Vol. XXXIV, No. 4 (August, 1953), was issued on 16.ix.53.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXXIV OCTOBER, 1953 NO. 5

A METHOD FOR COLLECTING BACTERIA AND THEIR PRODUCTS FROM INFECTIONS IN EXPERIMENTAL ANIMALS, WITH SPECIAL REFERENCE TO *BACILLUS ANTHRACIS*.

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Received for publication April 29, 1953.

THERE is abundant evidence that the complex metabolic activity of a pathogen in establishing a disease under the cultural conditions of the host's tissues is different from its metabolism *in vitro*. Thus, the change in morphology and colony appearance and the decrease in virulence which occurs on subcultivating recently isolated organisms is well known. The method of increasing the virulence of organisms by animal passage means that substances and processes connected with virulence are produced *in vivo* (possibly as a result of mutation), and not to any significant extent by culture *in vitro*. A similar conclusion can be drawn from the difficulty or impossibility of distinguishing between virulent and avirulent strains of certain species such as *Pasteurella pestis* (Wilson and Miles, 1948 ; Jawetz and Meyer, 1943 ; Pollitzer, 1952) by *in vitro* tests. Perhaps the most striking instances of the difference are those in which the use of living vaccines forms the only effective means of preventing the disease.

It seems, therefore, that chemical extraction of organisms and their products obtained from infected animals would be fruitful, particularly in connection with studies on virulence. Chemical fractionation of body fluids containing extracellular products of an infecting organism has been done before (*e.g.*, Cromartie, Watson, Bloom and Heckly, 1947). This is not so for the bacterial substance. Up to the present, organisms grown *in vivo* have not been obtained in large enough quantity and of sufficient purity for a chemical examination of their products and metabolism.

This paper describes a method for collecting *Bacillus anthracis* and its products from infected guinea-pigs for use in a study of virulence described in the next paper (Smith, Keppie and Stanley, 1953). The same method has been tried on a small scale for the separation of products from infections with several different species of pathogenic bacteria. It was equally successful as regards the collection of bacteria free from blood cells. Briefly, the method consists of collecting the blood and thoracic and peritoneal exudates from infected animals and removing the blood cells from the bacteria and their products by differential centrifugation

at low temperatures. The general problems in obtaining a sufficient quantity of materials and bacteria of adequate purity are as follows :

To ensure a sufficient quantity of bacteria, only those diseases which produce a large number of organisms in accessible locations of the host could be studied. It is inevitable that only a relatively small proportion of the organisms and their products present in an experimental animal can be collected (10–15 per cent for the anthrax infection described below). Large volumes of infected exudate were desirable in the thoracic and peritoneal cavities to facilitate removal of the maximum amount of material. Initial infection was therefore carried out in these cavities. To obtain the maximum yield of products, the largest available animal, *e.g.*, a sheep, would have been the best host for the infection, but, if the ultimate products of chemical extractions are to be adequately tested biologically in a homologous system, a species available in quantity has to be used. The guinea-pig was a successful compromise between these two requirements.

The purity of extracellular products from *in vivo* cultures must be low, since they are grossly contaminated with plasma constituents. It is hoped that chemical fractionation will remove these impurities. As regards the bacteria, however, the possibility of obtaining them almost free of blood cells presented itself. For subsequent chemical fractionation of bacterial extracts a contamination of less than 1 per cent of blood-cell substance could reasonably be called negligible ; it would probably be diluted out in most extraction procedures. Since the degree of contamination with blood cells was to be followed by haemocytometer counts during the differential centrifugation, the relative sizes of blood cells to the chains (4–8 bacilli) of anthrax organisms were estimated. Figures of 2·5–5 × for red blood cells and 8–16 × for white blood cells were obtained. This means that haemocytometer counts on the final product must indicate a contamination of less than 0·05 per cent for each type of cell, if the limit of purity stated above is to be maintained. For organisms smaller than these bacterial chains this figure would have to be less than 0·005–0·001 per cent.

To obtain this degree of purity careful attention must be given to the details of the method described below, especially those dealing with the removal of supernatants from lightly packed blood cell deposits.

METHODS AND RESULTS.

