CLARIFICATION OF HOG CHOLERA DEFIBRINATED BLOOD ANTITOXIN.

Thesis submitted by

R. R. (Robert) Henley,
B.S. 1906,
V.P.I.

(In partial fulfillment of the requirements for the Master of Science's degree, Virginia Polytechnic Institute, session 1921-1922).

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By R. R. Henley.

The method of preparing clear and sterile anti-hog-cholera serum described by Dorset and Henley[1] in 1916, and now in general use in the industry, is applicable only to fresh and nonphenolized defibrinated or citrated blood. For various reasons, it may often be desirable to clarify, concentrate, or sterilize old phenolized defibrinated blood antitoxin. In the past this has be been done frequently, either by a modification of the Banzhaf-Gibson[2] process, or by the heat-salt[3] process developed in these laboratories, but as these processes are rather expensive, and are difficult and tedious in operation, we have been endeavoring for several years to develop a process that would serve to clarify, concentrate, and sterilize old defibrinated blood antitoxin with the same relative practicability and simplicity with which the bean salt processes accomplishes these ends in the case of fresh defibrinated or citrated blood.

In fresh defibrinated blood antitoxin the cells, which are known to be inert, are intact, and on this account the entire cell, both the stroma and contents, consisting for the most part of hemoglobin, can be separated easily from the serum by the bean salt process. In old defibrinated

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1. Dorset, M. and Henley, R. R. Production of Clear and Sterilized Anti-Hog Cholera Serum. Jour. Agri. Research., v. 6., no.9., p. 333-338. 1916.

2. Banzhaf, E.J. and Gibson, R.B. Fractional Precipitation of Antitoxic Serum. Jour. Biol. Chem. v. 3., no.4., p 253-263.1907.

3. Report of The Chief of Bureau, Bureau Animal Industry. 1918.p.53.

blood antitoxin a very different condition exists because of the fact that the greater part, if not all, of the cells have been broken down, or hemolyzed and the liberated hemoglobin has passed into solution in the serum. Bean extract will agglutinate and thus facilitate the removal of the disrupted stroma, but it has no effect whatsoever upon the dissolved hemoglobin, and so, is of no avail in aiding its removal.

It was believed that a selective precipitant for hemoglobin could be found, and a search for such a reagent was instituted. In this search, the action of a great variety of materials, chiefly gasses and liquids of low boiling points, was studied, and simultaneously, an extended search of the literature was conducted. A statement by Mann[1] that chloroform would precipitate hemoglobin attracted attention and promised fulfillment of this quest.

The first attempts to precipitate hemeglobin were made on fresh and nonphenolized defibrinated-blood, and were very disappointing in that only small amounts of hemoglobin were precipitated, and even this could not be accomplished consistently. An extended study of the reaction showed that the completeness of precipitation was dependent upon several factors, the most important of which are (1) the temperature at which precipitation is effected - temperatures of 40° C. or less being necessary, and (2) the presence of phenol in amounts of 0.5% . While a low temperature is necessary for the precipitation of hemoglobin, it is only

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1Mann, Gustav. Chemistry of the Proteids. London 1906.

necessary while the reaction is taking place and its continued maintainance is not essential. When the factors governing the precipitation of hemoglobin were inderstood and subject to control, a process to employ this method for the removal of hemoglobin from old blood was devised.

As noted, the cells of old phenolized defibrinated-blood antitoxin have been disrupted, or hemolyzed, and the hemoglobin has been dissolved, and the cell walls, or stromata, distributed as a colloidal suspension throughout the fluid. It is necessary to remove the inert hemoglobin and cells without removing the globulins which carry the antitoxin. Chloroform will precipitate the hemoglobin without precipitating, or otherwise adversly affecting, the globulins and antitoxin, and this precipitate will occlude the greater part of the stroma. But in order to remove the stroma more completely, it has been found advisable to use an agglutinine, bean extract [1], in the same manner as in the case of fresh blood. Based upon these facts the following process was developed.

PROCESS FOR THE REMOVAL OF STROMA AND HEMOGLOBIN

FROM OLD DEFIBRINATED BLOOD ANTITOXIN.

Step 1. Addition of Precipitants.

a. Addition of agglutinine to remove stroma.

