one, two, four, eight, and 16 minutes, and their pH determined colorimetrically.

From the results of this experiment (Table 2) we found it was possible to obtain a solution of carbonic acid in distilled water, which had a pH value of 3.7 at 0°C. Less carbonic acid remained in solution in the physiological solutions of sodium chloride. This was probably due to the "salting out" effect of the sodium chloride. In the adjusted salt solution a carbonate buffer system was probably formed.

The pH values of all of these solutions were much too low when complete saturation with carbonic acid was attained. The addition of tissue material to these solutions may change the amounts of carbonic acid held in solution. This problem is dealt with in Experiment 3.

Experiment 3: The pH of carbonic acid solutions in one per cent dermo emulsions.

The object of this experiment was to determine the pH of carbonic acid solutions in emulsions made up in the three solutions tested in Experiment 2. The required time for noticeable clumping of the tissue and clarification of the supernatant fluid were also noted.

A one per cent emulsion was made in each of the three solutions. They were placed in an ice bath and carbon dioxide was passed through a bubbling tube into them. At the end of one, two, four, eight, 16 and 32 minutes samples were removed, centrifuged, and their pH value determined colorimetrically.

In this experiment, also, the pH values (Table 3) obtained in the saturated solutions of carbonic acid were much lower than the iso-electric point of the tissue. The rate of bubbling was a big factor in the time required to obtain saturation, and it could not be controlled to a satisfactory degree without special apparatus. Also, the pH dropped rapidly below the iso-electric point of the tissue. Therefore, the desired pH would be difficult to obtain by checking the flow of carbon dioxide through the solution at a certain time. The fact that gases are less soluble at higher temperatures gave us a possible method of controlling the pH of the saturated solutions of carbonic acid. With these points in view the next experiment was carried out. Distilled water was chosen for the suspending medium because saturation and clarification were obtained in a much shorter time than with the emulsions made up in salt solutions.

Experiment 4: The pH of carbonic acid solutions in one per cent dermo emulsion at 15°, 20°, and 25°C.

A one per cent emulsion was made in distilled water and divided into three parts. The three parts were placed, respectively, in water

[2] A satisfactory bubbling tube may be prepared by sealing a spider of 22 gauge iron wire in the joint of two straight glass tubes of the same diameter. After removing one end of the tube (about one-half inch from the joint) the tube is immersed in acid to remove the wire. The end of the tube near the holes may be sealed or it may be stoppered with a cork stopper which can be removed to facilitate cleaning of the tube.

TABLE 2. Showing pH of Carbonic Acid Solutions at Various Intervals of Time During Carbon Dioxide Bubbling at 0° C.

Emulsifying liquid	*pH of solutions at various time					
	0 min.	1 min.	2 min.	4 min.	8 min.	16 min.
Distilled water	5.6	4.0	3.8	3.7	3.7	3.7
Physiological solution of sodium chloride	5.7	4.4	4.1	4.0	3.9	3.9
Physiological solution of sodium chloride (neutralized with sodium carbonate)	7.4	5.4	5.0	4.8	4.6	4.5

*pH determined colorimetrically.

baths having temperatures of 15°, 20°, and 25°C. Carbon dioxide was passed at a slow rate into the emulsions, through a bubbling tube, and the pH values were determined potentiometrically at regular intervals during the bubbling. At the end of five minutes clarification was complete in all three samples.

TABLE 3. Showing pH, Clumping, and Supernatant Fluid Clearness at Various Intervals of Time During Carbon Dioxide Bubbling Through Dermo Emulsion at 0° C.

Time in minutes	*pH of supernatant fluid			Clumping			Supernatant fluid clearness		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
0	6.6	6.6	7.4	—	—	—			
1	5.0	5.2	5.4	+	—	—	turbid	turbid	turbid
2	4.6	4.8	5.0	+	—	—	clear	turbid	turbid
4	4.4	4.6	4.8	+	+	—	clear	turbid	turbid
8	4.4	4.5	4.6	+	+	+	clear	clear	turbid
16	4.4	4.5	4.6	+	+	+	clear	clear	clear
32	4.4	4.5	4.6	+	+	+	clear	clear	clear

No. 1 Emulsion made up in distilled water.
No. 2 Emulsion made up in physiological solution of sodium chloride.
No. 3 Emulsion made up in neutralized solution of sodium chloride.
*pH determined colorimetrically.
+Indicates visible clumping of the tissue.
—Indicates no visible clumping.