Abbreviations—red blood cell (R.B.C.), white blood cell (W.B.C.), chain of anthrax organisms (B.C.).

A Method used for the Collection and Separation of B. anthracis *and its Products from Infected Guinea-pigs.*

The experimental disease.

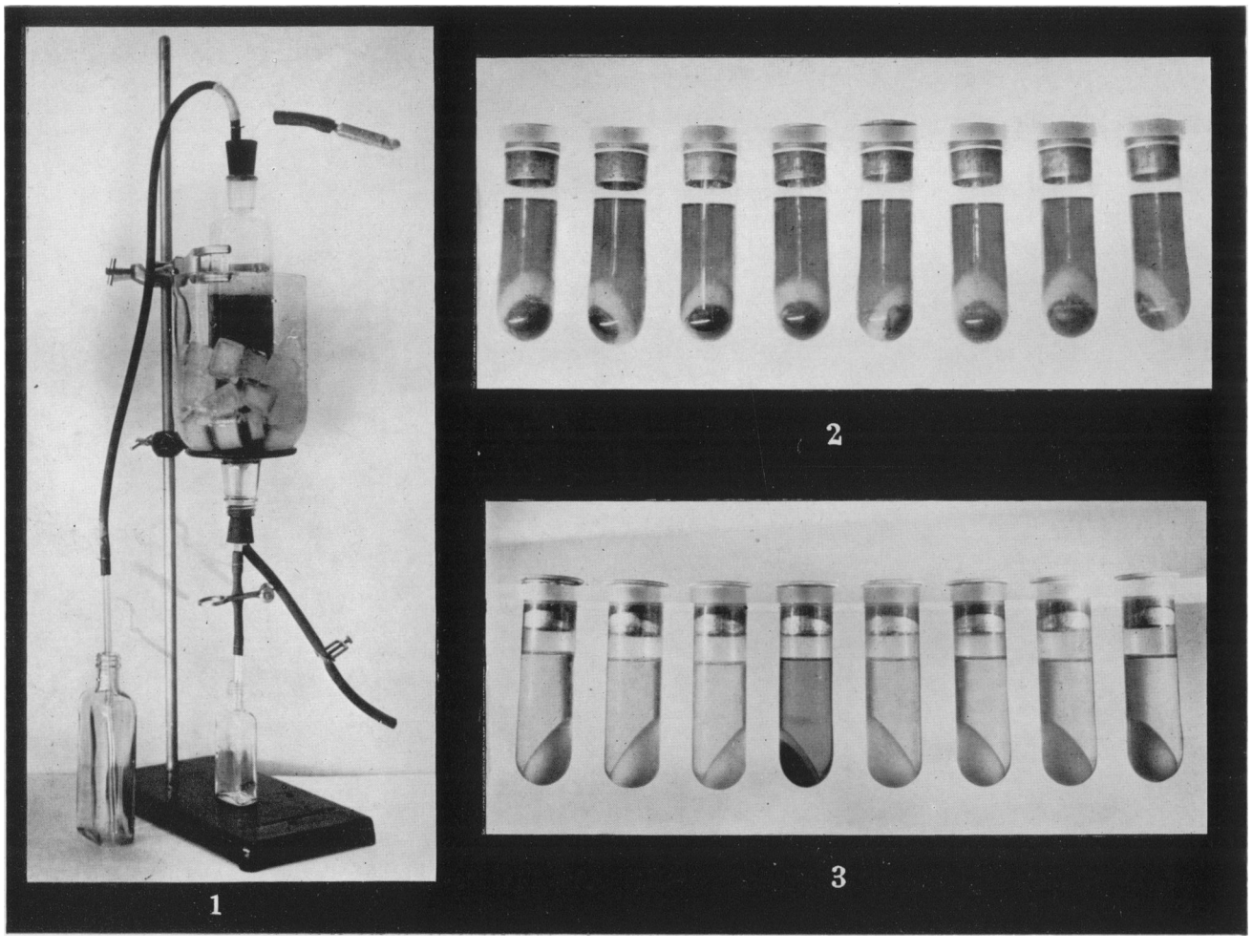
One hundred guinea-pigs (800–1000 g.) were given simultaneous intrapulmonary and intraperitoneal injections of 1×10^8 *B. anthracis* spores. The strain used (N.P.) was a non-proteolytic mutant of the Vollum strain obtained by Wright, Hedberg and Feinberg (1951).

