NOTES ON THE SPORULATION OF *B. SPOROGENES* AND OTHER ANAEROBES

(A REPORT TO THE FOOD INVESTIGATION BOARD)

By F. P. G. de SMIDT, M.R.C.S., L.R.C.P., D.P.H.

(*From the Pathological Department, Manchester University.*)

(With 1 Chart)

In the course of experiments on the cultivation of anaerobic bacilli, it was found advisable to be able to calculate with some certainty on the production of spores by the strains of anaerobes worked with. Observations were accordingly made of the conditions of cultivation by which sporulation appeared to be influenced. It was evident that in the case of *B. sporogenes* and other well-known types observed, the proportion of spores produced within a given period varied markedly in response to variations in the same factors that mainly influenced the rate of growth in cultures, viz. concentration of food material, incubation temperature, and reaction of medium. With the exception of cultures in media containing added carbohydrate, it was evidently also the rule that sporulation was most active in cultures commencing under conditions most favourable to rapidity of vegetation.

These points are perhaps too well known to need further emphasis, but a note on one aspect of their biological significance may not be out of place. If we consider two communities of a type of anaerobe—(*A*) multiplying slowly in poor environment, and (*B*) multiplying rapidly in a rich environment, it is evidently of advantage to the type that sporulation should be the more profuse in community (*B*); for then on the death of both communities the greatest number of spores are ensured to propagate the race.

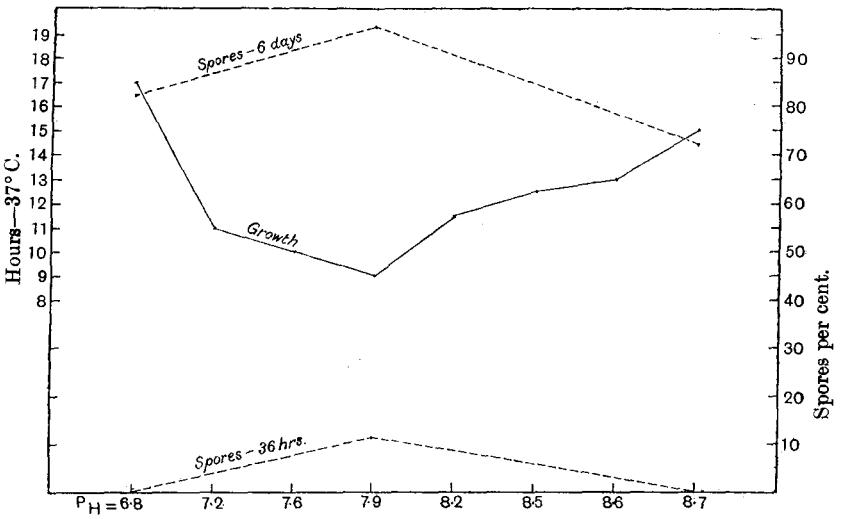
It is easily conceivable that thorough study of sporulation in anaerobes and the conditions governing it should furnish valuable knowledge especially perhaps in connection with the preservation of foods. Many notes on sporulation may be found in the literature dealing with type characters of anaerobes, and at least one series of experiments on the sporulation of a particular type has been published (Fitzgerald, 1911). But no work devoted to a general treatment of the subject has been met with; nor does any method appear to have been described for observation on an arithmetical basis.

Experiments were therefore made with a view to testing a simple method permitting of numerical estimation of the proportion of spores present in cultures. The method was considered to give sufficient accuracy to enable practical conclusions to be drawn; and it was accordingly applied to a preliminary investigation of the more important factors in cultivation that

influence sporulation. Although very little, if anything, of value may have been added to our knowledge in the following notes, it is hoped that these methods of observation may be useful as a basis to others wishing to carry out more important work on the subject.

METHOD OF ENUMERATING SPORES.