To 500cc of phenolized (0.5% phenol) old defibrinated blood antitoxin, previously chilled to 40°F. or lower, 10 to 15 cc of bean extract [1] are added and the contents thoroughily agitated by hand.

1. Dorset, M. and Henley, R. R. Jour. Amer. Vet Asso. v. 50. (n.s. v.3) no. 6. p.699-702. 1919.

b. Addition of Chloroform. Immediately following "a," 50cc of chloroform are added to each 500CC of blood and the contents of the flask agitated by hand.

c. Addition of Salt. This procedure should be used only when the serum is to be separated from the clot by filtration. Promptly following the completion of "b" add 5 gms. of sodium chlorid and immediately transfer the flask and contents to a mechanical shaker.

Step 2. SHAKING.

Unless shaking in the mechanical shaker immediately follows the addition of chloroform a clot, difficult to break, will form at the bottom of the flask. As chloroform is practically insoluble in water and blood, it is essential that the mixture of chloroform and blood be agitated thoroughily in order to secure the maximum precipitating effect. For this purpose a power driven Camp shaking machine is used, but doubtless any other shaking machine would answer the purpose. The machine that we have employed has a capacity of six one liter flasks, but on account of the danger of splashing, amounts greater than 600 cc are never placed in a flask. The mixture is shaken for ten minutes, following which the blood will be found to be in a semisolid clot from which a clear, light red serum will exude on standing.

Step 3. SEPARATION OF SERUM.

The serum may be separated from the clot by either (1) centrifugalization or (2) filtration.

(1) Centrifugalization.

Either the continuous or bucket type centrifuge may be used to separate the clear serum from the clotted hemoglobin and stroma. As the blood after shaking is usually clotted into a semisolid mass, it is necessary inorder to remove it from the container to break the clot. This may be accomplished easily if the clot is allowed to stand until the serum exudes and then is slightly shaken by hand until it is reduced to the desired fluidity. With this precaution the serum may be separated by either type machine. It may be mentioned, however, that it will be advisable to employ a continuous type machine only when it is of sufficient capacity to care for a considerable amount of sediment. Separation with the bucket type machine is accomplished in the same manner and with the same ease and rapidity that mark the separation of cells from fresh defibrinated or citrated blood by the bean salt process.

(2) Filtration.

The separation of the serum from the clotted hemoglobin may be accomplished also by filtration through paper, yielding a product of the same clearness and color that is obtained by centrifugalization. For filtering the serum from the clotted hemoglobin a filter tray[1] instead of the usual funnel has been employed advantageously. The clotted blood is filtered in lots of 1000 cc each. A yield

1. Ka-Ki filter tray

of at least 500 cc should be obtained from each 1000cc at this stage, but this does not represent all of the serum present, as, because of the bulkiness of the hemoglobin precipitate, a considerable portion of the serum remains mechanically held in the precipitate. In order to recover this, it is necessary to press it out of the precipitate, for which purpose the precipitate and paper are transferred to a muslin cloth, which after being folded into a baglike shape is transferred to a fruit press and the retained serum pressed out. The volume of pressings obtained should not be less than 150cc from the clot representing each 1000 cc of blood. The expresced serum is combined with the clear serum obtained by direct filtration as above described. In case the pressings are not entirely clear, they are clarified, previous to combination with the clear filtrate, by thoroughily incorporating into the pressings 1/2 to 2 % of powdered infusorial earth and filtering the mixture through paper. This will remove effectually any trace of cloudiness from the pressings.

Step 4. HEATING AND PHENOLIZATION.

The serum obtained as set forth above may be heated to 58 -60°C. for one half hour in the same manner and with the same results as attend the heating of serum obtained from fresh defibrinated blood.

Although a defibrinated blood antitoxin may contain 0.5% phenol, the amount usually added to insure keeping, it has been found that the clear serums obtained as a produst of the process contain only on an average about 0=2% phenol, an amount insufficient to prevent spoilage; so it is necessary to add sufficient additional

phenol to bring the phenol content to 0.5%. Assuming the presence of 0.2% phenol it is necessary to add of 5% phenol, one-fifteenth of the volume of the serum to be treated. Thus, if one has 950cc., of serm, add 1/15 x 950 or 63 cc., of 5% phenol.

Discussion of Steps.