The results show that the pH dropped very rapidly to pH 5.1, which is above the iso-electric point of the tissue. It continued to drop at a slower rate until saturation was complete. This led us to believe that the bubbling of carbon dioxide could be checked after a certain interval of time and fairly constant pH values could be obtained. At 15°C. the bubbling time required to reach the iso-electric point of the emulsion is two minutes; at 20°C. it is three minutes, and at 25°C. it is five minutes. Upon continued bubbling the pH does not go below 4.65, in

any case. It was not considered advisable to increase the temperature of the emulsion above 25°C. because higher temperatures may have a harmful effect on the virus.

Table 4. Showing pH of Dermo Emulsion at Various Intervals of Time During Carbon Dioxide Bubbling at 15°, 20°, and 25° C.

Bubbling time		*pH of dermo emulsion at various temperatures		
Min.	Sec.	15° C.	20° C.	25° C.
0	.....	6.3	6.3	6.3
0	30	5.4	5.5	5.8
1	.....	5.1	5.25	5.5
1	30	4.95	5.1	5.2
2	.....	4.85	4.95	5.1
2	30	4.75	4.9	5.05
3	.....	4.75	4.8	4.95
3	30	4.7	4.8	4.9
4	.....	4.7	4.75	4.9
4	30	4.7	4.75	4.85
5	.....	4.7	4.75	4.8
6	.....	4.65	4.7	4.75
7	.....	4.65	4.65	4.7
8	.....	4.66	4.65	4.7
9	.....	4.65	4.65	4.7
10	.....	4.65	4.65	4.7
12	.....	4.65	4.65	4.7
15	.....	4.65	4.65	4.7

*pH determined potentiometrically.

We now had adequate means of controlling the pH of the emulsion while carbon dioxide was being bubbled through it. It was only necessary to bubble slowly enough to keep foaming at a minimum, to keep the temperature 20°C. and 25°C, and to stop the bubbling at the end of four minutes. At these temperatures, and after a four minute interval of bubbling, the pH is very near the iso-electric point of the tissue.

The time required to displace carbonic acid from a solution, by bubbling nitrogen through it, was determined before proceeding with experimental work on animals.

Experiment 5: Nitrogen as a neutralizing agent.

The purpose of this experiment was to determine the time required to displace carbonic acid by bubbling nitrogen through the supernatant fluid.

A one per cent emulsion was made in each of the three solutions used in Experiment 3. Carbon dioxide was bubbled through the emulsions at 20°C. for four minutes. After centrifuging, nitrogen was bubbled through the supernatant fluid. Samples were removed at the end of one, two, four, eight, and 16 minutes and the pH determined colorimetrically.

From the results, found in Table 5, it is apparent that 16 minutes is sufficient time to bubble nitrogen through any of the three solutions, at room temperature, to restore the pH to 7.0.

TABLE 5. Showing pH of Carbonic Acid Solutions at Various Intervals of Time During Nitrogen Bubbling.

Emulsifying liquid	*pH of solutions at various times					
	0 min.	1 min.	2 min.	4 min.	8 min.	16 min.
Distilled water	4.7	4.9	5.1	5.5	6.5	7.0
Physiological solution of sodium chloride	4.8	5.0	5.2	5.6	6.5	7.0
Physiological solution of sodium chloride (neutralized with sodium carbonate)	4.8	5.1	5.2	5.5	6.6	7.1

*pH determined colorimetrically.

Experiments With Purified Vaccine

The following experiments were carried out to determine the percentage and the virulence of the virus in the purified vaccine.

One Per Cent Dermo Emulsion

Experiment 6: Purification by bubbling carbon dioxide for four minutes at room temperature.

A one per cent emulsion was made in distilled water and carbon dioxide was passed through it at a slow rate for four minutes. The

TABLE 6. The Effect of Bubbling Carbon Dioxide Through the Dermo Emulsion for Four Minutes.

Rabbit number	Prepared virus	Reactions with various dilutions*								"Days"
		5	10	15	20	25	30	35	40	
3	Original emulsion	+	+	+	+	+	+	+	+	5
		++	++	++	++	++	++	++	++	7
3	Purified	+	+	+	—	—	—	—	—	7
		+	++	+	+	+	+	—	—	11
4	Original emulsion	+	+	+	+	+	+	+	—	5
		++	++	++	++	++	++	++	+	7
4	Purified	+	+	—	—	—	—	—	—	7
		++	++	+	+	+	+	+	—	7

*pH of the Solutions Throughout the Experiment: (Determined Colorimetrically.)