The principle is simply that of "projecting" microscopic fields on to squared paper, and marking off spores, spore-bearing bacilli, and vegetative bacilli on small areas to facilitate counting. This was done by means of an ordinary camera lucida attached to the eye-piece of the microscope in the



Rate of Growth and Sporulation—*B. sporogenes*

Chart 1

horizontal position. Graph paper was used printed in 1/10 in. squares, and further divided with black ink into 1/2 in. squares. It was arranged that a microscopic field viewed in projection on the graph paper embraced 36 of the 1/2 in. squares. Film preparations for counting were made on cover glass slips, which were mounted in a drop of 1–5 dilution in water of the ordinary 1 per cent. methylene blue stain; to the diluted methylene blue sufficient caustic soda had been added to procure immediate intense staining of healthy bacilli. After mounting, the edges of the cover slip were sealed with vaseline. It is noteworthy that if film preparations are over-fixed by slightly prolonged heating, mature spores are stained with considerable intensity by the above or other ordinary methods: as I believe, first pointed out by P. N. Panton (1908). It is unnecessary, however, for the present purpose that the spores should be stained at all.

In using the camera lucida, the lighting must be controlled so that the reflection of the microscopic field seen in projection on the squared paper is not too bright for the process of marking to be clearly visible.

Such microscopic fields were chosen that at least 300 organisms were available for counting. Spores and spore-bearing bacilli were dotted off with red ink, vegetative bacilli with black ink. A bacillus was not marked as a spore-bearer unless that spore was mature enough to be at once recognisable either by the presence of a swelling, or by its "refractility," in types where the spore produces no swelling.

In the complete chart, the red and black dots, representing spores and bacilli, were counted, and the proportion of spores recorded as a percentage of the total organisms enumerated. At first, results were expressed as a "sporulation index" represented by the quotient obtained by dividing the number of spores by the number of bacilli. Thus with 92 spores and 318 bacilli, the "sporulation index" is 0·3 approximately. This device was not found to be generally convenient. The error involved in the method was gauged by estimating the proportion of spores counted in homologous films and cultures.

Method of Cultivating Anaerobes for Spore-Estimation.

Cultures for sporulation were inoculated in the broth known as P 3, which is a filtered extract of pancreas, first prepared by G. S. Graham-Smith, by a method described in another communication (de Smidt, 1923). P 3 affords an exceptionally rich nutrient medium for the types of anaerobes experimented with, so that if needed a poor nutrient environment can be readily produced by dilution.

The methods of obtaining anaerobic cultures were based on McIntosh and Fildes' invaluable method for surface cultivation in single tubes, depending for the removal of oxygen, on the use of hydrogen and "palladium-asbestos-wool" (McIntosh and Fildes, 1917).

In earlier experiments, broth containing 0·5 to 1·0 per cent. of glucose was used, and was strained and cooled immediately before inoculation. Later, plain broth, containing no added carbohydrate or solids, was rendered sufficiently oxygen-free for anaerobic growth by means of a simple procedure of re-heating in an oxygen-free atmosphere—more convenient than, and probably as reliable as the more cumbersome methods depending upon exhaustion apparatus.

Details of the method are given below.

Cultures were made either in duplicate or in separate anaerobic tubes, the contents of which were thoroughly mixed together before spore-enumeration; or they were prepared in small tubes each containing 2 c.c. of broth, which were incubated within large anaerobic containing tubes each holding four or five cultures.

Experimental Error in Enumerating Spores.

In these and all subsequent experiments, the broth cultures were subjected to prolonged shaking before preparing films for spore counting.

A. *Estimation of spores in films from same culture*—B. sporogenes.

P 3 broth, reaction $pH = 7{\cdot}5$. Incubation 5 days at 37° C.

Total organisms counted	Spores	Spores %
334	274	82
370	314	85
417	345	83
380	316	83
385	301	78
		Error = 7

B. *Estimation of spores in films from uniform cultures*—B. sporogenes.

One film from each of five cultures, uniformly inoculated with a loopful of spore suspension transferred to 2 c.c. of P 3 broth, reaction $pH = 7{\cdot}5$. Cultures in same anaerobic container, 96 hours at 37° C.

Total organisms	Spores	Spores %
453	76	17
338	70	21
410	72	22
449	78	17
586	63	11
		Error = 11

The importance of the error was reduced in the following experiments by repeating them as many times as was considered necessary for reliable conclusions to be drawn from the results.

Relation of Sporulation to Rate of Growth.

Experiments were made to gauge the influence on sporulation of variations in certain primary factors that govern the rate of growth of organisms in culture. Throughout, except when otherwise mentioned, tubes containing 2 c.c. of broth were inoculated with one standard loopful of a shaken suspension of spores in pure culture.

I. *Influence of reaction of medium*—B. sporogenes.

(*a*) P 3 broth, containing 1 per cent. glucose, in duplicate cultures, incubated 96 hours at 37° C.