While the process as given in the steps described may be depended upon to give a complete separation of clear serum from cellular constituents, a close observation of the following precautions and suggestions is requisite to smooth operation:

1° The blood should always be chilled to a temperature of 40°F, or lower, before adding the bean extract and chloroform.

2. After the addition of the precipitants in step 1. the mixture should not be allowed any longer than necessary, but should be immediately be transferred to the shaker.

3. Shaking in the mechanical shaker should be only sufficient to form a clot. In order to determine this, the machine is stopped for a minute, after which a flask is removed and examined to see whether the blood has clotted into a semi-solid mass that will not flow in the flask. 10 minutes of shaking is usually sufficient. Shaking for a longer time than 10 or 15 minutes will break the clot in such a manner that the subsequent step of separation will be rendered more difficult. Too little rather than too much shaking is preferable.

4. After the clot has formed and the flask has been removed from the shaker, the flask may be allowed to stand until a clear serum exudes from the clot. The flask should then be slightly shaken by hand, as this will break the clot slightly and greatly facilitate its transfer to filter paper or centrifuge cup.

5. Phenol should be present. As this process is designed primarily for the treatment of phenolized bloods the further addition of phenol is not required.It has been found, however, that the further addition of small amounts, for example 40cc of 5% phenol per 1000cc of blood, facilitates separation and gives a slightly less colored product, but products obtained in this manner have not been tested for potency, so its use cannot be advised.

6. The amount of hemoglobin present in different bloods, even in different lots of the same blood, varies, so the proportion of chloroform to be employed cannot be definitely formulated. It is of course economy to use the minimum amount of chloroform consistent with good results.

7. The clot, after separation of the serum by centrifuging, or by filtration and pressing, should be suffiviently dry to crumble readily when handled.

RESULTS OF THE PRACTICAL APPLICATION OF THE PROCESS TO OLD DEFIBRINATED BLOOD ANTITOXINS.

Yields.

The yields obtained by this process vary rather widely, due no doubt to an uneven distribution of the red blood cells in the containers. Tables I to IV exhibit yields that may be anticipated.

Table I.
Separation by Centrifugalization.

1. Serum	Precipitants	Type of centrifuge	Yield per cent	Final 2 Yield
230	Bean extract and chloroform	Bucket	56	59.7
231	do	do	64	68.3
231	do	do	67	71.5
231	do	do	61	65.0

TableII.
Separation by filtration, no salt used.

Serum	Precipitamt	Yield nper cent	Final Yield percent 2
231	Bean extract, chloroform	64	68.3
231	do	61	65.0
262	do	71	75.7

1. It is a common commercial custom to refer to defibrinated blood antitoxin as "serum". In this paper the term "serum" associated with appropriate identifying numbers is used to refer to defibrinated-blood antitoxin.

2. After phenolization.

Table III.

Separation by Filtration. Comparison of Yields with and without Salt.

Serum	Treatment	Yield	Final Yield.[1]
231	with salt	65.5%	69.9%
	without salt	63.5	67.7
109	with salt	64.0	68.3
	without salt	66.0	70.4
259	with salt	75.5	80.5
	without salt	71.0	75.7
262	with salt	66.5	70.9
	without salt	56.5	60.2
230	with salt	67.5	72.0
	without salt	65.5	69.9

Table IV.

Comparison of Yield by Regular Bean Salt Method on Fresh Blood with Yield by Modified Method on same Blood, Phenolized one Week Old.

Serum	Process	Yield	Final Yield 1
Fresh	Bean Salt	68.0%	72.5%
One week old.	Bean Chloroform Salt	63.0	67.2

1. After phenolization.

Effects of Age.

The process herein described has been applied to bloods varying in age from one week to four years, with consistently good results. The serum from the blood one week old was more highly colored than that from the older bloods, that is, the removal of the hemoglobin was less complete in the fresh than in the old bloods.

Change in Phenol Content.

All the bloods used in these tests were the ordinary phenolized defibrinated-blood antitoxins prepared in the Bureau of Animal Industry laboratories and contained o.5% phenol in the beginning. As there were indications that phenol played an important part in the precipitation of hemoglobin, it was believed that part of the phenol might be removed in the removal of the hemoglobin. In order to ascertain the correctness of this opinion, the amounts of phenol remaining in a number of clarified serums were determined with the results shown in Table V.