DESCRIPTION OF PLATE.

FIG. 1.—Apparatus for collection of supernatants and for their re-distribution into fresh centrifuge pots.

FIG. 2.—The deposit after high-speed centrifugation of successive batches of material showing the single layer of blood cells beneath the bacteria.

FIG. 3.—The final bacterial deposits together with the blood cells which have been removed in the washing procedure.



Smith, Keppie and Stanley.

It was selected from a group of 10 virulent strains which were tested in preliminary experiments for their ability to produce large volumes of thoracic and peritoneal exudate. A dense stock suspension of heated spores was stored at 0° and was the single source of *B. anthracis* employed throughout this work. All the 100 guinea-pigs infected in this manner died 36–60 hr. later. As the main septicaemia begins within a few hours of death and increases hourly the body fluids were collected immediately after death.

Collection of infected body fluids.

The dead animal was secured on a sloping board with the head higher than the hind quarters. This allowed the exudate to drain to the bottom of the cavities and aided its subsequent removal. The chest was opened and the exudate (15–30 ml.) collected in a syringe and expelled into centrifuge pots (250 ml.) standing in ice. The latter had caps made of lint to prevent the escape of aerosol, and they contained sufficient sodium citrate to give a final concentration of 0·6 per cent w/v. The blood was collected after the chest had been opened and the lungs removed, by puncturing the heart and severing the veins and arteries. The blood and the peritoneal exudate were added to the thoracic exudate. Each animal gave 20–30 ml. of this mixture which contained $1{\cdot}2–2{\cdot}6 \times 10^8$ chains of anthrax bacilli (4–8 per chain) per ml.

Low temperature manipulation of the material.

As described above, the infected body fluids were cooled in ice immediately after collection. From then on all the procedures described below such as centrifugation and filtration were carried out at 0–3°. Fig. 1 shows an apparatus which simplified the careful removal of large volumes of supernatants from cell deposits. The material was drawn into the ice-cold reservoir by a slight negative pressure produced by a water pump. It could then be dispensed into fresh centrifuge pots when necessary.

Separation of the bulk of the blood cells from bacteria by differential centrifugation.

The infected body fluids were processed continuously as they were collected. Three full centrifuge pots (250 ml.) constituted the first batch and two pots each subsequent batch.

First slow centrifugation.—The initial material was centrifuged at 500 r.p.m. (60 **g**) for ½ hr. The supernatant containing the bacteria was removed to within ½ in. (1·25 cm.) of the blood-cell deposit and distributed into two fresh pots (250 ml.). The impure supernatant remaining was added to the next sample of initial material. The deposit of blood cells was discarded.

Second slow centrifugation.—The supernatant was again centrifuged at 500 r.p.m. for ½ hr. Only two-thirds of the supernatant was removed to avoid disturbing the small blood-cell deposit. The same two pots were used for this centrifugation of subsequent batches. When all the infected fluids had been treated, the material remaining in these two pots (160–180 ml., B.C. count $2{\cdot}3–2{\cdot}7 \times 10^8$/ml.) constituted 10–15 per cent of the total yield. To avoid losing this material and to reduce its content of blood cells to the required level it was twice centrifuged at 500 r.p.m. for ½ hr. Each time almost all the supernatant was carefully removed from the cell deposits which were discarded.

High speed centrifugation.—The supernatant was centrifuged at 10,000 r.p.m. (12,000 **g**) for 20 min. in the sealed head of an angle centrifuge. The supernatants of plasma/exudate were removed but the bacteria from 100 guinea-pigs were accumulated in the same tubes.

The cell counts recorded in Table I show the progressive removal of blood cells by this differential centrifugation without loss of bacteria.

Removal of the remainder of the blood cells while washing the bacterial deposit.

At the end of differential centrifugation the total bacteria were contaminated with 1·4 per cent of R.B.C. and 0·2 per cent of W.B.C. As successive batches were centrifuged the blood cells did not form a layered arrangement with the bacteria as one might have expected, but slipped through the deposit to form a single red-cell layer at the bottom of the tube. Fig. 2 shows the cell deposits at this stage in the process.