Reaction	Spores
$pH = 6{\cdot}4$	1·8 %
$pH = 7{\cdot}7$	77·7
$pH = 8{\cdot}7$	58·0

(*b*) P 3 broth, plain, cultures in same anaerobic container, incubation (1) 6 days at 37° C., (2) 36 hours at 37° C.

Reaction	(1) Spores	(2) Spores
$pH = 6{\cdot}8$	82·8 %	Nil
$pH = 7{\cdot}9$	96·8	11·5 %
$pH = 8{\cdot}7$	73·6	Nil

The accompanying chart shows the rate of growth of a strain of *B. sporogenes* in P 3 agar at various hydrogen ion concentrations. The curve is based on the

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times of appearance of growth in a duplicate series of stab cultures. Disregarding any discrepancies arising from slight variations in quality of the samples of P 3 used, two of the above results are superimposed for comparison on the rate of growth curve.

II. *Influence of temperature*—B. sporogenes.

P 3 broth, containing 1 per cent. glucose, reaction pH = 7·8, in duplicate cultures; (1) incubated 96 hours at 37° C., (2) incubated 18 hours at 37° C. then transferred to electric incubator at 25° C. for 88 hours.

	Temperature	Spores
(1)	37° C.	69·3 %
(2)	25° C.	2·9

Rate of growth was judged by observing the appearance of similar cultures after 24 hours' incubation at 37° C. and 25° C.

	Temperature	Growth	Spores, 96 hours
(1)	37° C.	Turbid, frothing	Very abundant
(2)	25° C.	Opalescence	Scanty

III. *Influence of concentration of food material*—B. sporogenes.

Comparison was made of sporulation in P 3 broth diluted with water, and full strength P 3 broth. Cultures were inoculated both with small and very heavy sowings.

(*a*) P 3 broth, reaction pH = 7·7, (1) 20 per cent. P 3 and 1 per cent. glucose, (2) full strength P 3 and 1 per cent. glucose. Duplicate cultures inoculated with one loopful of spore suspension, incubated 72 hours at 37° C.

	Medium	Spores, 72 hours	Growth, 20 hours
(1)	P 3 20 %	0·9 %	Opalescence
(2)	P 3 full strength	12·5 %	Turbidity, gas

(*b*) P 3 broth, reaction pH = 7·7 in strengths (1) 15 per cent. P 3 and 0·5 per cent. glucose, (2) full strength P 3 and 0·5 per cent. glucose. Duplicate cultures inoculated with one loopful of spore suspension, incubated 96 hours at 37° C.

	Medium	Spores, 96 hours	Growth, 20 hours
(1)	P 3 15 %	Nil	Opalescence
(2)	P 3 full strength	79·2 %	Turbidity, gas

(*c*) P 3 broth, reaction pH = 7·7 in strengths (1) 20 per cent. P 3 and 1 per cent. glucose, (2) full strength P 3 and 1 per cent. glucose. Duplicate cultures in 2 c.c. broth inoculated with 0·5 c.c. of a vigorously growing 20 hours' broth culture. Incubated 72 hours at 37° C.

	Medium	Spores, 72 hours	Growth, 20 hours
(1)	P 3 20 %	0·4 %	Cloudiness
(2)	P 3 full strength	16·6 %	Turbidity, gas

P 3 broth, reaction pH = 7·7 in strengths (1) 15 per cent. P 3 and 0·5 per cent. glucose, (2) full strength P 3 and 0·5 per cent. glucose. Duplicate cultures in 2 c.c. broth, inoculated as above; incubated 96 hours at 37° C.

Medium	Spores, 96 hours	Growth, 20 hours
(1) P 3 15 %	Nil	Cloudiness
(2) P 3 full strength	87·7 %	Turbidity, gas

On the assumption that sporulation proceeds in relation to the exhaustion of food material, it might be supposed that dilute broth should become exhausted earlier than full strength broth, and the above results should be reversed. It is probable, however, that the rich medium is more rapidly used up than the poor, by reason of the rate of growth in the former being enormously greater in proportion.