Rabbit Number	Emulsion pH	Mixture pH	Supernatant fluid pH	Neutralized supernatant fluid: pH	Exposure to low pH
3	6.3	4.7	4.8	7.0	20 min.
4	6.3	4.8	4.9	7.0	20 min.

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution).
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.

mixture was centrifuged for 10 minutes and nitrogen was passed through the supernatant fluid for 16 minutes. This procedure resulted in a transparent preparation having a pH of 7.0.

The results of the titre are found in Table 6.

A high percentage of the virus was present in the purified preparation, but it did not have the virulence to produce good reactions in seven days, as did the original emulsion. The reduced virulence must have been due to exposure of the virus to the acid for so long a time.

Experiment 7: Purification by bubbling carbon dioxide at a rapid rate for one minute.

As the virus was exposed to pH 4.8 for at least 20 minutes in Experiment 6, an attempt was made to reduce this time to a minimum.

TABLE 7. The Effect of Bubbling Carbon Dioxide through the Dermo Emulsion for One Minute.

Rabbit number	Prepared virus	Reactions with various dilutions*							"Days"
		10	20	30	40	50	75	100	
5	Original emulsion	++	++	++	++	++	++	++	5
		++	++	++	++	++	++	++	7
5	Purified	++	++	++	++	++	++	+	7
		++	++	++	++	++	++	+	9
6	Original emulsion	++	++	++	++	++	++	++	5
		++	++	++	++	++	++	++	7
6	Purified	++	++	++	++	++	++	+	7
		++	++	++	++	++	++	+	9

pH of the Solutions Throughout the Experiment:
(Determined potentiometrically.)

Rabbit Number	Emulsion pH	Mixture pH	Supernatant fluid pH	Exposure to low pH
5	6.4	4.9	4.9	6 min.
6	6.4	4.8	4.9	6 min.

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution.)
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.

A recently harvested sample of pulp was obtained which had a high emulsion titre. Emulsion made up with the new pulp did not foam so badly as those made up from old pulp. This permitted more rapid bubbling.

Carbon dioxide was bubbled through the emulsion at a rapid rate for one minute and the mixture was centrifuged for five minutes. This procedure resulted in a transparent preparation. Dilutions were immediately made in adjusted salt solution.

The results of this experiment (Table 7) show that the virulence of the virus was increased by this procedure, but the reactions did not appear as soon as those from the emulsion.

Experiment 8: Purification by the addition of a carbonic acid solution to a 20 per cent dermo emulsion.

In Experiment 2 it was noted that at 0°C. a saturated solution of carbonic acid in distilled water may reach pH 3.7. From Experiment 4 it became apparent that a temperature change affects the solubility of the carbon dioxide. So it seemed possible to obtain a solution of carbonic acid of such pH that when it was added to a concentrated emulsion the resulting pH would be 4.8.

Distilled water was saturated with carbonic acid at 20°C. and found to be pH 4.2. This solution was then added to a 20 per cent emulsion, the combination making a one per cent emulsion. Small clumps of the tissue appeared at once. After the mixture underwent centrifugation for five minutes it was clear. The mixture pH was found to be 4.8. The supernatant liquid was immediately diluted in adjusted salt solution.

TABLE 8. The Effect of the Addition of a Carbonic Acid Solution to a 20 per cent Dermo Emulsion.

Rabbit number	Prepared virus	Reactions with various dilutions*							"Days"
		10	20	30	40	50	75	100	
7	Original emulsion	++	++	++	++	++	+	+	5
		++	++	++	++	++	++	++	7
7	Purified	+	+	+	+	+	+	—	7
		+	+	+	+	+	+	+	11
8	Purified	+	+	+	+	+	—	—	7
		++	++	+	+	+	+	+	11

pH of the Solutions Throughout the Experiment:
(Determined potentiometrically).

Rabbit number	Emulsion pH	Mixture pH	Supernatant fluid pH	Exposure to low pH
7	6.3	4.9	5.0	5 min.
8	6.3	4.8	4.9	5 min.

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution).
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.

The results (Table 8) show that a high percentage of the virus was retained in suspension, but the virulence was decreased.

The time of exposure of the virus to the low pH had been cut to a minimum, so we turned to the neutralization of the virus suspension as a means of retaining its virulence.