Table V.

Amounts of Phenol Remaining in Clarified Serums.

Serum	Date prepared	Phenol added at preparation	Date clarified	Percentage Phenol remaining
231	1916	0.5%	1920	0.16
259	1919	do	1920	..24
261a	Aug.1920	do	Feb.1920	.24
230	1915	do	1920	.2o
109		do	192o	.18
262	Aug.1920	do	Feb.1920	.17
259	1919	do	1920	.18
Fresh	Feb.1920	do	Feb.1920 b	.17.

a. Added 40cc of 5% phenol to each 1000cc serum in addition to original 0=5%
b. This blood was one week old when analyzed.
Above analyses by the kindness of Messrs. R. M. Chapin and J. M. Schaffer.

Globulin Content of Clarified Serums.

As it is known that the globulins of anti-hog-cholera serum contain the antitoxin, an attempt was made to study the loss in globulins that would take place in a blood treated by this process. This problem seemed to be more easily attacked by treating the blood by the process described, determining the globulin content of the clarified serum product, and then removing the remaining globulins from the clot by farther washing and determining these separately. With this method, results shown in Table VI were obtained.

Table VI.

Globuline in Clarified Serum and Clot from 1000cc Defibrinated Blood.

Serum	Globulins in Clarified Serum	Globulins in Clot by Washing	Indicated Loss by retention in clot.
262	15.06 gms.	1.50 gms.	10%
231	14.43	1.67	11

As stated previously, the loss of globulins due to retention in the clot may be minimized by the use of a more powerful press. The press used was a very small fruit press, and it is believed that the serum was never completely expressed although after pressing the clots invariably crumbled when handled.

Further, the indicated losses of 10 and 11 percent may not be actual losses as there may have been some extraction of the stroma from the clot and this, in the process of analysis used, would have been determined as globulins.

Effect on Bacterial Count.

As old defibrinated blood antitoxins may be contaminated with numerous organisms, and as one of the primary purposes of this process is toe effect a sterilization of the treated serums, it was considered advisable to investigate the bacterial content of serums before and after, and at different stages of the treatment. For this purpose two antitoxins, No=230 and 259, both ordinary defibrinated blood antitoxins prepared in the laboratories of the Bureau of Animal Industry at Ames, Ia. in 1919 were selected for investigation. Agar plates were made[1] of the original untreated antitoxin, using 0.01cc portions, of the clear serum obtained after filtration from that antitoxin, of the same serum after heating, and after phenolization. Plates from the various products of refinement were all made with 1 cc portions. The heated portion had been subjected to a temperature of 58 - 60 ° C. for 1/2 hour before the samples were taken to determine the bacterial content.

Accordingly, plates were made from (1) the untreated old defibrinated blood antitoxin, (2) the clear serums derived from that antitoxin by precipitation and clarification by filtration, (3) the clear serum after heating, and (4) the clear serum after heating and phenolizing. Results of the count are shown in Table VLL.

1. The writer is indebted to Dr. F. W. Tilley for the plates and counts.

Table VII. [1]
Bacteria per cubic centimeter.

Serum	Original	Clarified	Heated	Phenolized
230	18,300	0	0	0
259	10,700	0	0	0

1. Agar plates incubated at 37.5°C for 48 hours.

Results of Tests on Hogs.

As it is recognized that the bean-salt process is an effective process for the separation of a clear, potemt serum from defibrinated-blood antitoxin, and as the process described herein differs from that process only in that chloroform is used, it was not considered necessary to carry on an extensive investigation as to the effect of the process on the potency of the product. Howeveer, in order to determine whether the addition of chloroform was in any way injurious, serum 262, a defibrinated blood antitoxin prepared in the Bureau of Animal Industry's laboratories, was treated by the process. Two products were obtained:(1) a clear, unheated, phenolized serum, and (2) a clear, heated, phenolized serum. After bottling, these two products and a sample of the original untreated defibrinated blood antitoxin No. 262 were tested for potency on hogs.[2] Table VIII shows the volume yields in the different steps of the process. It will be noticed that the volume yield after phenolization was 80% of the original volume The original serum was tested in doses of 10 and 15 cc of serum against 2 cc of virus. As The vol-

2. Tests condusted by Dr. W. B. Niles

ume of clear serum obtained as a final product was 80% of the original volume, 8 and 12 cc doses of the clarified serums were administered, these doses being 80% of the 10 and 15 cc. doses of the original serm. Thus no allowance was made for the loss of any antibodies that may have occurred during clarification. Reseults of the potency tests are shown in Table IX.