The bacteria were freed from adhering plasma/exudate by washing them three times with Locke's solution, and during this process the remaining blood cells were removed in the following manner. By the careful use of a Pasteur pipette, first on the undiluted sludge

TABLE I.—*Summary of Cell-counts during Differential Centrifugation of Infected Blood and Exudate.*

Nature of sample.	Bacterial chains: Number per ml. ($\times 10^8$).	Percentage contamination with blood cells. R.B.C.	W.B.C.
Initial material	1·7 (1·2–2·6)	Large numbers	Large numbers
a. First slow centrifugation:			
Supernatant	2·0 (1·4–3·2)	4 (3–6)	0·6 (0·4–1·0)
Deposit	0·3 (0·1–0·6)	Large numbers	Large numbers
b. Second slow centrifugation:			
Supernatant	1·8 (1·3–2·5)	1·4 (0·7–3·0)	0·2 (0·06–0·4)
c. Removal of bacteria by high speed centrifugation:			
Supernatant	0·001 (0·0005–0·002)	*nil*	*nil*

These figures are a summary of haemocytometer counts for B.C., R.B.C., and W.B.C. carried out on 12 different batches of material; average figures are quoted and the range is given in brackets.

and then with the use of a little of the washing fluid, most of the bacteria could be removed from the red cell layer. The bacteria were distributed into 7 tubes and the blood cells containing some adhering bacteria were collected into one tube. The material in all tubes was then washed with Locke's solution (approx. 30 ml. per tube). After high-speed centrifugation as described above the supernatants were collected and more bacteria could now be removed from the single red layer. This procedure was repeated twice. After the final washing, few bacteria remained to be removed from the blood cells, which were discarded (see Fig. 3).

The counts recorded in Table II confirm the success of the washing procedure in removing the remaining blood cells to give the final product.

TABLE II.—*Summary of Cell-counts during the Removal of Remaining Blood Cells when the Bacteria were Washed.*

Nature and Volume of sample.	Bacterial chains: Number per ml. ($\times 10^9$).	Percentage contamination with blood cells. R.B.C.	W.B.C.
Initial material (approx. 21.)	0·18 (0·13–0·25)	1·4 (0·7–3·0)	0·2 (0·06–0·4)
Final material (225 ml.)	2·1 (1·7–2·4)	0·02 (0·01–0·03)	0·02 (0·005–0·03)
The blood-cell layer (10 ml.)	0·4 (0·18–1·0)	Large numbers	Large numbers

These figures are a summary of haemocytometer counts for B.C., R.B.C., and W.B.C. carried out on 6 different batches of material; average figures are quoted and the range is given in brackets.

Treatment and total yield of plasma/exudate and bacteria.

The plasma/exudate collected from the high-speed centrifugation was sterilised by filtration. Berkfeld candles originally used have recently been replaced by "Millipore" filters of the Lovell Chemical Company, Watertown 72, Massachusetts, U.S.A., with excellent results. They are composed of cellulose acetate and are of the thickness of normal laboratory filter-paper; hence there is no possibility of large scale absorption of products. To obtain a good filtration rate, the somewhat viscous plasma/exudate was diluted with an equal quantity of Locke's solution.

The bacterial sludge was immediately extracted by the methods described later. The three washings of the bacteria were collected separately from the plasma/exudate since they might contain materials washed from the surface of the bacteria.

Table III summarizes the yield of plasma/exudate and bacteria obtained from five different batches of 100 guinea-pigs. The small loss of bacteria in the total discarded blood cell deposits is included.

TABLE III.—*Yields of Plasma/exudate and* B. anthracis *Obtained from Batches of 100 Guinea-pigs.*

Initial material from 100 guinea-pigs. (B.C. count 1·2–2·6 × 10^8 per ml.) Vol. (l.).	Recovered plasma/exudate. (Vol. (l.).)	Recovered bacteria (Volume of final suspension 225 ml.). Number (× 10^9) of B.C. per ml.	Wt. (g.) dried bacterial extract* obtained.	Discarded R.B.C./W.B.C. deposits. Vol. (ml.).	Number (× 10^8) B.C. per ml.	Loss of B.C. as per cent of recovered B.C.
2·8	2·3	1·8	2·2	500	—	—
2·2	1·8	1·7	1·8	400	1·0	11
2·2	1·7	1·7	1·7	450	0·9	11
3·0	2·5	2·2	2·1	500	1·3	13
3·2	2·6	2·2	2·5	600	1·1	13

* For details of preparation see next paper.