IV. *Influence of carbohydrates.*

It is well known that with certain types of anaerobes sporulation is less profuse in media to which glucose has been added. The following experiment with *B. sporogenes* shows that sporulation is inhibited by increasing the quantity of glucose added to broth. P 3 broth, 90 per cent., reaction pH = 7·8, in duplicate cultures containing (1) 0·5 per cent. of glucose, (2) 2 per cent. of glucose. Inoculated with one loopful of spore suspension, incubated 96 hours at 37° C.

	Glucose	Spores
(1)	0·5 %	60 %
(2)	2·0 %	9 %

Inhibition of sporulation by carbohydrate may be due to production of acid by fermentation, with the result that the reaction of the medium is rapidly altered to an extent that greatly lessens the rate of growth.

Fermentation of carbohydrates in its effect on sporulation. The following experiments indicate that sporulation is inhibited only when the carbohydrate present is fermented by the type of organism concerned.

Medium. P 3 broth, 50 per cent. strength, coloured with litmus, containing (1) 1 per cent. of glucose, (2) 1 per cent. of lactose, (3) no added carbohydrate. The plain dilute broth did not contain sufficient sugar to be demonstrable by Fehling's reagent.

Types of anaerobe. B. sporogenes, which ferments *glucose*, but not *lactose*; McIntosh's *Type* 3 *c* ("round spored V. Hibler") which ferments neither of these sugars; and *B. butyricus* (Adamson) which ferments both *glucose* and *lactose*.

Cultures in 2 c.c. of above media, uniformly inoculated with spore suspension, and incubated in same anaerobic container for 6 days at 37° C. Acid reactions when present were strongly marked in 24 hours.

(*a*) *B. sporogenes.*

	Glucose	Lactose	Plain broth
Reaction	Acid	Unchanged	Unchanged
Spores	Nil	46 %	42 %

(*b*) *Type* 3 *c.*

	Glucose	Lactose	Plain broth
Reaction	Unchanged	Unchanged	Unchanged
Spores	63·2 %	61 %	60·9 %

(*c*) *B. butyricus.*

	Glucose	Lactose	Plain broth
Reaction	Acid	Acid	Unchanged
Spores	Nil	Nil	70·3 %

Error in sugar experiments. Using Type 3 *c*, five cultures were prepared in 2 c.c. of 50 per cent. P 3 broth containing 1 per cent. of glucose: these were incubated in the same anaerobic container, and the spores estimated after 6 days at 37° C. Results: 63, 65, 66, 72 per cent. respectively.

The greatest difference between two counts = 9 per cent.

Constancy of reaction in sugar-free broth cultures. As shown above, no alteration of reaction was indicated by litmus in 50 per cent. P 3 broth containing no sugar.

To decide whether any slight or gradual change in hydrogen ion concentration occurred in cultures in plain broth, to be considered as a possible factor influencing sporulation, the hydrogen ion concentration of a sample of P 3 broth was estimated before and after prolonged cultivation of *B. sporogenes*.

A sample of P 3 broth containing no added carbohydrate was adjusted to pH = 7·4 by the colorimetric method. Of this two tubes containing 10 c.c. were heavily inoculated with *B. sporogenes* and became turbid with growth within 20 hours at 37° C. Incubation at this temperature was continued for 9 days, when examination of films showed a large majority of free spores, the remainder consisting of bacilli bearing mature spores, with occasional sporeless bacilli of degenerate appearance. The cultures were centrifugalised, the clear fluids pipetted off and mixed, and the hydrogen ion concentration again taken. The result was pH = 7·4. No difference was appreciable by the colorimetric method between the cultivated broth and a sterile sample of the original broth which had been steamed, for the same length of time (10 mins.) as the former before inoculation, and incubated in sealed tubes for the same length of time along with the cultures.

V. *Problem of the determining factor in sporulation.*

The foregoing experiments indicate that sporulation is dependent upon rate of growth, and support the view that, provided no disturbing factor arises, the more vigorously a number of bacilli inoculated in a culture are enabled to multiply, the larger will be the proportion of spore-bearing individuals present within a given period. The growth of an organism, for example *B. sporogenes*, in a nutrient medium causes alteration of that medium. The alteration consists broadly in (1) exhaustion of the food material required by the organism, (2) chemical change due to products of growth of the organism. The external factor that determines the production of a spore by a bacillus must be, normally, the stimulus of one or the other of these conditions of its environment. Abnormal influences such as the introduction of free oxygen into an actively growing anaerobic culture, may and probably do stimulate sporulation. Alteration of hydrogen ion concentration, such as is caused by acid fermentation of carbohydrate, has been shown to be an inimical factor. No appreciable alteration in the hydrogen ion concentration of a sugar free medium is brought about by the vigorous growth in it of *B. sporogenes* up to the point at which vegetation has ceased and only free spores remain. Alteration in hydrogen

ion concentration can therefore be eliminated as a possible stimulus. The determining cause must therefore be sought for either in the effect upon the bacilli of a diminution of their nutriment; or in the presence in sufficient amount of some chemical body introduced into the environment by means of their own growth therein.