Experiment 9: Neutralization of the virus suspension with sodium carbonate.

Purified vaccines were prepared following the procedures in Experiments 7 and 8. Tenth normal sodium carbonate was added to each sample, the amount having been previously determined by potentiometric titration.

The results (Table 9) show that the virulence of the virus was increased by this procedure. The reactions appeared on the fourth or fifth day and were comparable to those produced by the original emulsion.

The possibility of neutralizing the acid in the mixture, before centrifugation, presented itself as a means of shortening the time the virus was exposed to the action of the acid.

Experiment 10: Neutralization of the mixture.

If the clumping of the tissue is complete when the pH reaches 4.8, it would seem possible to neutralize the acid, and, after centrifugation, to obtain a clear preparation.

An emulsion was prepared and the tissues clumped by the procedures followed in Experiments 7 and 8. A sample of each of these mixtures was neutralized by the addition of a previously determined amount of tenth normal sodium carbonate solution. In the remaining samples the acid was displaced by bubbling nitrogen through them for 16 minutes.

The results of the experiment are shown in Table 10.

The results of Experiment 10 give no evidence of an increase in the virulence of the virus. A clear vaccine was very difficult to prepare by this method. Often the result was a translucent preparation which could not be clarified by centrifuging for as long as one hour. Clumping of the tissue was not complete until the mixture was allowed to

TABLE 9. The Effect of Neutralizing the Virus Suspension with Sodium Carbonate.

Rabbit number	Purification method	Reaction with various dilutions*							"Days"
		10	20	30	40	50	75	100	
9	Bubbling CO_2 through the emulsion for one minute.	+	+	+	+	—	++	—	5
		++	++	++	++	++	++	+	7
		++	++	++	++	++	++	+	9
10	Bubbling CO_2 through the emulsion for one minute.	+	+	+	+	+	+	+	4
		++	++	++	++	++	++	+	6
		++	++	++	++	++	++	++	8
		+	++	++	++	+	+	++	10
11	Add carbonic acid solution to 20% emulsion.	+	+	+	+	+	—+	+	4
		++	++	++	++	++	++	++	6
		++	++	++	++	++	++	++	8
		+	+	++	+	++	++	++	10
12	Add carbonic acid solution to 20% emulsion.	++	++	++	+	+	+	+	5
		++	++	++	++	++	++	+	7
		++	++	++	++	++	++	++	9
		+	+	+	++	+	+	+	11

pH of the Solutions Throughout the Experiment:
(Determined potentiometrically).

Rabbit number	Emulsion pH	Mixture pH	Supernatant fluid pH	Neutralized supernatant fluid pH	Exposure to low pH
9	6.3	4.8	4.9	7.0	6 min.
10	6.3	4.9	5.0	7.2	6 min.
11	.....	4.9	5.1	7.3	5 min.
12	.....	4.8	5.0	7.2	5 min.

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution.)
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.
Emulsion titre: 1:100,000

TABLE 10. The Effect of Neutralizing the Clumped Dermo Emulsion with Nitrogen and Sodium Carbonate.

Rabbit number	Purification method	Neutralizing agent	Reaction with various dilutions*							"Days"
			10	20	30	40	50	75	100	
13	Bubbling CO_2	Nitrogen	+	+	—	—	—	—	—	5
	through the		+	+	+	+	+	+	—	7
	emulsion for		++	++	++	+	+	+	+	9
	one minute.									
14	Bubbling CO_2	Sodium	++	++	++	+	+	+	+	5
	through the	carbon-	++	++	++	++	++	++	++	7
	emulsion for	ate	++	++	++	++	++	++	++	9
	one minute.									
15	Add carbonic	Nitrogen	+	+	+	+	—	—	—	5
	acid solution		+	+	+	+	+	+	+	7
	to 20% emul-		+	++	++	++	+	+	+	9
	sion.									
16	Add carbonic	Sodium	++	++	+	+	+	+	+	5
	acid solution	carbon-	++	++	++	++	++	++	+	7
	to 20% emul-	ate	++	++	++	++	++	++	++	9
	sion.									

pH of the Solutions Throughout the Experiment:
(Determined potentiometrically).

Rabbit number	Emulsion pH	Mixture pH	Supernatant fluid pH
13	.....	4.8	6.8
14	.....	4.9	7.2
15	.....	4.9	7.0
16	.....	4.8	6.9

*The numbers repreesnt dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution).
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.
Emulsion titre: 1:100,000.