Application of the Above Method to the Products of Other Infections.

To test whether the method could be generally applied to obtain bacteria of sufficient purity, small numbers of guinea-pigs were infected with five different organisms. These were recently isolated strains of *Streptococcus pyogenes* (2 strains), *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Listerella monocytogenes* and a virulent strain of *Past. pestis*. Dense suspensions of the first five organisms were prepared by washing off cultures on blood agar with Locke's solution. *Past. pestis* was grown on one tryptic meat agar slope, washed off with Locke's solution (10 ml.) and diluted 1/100. Each suspension was injected intrapulmonarily (2 ml.) and intraperitoneally (8 ml.) into two guinea-pigs (over 800 g.); the *Str. pneumoniae* suspension was mixed with an equal amount of 2 per cent w/v hog gastric mucin. All the animals died in 20–24 hr. and with the exception of those infected with *List. monocytogenes*, contained large numbers of organisms in their body fluids. The thoracic exudate, peritoneal exudate (washed out with Locke's solution (10 ml.) containing 0·6 per cent citrate) and blood were collected, and treated as described above. The cell counts recorded in Table IV show that blood-cell contamination is very low at the end of differential centrifugation. Counts were difficult on the final deposits owing to the presence of clumps formed on high-speed centrifugation. These counts are not included in Table IV, but they indicated an even lower contamination with blood cells.

TABLE IV.—*Cell-counts after Differential Centrifugation of the Products Harvested from Other Infections in Guinea-pigs.*

Organism producing the infection.	Number (per ml.) of cells present after differential centrifugation. Bacteria.	R.B.C.'s (× 10^4).	W.B.C.'s (× 10^4).
Str. pyogenes (1)	8 × 10^6	Less than 1	Less than 1
„ „ (2)	4 × 10^8	„ „ 2	„ „ 1
Str. pneumoniae	1 × 10^8	„ „ 1	„ „ 1
Staph. aureus	2 × 10^9	„ „ 1	„ „ 1
Past. pestis	3 × 10^9	„ „ 3	„ „ 3
List. monocytogenes	1 × 10^6	„ „ 1	„ „ 1

The products (approx. 50 ml.) were the bulked thoracic exudate, peritoneal washings and blood from two guinea-pigs (over 800 g.) infected with the organism under test.

DISCUSSION AND SUMMARY.

Using *B. anthracis* in the guinea-pig, a method has been established for the separation of bacteria and their products from infections in experimental animals. It has been possible to collect from 100 infected guinea-pigs 1·5–2·0 g. of *B. anthracis* and 1·5–2·5 l. of body fluids containing the extracellular products of this organism. These quantities were sufficiently large to allow a chemical study of the factors involved in the virulence of *B. anthracis* in guinea-pigs (see following paper).

This is the first time that large quantities of bacteria have been collected from culture *in vivo*. A high degree of freedom from blood cells has been obtained by a process involving only a small overall loss (10–13 per cent) of the total bacteria collected. The maximum contamination (0·03 per cent) with both types of blood cell was well below the desired limit indicated in the introduction to this paper.

As regards the almost complete separation of blood cells from bacteria, this method has been shown to be equally effective for the body fluids obtained from five other infections in guinea-pigs. Since the experiments were on a small scale, no definite information other than the counts given in Table IV has been gained on the amount of material that could be obtained from these infections.

Our thanks are due to Dr. D. W. Henderson and Prof. W. T. J. Morgan for encouragement, particularly in the early stages of this work. We are also indebted to Mr. T. Snape for excellent technical assistance, to our colleague Mr. H. T. Zwartouw for the photographs, and to the M.O.S. Animal Farm for the supply of guinea-pigs.

Acknowledgment is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

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