PRODUCTS OF GROWTH IN CONNECTION WITH SPORULATION.

Attempts were made to determine whether spent broth in which *B. sporogenes* had grown vigorously, contained any element capable of stimulating sporulation in the same organism. Broth cultures 7 days old, in which sporulation had reached a very advanced stage were centrifugalised without interrupting anaerobiosis. From the clear top fluid both aerobic and anaerobic tubes of medium were inoculated to control its sterility. These cultures were negative. Of the spent broth, volumes of 0·25 c.c. were added to two of four 24 hours' broth cultures of *B. sporogenes* in 2 c.c. of medium. To the remaining two cultures, the same volumes of sterile boiled water were added. The transfers of spent broth and water were performed rapidly, and the cultures treated were at once rendered anaerobic again, so that no oxygen sufficient to interrupt growth was introduced. A further set of cultures was treated with 0·5 c.c. volumes of spent broth and water. Both sets of cultures were incubated at 25° C. for 6 days, after which film preparations were examined for spores. Unfortunately, the enumeration method could not be used here, and ordinary judgment was relied upon for comparing results.

In both experiments it was found that a moderate proportion of spores had appeared in all four cultures; but no difference could be discovered between the films from the cultures treated with spent broth and those to which water had been added. It was concluded therefore that the spent broth had exercised no influence on the ordinary progress of sporulation in the cultures treated with it.

REINOCULATION OF CULTURES WITH THE SAME STRAIN OF ORGANISM.

Experiments were made to obtain evidence of the relation of sporulation to exhaustion of food material, as judged by the capacity of the medium to support growth at various stages of a culture therein. A number of stab cultures were inoculated with *B. sporogenes* in large deep tubes of nutrient agar, and incubated at 37° C. At intervals of 48 hours, secondary inoculations of the same organism were made in stabs parallel to the original in each culture in succession. At the same time, film preparations from the original inoculation were examined for spores.

After 48 hours, spores amounted to about 30 per cent.; secondary inoculation yielded a visible growth. At 96 hours, about 70 per cent. of spores were present; secondary inoculation showed no visible growth.

It is conceivable that when bacilli of spore-bearing type are fully supplied with nutrient material, and with the conditions that enable them to make

use of it, all energy derived from the food is directed towards vegetation. When the food supply fails by exhaustion, insufficient energy is obtainable for multiplication, and what remains is directed towards spore-formation.

It has been shown that of two communities in the same environment, one at the favourable temperature of 37° C. grows and spores vigorously, while the other at the unfavourable temperature of 25° C. grows and spores feebly. On the above tenets, it might be supposed that at the low temperature, the bacilli, being unable to make full use of their food, should direct what energy is available to producing spores. As shown, however, sporulation in these conditions is greatly delayed, probably because multiplication proceeds, however slowly, as long as sufficient energy can be got to maintain it. At length the stage is reached for each individual bacillus when food energy is so far deficient that division is impossible; sporulation then commences. The cycle is thus precisely the same as that proceeding at the favourable high temperature, but covers a longer period.

Similarly, where equal numbers of bacilli are introduced into a poor and a rich environment respectively, other conditions being equal, multiplication is proportionately vastly more rapid in the rich medium; with the result that the stage is reached earlier at which food energy is insufficient for multiplication; but is still sufficient for spore production.

Abnormal Influences in Sporulation.

By abnormal influences is implied those of conditions which do not as a rule arise in the environment of a community of anaerobic bacilli. One of these perhaps is the intermittent exposure of the organisms to free oxygen.