TABLE 11. Summary of Experiments in Purifying the One Per Cent Dermo Emulsion.

Rabbit number	Emulsion titre*	Mixture pH	Exposure to pH	Purified titre*	Virulence	
					Reaction	"Days"
1	40		35 min.	30	+	11
2	40		35 min.	30	+	11
3	40	4.7	20 min.	30	+	11
4	40	4.8	20 min.	35	+	11
5	100	4.9	6 min.	100	++	7
6	100	4.8	6 min.	100	++	7
7	100	4.9	5 min.	100	+	7
8	100	4.8	5 min.	100	+	7
9	100	4.8	6 min.	100	++	5
10	100	4.9	6 min.	100	++	4
11	100	4.9	5 min.	100	++	4
12	100	4.8	5 min.	100	++	5
13	100	4.8	10 min.	100	+	7
14	100	4.9	2 min.	100	++	5
15	100	4.9	10 min.	100	+	7
16	100	4.8	3 min.	100	++	5

*The numbers represent dilutions in thousands.
++Indicates strong reaction.
+Indicates reddening of the skin.
"Days" is the number of days after injection that the reactions appeared.

stand for four or five minutes. This exposed the virus to the low pH for nearly as long a time as the methods followed in Experiment 9.

Experiment 11: The use of physiological solution of sodium chloride as a suspending medium.

Because the virus becomes attenuated if it remains long in distilled water, the use of physiological solution of sodium chloride should be considered in making the emulsion for purification. On the other hand, the attenuating action of distilled water is not very rapid and a sodium chloride solution can be added after the purification process is completed. But each solution added dilutes the vaccine, and the final purified vaccine becomes more dilute than the original emulsion.

A physiological solution of sodium chloride was saturated with carbonic acid at room temperature. It was added to a 20 per cent emulsion in sufficient quantity to make a one per cent emulsion. The resulting mixture was centrifuged for five minutes. Even though the mixture pH was 4.8, clarification was not complete and the suspension remained turbid after repeated centrifuging.

Similar results were obtained by bubbling carbon dioxide rapidly for one minute through a one per cent emulsion made in physiological solution of sodium chloride. The pH of the mixture was 4.8 and clarification was not complete after 15 minutes of centrifuging.

So the idea of using physiological solution of sodium chloride in making emulsions for purification was discarded.

Recaptiulation of the Carbon Dioxide Purification of the One Per Cent Dermo Emulsion (Table 11)

In the purification of the one per cent emulsion the best results were obtained by the following procedure:

1. Making the emulsion in distilled water,
2. Bubbling carbon dioxide through the emulsion rapidly for one minute, or,
3. By adding a saturated solution of carbonic acid to a 20 per cent emulsion.
4. Centrifuging at high speed for five minutes and immediately neutralizing with a sodium carbonate solution.
5. Carrying out the experiments at 20°C. to 25°C.

Four Per Cent Dermo Emulsion

These experiments were carried out at the same time that those with the one per cent emulsion were being run; for the sake of continuity they are placed under a separate heading.

Experiment 12: Carbon dioxide as a purifying agent of a four per cent dermo emulsion.

As commercial vaccine is much more concentrated than the one per cent emulsion usually used in experimental work, we endeavored to purify a more concentrated emulsion.

A four per cent emulsion was made in distilled water and carbon dioxide was passed through a capillary tube into the emulsion for 20 minutes. The mixture was centrifuged for 10 minutes and nitrogen was

TABLE 12. Purification by Bubbling Carbon Dioxide Through the Four Per Cent Dermo Emulsion for 20 Minutes.

Rabbit number	Prepared virus	Reactions with various dilutions*							"Days"
		10	20	30	40	50	75	100	
17	Original emulsion	++	++	++	++	++	+	+	5
		++	++	++	++	++	++	++	7
17	Purified	+	+	+	—	—	—	—	5
		++	+	+	+	—	—	—	7
18	Original emulsion	++	++	++	++	++	+	+	5
		++	++	++	++	++	++	+	7
18	Purified	—	—	—	—	—	—	—	5
		+	+	+	+	—	—	—	7

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution).
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.

bubbled through the supernatant liquid for 20 minutes. The resulting preparation was slightly opalescent, but it was diluted and injected intradermally into a normal rabbit.

The results recorded in Table 12 show that a four per cent emulsion may be purified by the carbon dioxide method. The percentage of virus in the purified suspension was very low, but some of the reactions appeared on the fifth day after inoculation.