Table VIII.

Yields of Serum 262 at Different Stages of Process.

Serum	Amount	Volume Filtrate	Volume Pressings.	5% Phenol added	Vol. Product	Yield
262	2,000cc	1169 cc	343 cc	100 cc	1,612 cc	80%

Table IX.
see insert following.

Table IX. Results of Potency Tests on Treated Serums.

Weight of Pigs.	Material injected. 12 - 11 - 20= Serum		Virus	Temperature Record. 12/13	12/14	12/15	12/=6	12/17	12/1	12/20	12/21	12/22	12/24.	
pounds		cc	cc											
95	Original 262	15	2	102.6	104.0	103.0	104.0	103.8	10a.	101.8	102.0	102.0	102.0	Remained normal to Jan., 6, 1921.
95	do	15	2	102.0	103.2	102.6	103.4	102.8	103.	103.0	102.6	102.4	102.0	do
85	Clarified Unheated.	10	2	103.0	102.0	102.0	103.8	102.6	103.	102.2	102.2	102.2	101.8	do
85	do	10	2	102.4	103.0	101.8	103.2	102.6	103.	102.0	102.0	101.4	103.2	do
95	Clarified Unheated.	12	2	102.2	102.5	102.6	102.0	102.0	103.	101.6	101.6	102.0	103.0	do
90	do	12	2	102.0	102.0	103.0	102.6	102.0	103.	102.0	102.0	101.4	103.2	do
75	do	8	2	103.0	102.2	102.6	104.8	103.0	101.	101.6	102.4	103.6	103.4	Remained normal to Jan., 6, 1921, except slight diarrhoea on the 13th.
80	do	8	2	103.2	103.6	103.0	103.0	104.0	103.	102.0	102.2	102.2	103.0	Remained normal to Jan., 6, 1921.
80	Clarified, Heated.	12	2	103.0	103.6	102.8	103.4	103.0	102.	102.0	101.6	103.0	103.0	Remained normal to Jan., 6, 1921, except slight diarrhoea on 13th.
80	do	12	2	103.2	104.4	103.8	103.2	104.0	102.	103.4	102.8	102.0	102.0	Remained normal to Jan., 6, 1921.
70	do	8	2	102.6	101.8	102.4	103.0	102.0	103.	102.0	101.6	102.6	102.6	do
80	do	8	2	103.0	103.0	104.0	103.0	102.6	102.	102.4	102.2	10o.8	102.0	do
	Control		2	101.8	104.8	104.4	104.2	104.8	103.	103.8	106.6	105.8	103.8	Off feed from Dec., 16th. Weakness and diarrhoea to Jan. 6th. May recover
	do		2	101.6	103.6	104.8	105.0	104.0	107.	106.6	106.6	105.0	102.6	Off feed and diarrhoea from Dec. 16th. Died Dec., 28th.

Caution.

Of the various processes for the refinement and sterilization of old phenolized defibrinated blood antitoxin which this laboratory has tried out some on a large commercial scale, the process herein described appears to be by far the simplest and most practicable. This process, however, has never been applied on a commercial scale: therefore anyone considering its employment is cautioned to familiarize himself with the various steps of the process by first applying it to small quantities of blood. Furthermore, products obtained from such small quantities of blood should be given rigid potency tests in order to check up any losses in potency that may occur.

Summary.

The factors governing the reaction between chloroform and hemoglobin by which the hemoglobin of the blood may be precipitated were studied, and a process, based upon this reaction, for the separation of a clear sterile serum from old defibrinated blood antitoxin was devised.

It is shown that the yield of clear serum separated by this process approximates 70% of the original volume and that the product of the process is free from bacterial contamination. While it is shown by analysis that the globulin content of the serum suffers a slight loss, when the serum is treated by this process, potency tests indicate that the loss in antibodies that occurs during clarification may be disregarded.