Experiments in this direction were made by aerating broth cultures of *B. sporogenes* at intervals of 24 hours by means of a two-way syringe and a sterile pipette passing through the plugs of the culture tubes. The cultures so treated were incubated at 25° C.: it was found that up to a period of 5 or 6 days sporulation appeared to be markedly increased as compared with similar cultures not aerated. No enumerations of spores were made in these experiments, and their results are therefore not considered conclusive. The probability that spores may be produced in response to exceptional conditions does not interfere with the conclusion that sporulation normally proceeds in response to failure of the food supply; among higher organisms, certain processes such as germination of the ovum may work independently of normal stimuli, or in response to artificial ones.

It is not presumed that the experiments here described are sufficient for their purpose. Possibly the determining factor in sporulation may be a combination of exhaustion of nutriment with the arrival at a certain concentration of growth products. Nor is any account taken of physical and physiological factors that may be at work. The bearing of specific differences in the behaviour of types must be considered. For example, a strain of *B. tertius* produced spores so rapidly in P 3 broth that within 24 hours

scarcely a sporeless bacillus remained; while *B. sporogenes* under the same conditions showed few signs of spore-swellings in this time.

There is no doubt that the problems presented by the sporulation of anaerobes are thoroughly worthy of investigation: and are such as to tax to the utmost the resources of the biochemist and biologist. Not the least of these is the problem offered by the spore itself, which enclosed within a wall of extraordinary powers of resistance to physical and chemical agents, is yet so delicately sensitive to external conditions that it germinates at once if placed in contact with a suitable nutrient surface.

METHODS OF ANAEROBIC CULTIVATION IN BROTH.

(1) *Single tubes* were rendered anaerobic by McIntosh and Fildes' method (1917), depending upon hydrogen delivered from a Kipps apparatus or modification, and palladium-asbestos-wool for removal of residual oxygen.

With glucose broth, the tubes of medium were inoculated after steaming and cooling in the usual way.

With plain broth, the tube was first steamed for 10 minutes, then while hot, rendered anaerobic and sealed, and the junction of stopper and tube painted with collodion. With the palladium-wool capsule still hot, the tube was replaced in boiling water for 5 minutes. It was then transferred to cold water without unsealing. When cold, the tube was unsealed, the broth rapidly inoculated without shaking and the tube at once rendered anaerobic and sealed again, painting with collodion as before.

When inoculating cultures, the corks as well as the mouth of the tubes were flamed for a second in the bunsen burner.

The capsule referred to consists of a small piece of "palladium-wool" folded in a single layer of copper gauze for safety, and to prevent pieces dropping into the culture.

Further details of McIntosh and Fildes' well-known method are given in their report as above.

(2) *Small tubes under the same anaerobic conditions.* Large, stout glass tubes 8 inches in length by about 3 centimetres of *internal* diameter, were used as containers. These, as described by McIntosh and Fildes for small single tubes, are fitted with rubber bungs bored with two holes to fit quill tubing. Two short pieces of quill tubing, drawn out pipette-wise at one end, are inserted into the holes, after lubrication with a little vaseline, by passing them point first through the bottom of the bung, and forcing them through by pressure on a table.

After insertion, one of the pipettes is bent to a right angle for the hydrogen outlet. The pipettes can be easily removed when necessary by pushing them out from below with a metal rod. A small capsule about 1 in. square of palladium-asbestos-wool folded in a single layer of gauze is fastened to the centre of the bottom of the bung with a pin pushed in while red hot. The culture tubes were 11 centimetres long by about 12 millimetres in *external*

diameter. Containing 2 or 3 c.c. of plain broth, and tightly plugged with wool, these are steamed for 10 minutes to expel air. Four or five at once are then transferred while hot into the container, which should contain a little hot water sufficient to immerse the ends of the culture tubes, and act as a conductor of heat. The container is at once rendered anaerobic, sealed, painting the bung at its insertion with collodion, and then the whole placed in boiling water while the capsule is still hot; there is no danger of the bung being forced out. After some minutes' steaming, the container is transferred to cold water without unsealing. When cold, the sealed tips of the pipettes are cut off, and the bung removed; the culture tubes are extracted by their plugs with forceps, quickly inoculated, and replaced, and anaerobiosis again set up in the container. The whole procedure, after unsealing the container, must be as rapid as possible without shaking the culture tubes. Although this form of anaerobic container is best suited for surface cultivation in small tubes of agar, the method described here is convenient and fairly rapid, besides being inexpensive; and it was found to be sufficiently dependable for the experiments detailed above.

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