The pH obtained by bubbling carbon dioxide through the four per cent emulsion was found to correspond very closely to that of the one per cent emulsion recorded in Table 4, when a bubbling tube was used.

Therefore, five minutes of bubbling at a slow rate were deemed sufficient to produce the pH required for complete clumping of the tissue.

Experiment 13: Purification by bubbling carbon dioxide for five minutes.

A four per cent emulsion was made in distilled water and carbon dioxide was bubbled through it for five minutes. The mixture was centrifuged at high speed for 10 minutes. The supernatant liquid was clear and it was immediately diluted in adjusted salt solution and injected.

The results (Table 13) show an increase in the percentage of the virus in the purified suspension over the percentage obtained in the preceding experiment, but no increase in the virulence of the virus.

TABLE 13. Purification by bubbling Carbon Dioxide Through the Four Per Cent Dermo Emulsion for Five Minutes.

Rabbit number	Prepared virus	Reactions with various dilutions*							"Days"
		10	20	30	40	50	75	100	
19	Original emulsion	++	++	++	+	+	+	+	5
		++	++	++	++	++	+	+	7
19	Purified	+	+	—	—	—	—	—	5
		+	+	+	+	+	—	—	7
20	Original emulsion	++	++	++	++	+	+	+	5
		++	++	++	++	++	++	+	7
20	Purified	+	+	+	—	—	—	—	5
		+	+	+	+	+	+	—	7

pH of the Solutions Throughout the Experiment:
(Determined colorimetrically).

Rabbit number	Emulsion pH	Mixture pH	Supernatant fluid pH	Exposure to low pH
19	6.5	4.8	5.0	10 min.
20	6.5	4.9	5.1	10 min.

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution).
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.

Experiment 14: Purification by bubbling carbon dioxide for two minutes.

Bubbling carbon dioxide rapidly through the one per cent emulsions gave good results, but the difficulty of foaming arose when the method was applied to the four per cent emulsion. For this reason the bubbling was interrupted when the foam reached the top of the container and was resumed after it had subsided. This procedure was carried out for a period of two minutes and clarification resulted. The purified suspension was neutralized with a previously determined amount of

TABLE 14. Purification of the Four Per Cent Emulsion by Bubbling Carbon Dioxide Two Minutes.

Rabbit number	Prepared virus	Reactions with various dilutions*							"Days"
		10	20	30	40	50	75	100	
21	Original emulsion	+	+	+	+	+	+	+	5
		++	++	++	++	++	++	++	7
21	Purified	+	+	+	+	—	—	—	5
		++	++	++	++	++	+	+	7
		++	++	++	++	++	++	+	9
22	Purified	+	+	+	—	—	—	—	5
		++	++	++	+	+	+	—	7
		++	++	++	++	++	+	+	9

pH of the Solutions Throughout the Experiment: (Determined Colorimetrically).

Rabbit number	Emulsion pH	Mixture pH	Supernatant fluid pH	Neutralized supernatant fluid pH	Exposure to low pH
21	6.5	4.8	4.9	6.8	7 min.
22	6.5	4.7	4.8	7.0	7 min.

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution).
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.

tenth normal sodium carbonate solution, and diluted in salt solution for inoculation.

Experiment 15: Purification of the four per cent emulsion by the addition of a carbonic acid solution.

Distilled water was saturated with carbonic acid at 25°C. This solution was added to a 20 per cent emulsion in sufficient quantity to make a four per cent emulsion. The mixture was centrifuged for five minutes, and a transparent preparation resulted. It was neutralized with sodium carbonate.

The results, found in Table 15, show that a high percentage of the virus was present in the purified vaccine. The virulence of the virus was comparable to that in the original emulsion.

The highest dilution of the emulsion has consistently produced a strong reaction. The highest dilutions in many of the purified vaccines have also produced strong reactions. To obtain a true percentage of the virus in the purified vaccine the highest dilution must surpass that expected to produce a reaction. This experiment was repeated, carrying the dilutions past the expected "end point."

Both of the purified vaccines produced strong reactions in the dilutions of 1:100,000 in seven days. One of the emulsions produced a

TABLE 15. Purification of the Four Per Cent Emulsion by the Addition of a Carbonic Acid Solution.

Rabbit number	Prepared virus	Reactions with various dilutions*							"Days"
		10	20	30	40	50	75	100	
23	purified	+	+	+	+	+	+	+	2
		++	++	++	++	++	+	+	4
		++	++	++	++	++	++	++	7
		++	++	++	++	+	+	—	10
24	purified	+	+	+	+	+	+	—	4
		++	++	++	++	++	++	+	6
		++	++	++	++	++	++	++	8
		+	+	++	++	+	+	+	10

pH of the Solutions Throughout the Experiment: (Determined Colorimetrically).

Rabbit number	Mixture pH	Supernatant fluid pH	Neutralized supernatant fluid pH	Exposure to low pH
23	5.0	5.2	7.0	5 min.
24	4.9	5.0	6.8	5 min.

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution.)
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.

strong reaction in the dilution 1:150,000 and the other produced a strong reaction in the dilution 1:200,000.

Results are tabulated in Table 16.

Twenty Per Cent Emulsion

We have been striving to develop a method of purification which will be applicable to the concentrated commercial vaccine. The commercial vaccine is comparable to a 20 per cent emulsion.

Experiment 16: Purification of the 20 per cent emulsion by bubbling carbon dioxide for two minutes.

A 20 per cent emulsion was prepared in distilled water and centrifuged at slow speed to remove the coarse particles. The supernatant emulsion was placed in a test tube, which was in a larger container to catch the overflow. Carbon dioxide was bubbled through the emulsion at a rapid rate until all of the emulsion had foamed out of the test tube into the larger container. A transparent preparation was produced after centrifuging for five minutes. It was neutralized with sodium carbonate.

The results (Table 17) show that much of the virus was lost and the virulence of the virus in the purified vaccine was not equal to that in the emulsion.

TABLE 16. Purification of the Four Per Cent Emulsion by the Addition of a Carbonic Acid Solution.

Rabbit number	Prepared virus	Reactions with various dilutions*							"Days"
		40	50	75	100	150	200	250	
25	Original emulsion	++	++	++	+	+	+	—	5
		++	++	++	++	++	+	—	7
		+	+	+	+	—	—	—	11
25	Purified	+	+	+	+	—	—	—	5
		++	++	++	++	+	—	—	7
		+	+	+	—	—	—	—	11
26	Original emulsion	++	++	++	++	++	+	—	5
		++	++	++	++	++	++	+	7
		+	+	+	+	+	—	—	11
26	Purified	+	+	+	+	+	—	—	5
		++	++	++	++	+	+	—	7
		+	+	+	+	—	—	—	11

pH of the Solutions Throughout the Experiment: (Determined Colorimetrically).

Rabbit number	Mixture pH	Supernatant fluid pH	Neutralized supernatant fluid pH	Exposure to low pH
25	5.0	5.1	7.2	5 min.
26	5.1	5.2	7.2	5 min.

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution).
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.

Comment

Transparent preparations have been prepared from one per cent, four per cent, and 20 per cent emulsions. The most successful results were obtained with one and four per cent emulsions. The loss of virus in the 20 per cent preparation can be attributed to the occlusion of the virus in the heavy precipitate.

Conclusions

A method of purifying vaccinia vaccine has been developed.

This procedure is based upon the iso-electric precipitation of the suspended tissue by means of carbon dioxide.

These purified preparations are water-clear and contain a high percentage of virulent virus.

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TABLE 17. Purification of the 20 Per Cent Emulsion by Bubbling Carbon Dioxide for Two Minutes.

Rabbit number	Prepared virus	Reactions with various dilutions*							"Days"
		40	50	75	100	150	200	250	
27	Original emulsion	++	++	++	+	+	+	—	5
		++	++	++	++	+	+	—	7
27	Purified	+	+	+	+	—	—	—	5
		++	+	+	++	+	—	—	7
28	Original emulsion	++	++	++	+	+	—	—	4
		++	++	++	++	++	+	—	7
28	Purified	+	+	+	—	—	—	—	5
		++	++	++	+	—	—	—	7

pH of the Solutions Throughout the Experiment: (Determined Colorimetrically).

Rabbit number	Supernatant fluid pH	Neutralized supernatant fluid pH	Exposure to low pH
27	5.4	6.8	7 min.
28	5.6	7.0	7 min.

*The numbers represent dilutions in thousands (Injected intradermally, 0.2 cc. of each dilution).
++Indicates a strong reaction.
+Indicates reddening of the skin.
—Indicates no reaction.
"Days" is the number of days after injection.

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