THE GROWTH OF *H. PERTUSSIS* ON MEDIA WITHOUT BLOOD.

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THE development of a vaccine to protect against whooping-cough has led to the need for an artificial medium which will grow *H. pertussis* satisfactorily. Little work has been done on the growth requirements of this organism, and the original medium first devised by Bordet and Gengou (1906; 1907) 41 years ago is still in general use with few modifications, although widely admitted to be far from ideal.

The modified Bordet-Gengou (B-G) medium most commonly used consists of 30 to 50 per cent whole blood and potato extract as well as a basis of peptone or tryptic digest of meat. Its composition suggests that the organism is highly fastidious; but even on this enriched medium single colonies do not reach countable size for 3 days, and on primary isolation growth may be even slower. Any attempt to simplify the medium has tended to cause a gradual change in the organism leading, after repeated subcultures, to loss of virulence and antigenicity, and change of phase from S to R. It is even possible that some loss of antigenic potency occurs very rapidly on the unsimplified B-G medium itself, leading to unsatisfactory batches of vaccine. Ability to grow on plain nutrient agar or in broth, a property which is usually only acquired after considerable training or laboratory storage, is invariably associated without complete loss of virulence and protective antigenicity for laboratory animals, as well as colonial and morphological changes which are more or less permanent (Lawson, 1939).

The nearest attempt to develop a synthetic medium for *H. pertussis* is that of Hornibrook (1939; 1940), who found that the required "Phase I" organism would grow in a mixture of amino-acids, salts and nicotinic acid and 0·1 per cent soluble starch. The main interest of his papers is that no growth would occur, even with a heavy inoculum, in the absence of starch or dextrin.

Our approach to the problem has been different from that of Hornibrook. Starting with a derived strain of *H. pertussis* that would grow well on ordinary 10 per cent blood agar, but not on plain nutrient agar or in broth, we have attempted to determine directly what factors in blood were responsible for its growth-promoting effect. It was discovered that a freshly isolated strain of *H. pertussis* (Phase I) could be trained to grow on 10 per cent blood agar as heavily as on the B-G medium, by two successive subcultures on starch-nutrient agar

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slopes containing diminishing quantities of blood. Repeated attempts to train the organism to dispense entirely with blood failed; and it was accepted as a suitable test strain for determining the role of blood in the growth of *H. pertussis*. Although the organism could not be regarded as typical "Phase I," it was considered to be a satisfactory indicator of the function of blood, because all attempts to induce it to grow without blood failed. Further detailed characteristics of this derived strain are given below.

METHODS.

1. *Media.*—The basal medium used throughout was a tryptic digest of beef. All blood fractions, except where otherwise stated, were human. Bordet-Gengou medium was prepared as described by Mackie and McCartney (1946).

2. *Organism.*—A derived, partially trained strain was obtained as follows: A freshly isolated Phase I strain of *H. pertussis* was inoculated from B-G medium on to a large agar slope of tryptic meat medium containing 0·1 per cent starch and 30 per cent blood. After 2 days' incubation at 35°, the growth, which was as good as on the original B-G medium, was subcultured on to a similar starch-agar slope containing 15 per cent blood, and the ensuing growth further subcultured on to a slope containing only 5 per cent blood. The organisms grew well after 5 days' incubation on this last medium, and were then found to grow on ordinary 10 per cent blood plates without starch as well as they did on the original B-G medium, even with small inocula. They were morphologically indistinguishable from the parent strain, and were agglutinated to titre by three different anti-Phase I sera. Absorption tests, however, with an anti-serum prepared from the parent strain showed that the derived strain had lost, completely or nearly completely, one of the antigens originally present. Virulence tests on the derived strain (undertaken by Mr. Proom) indicated a somewhat reduced virulence and toxigenicity for laboratory animals:

Route of injection.	M.L.D. for mouse (20 g.).	
	Normal Phase I.	Derived strain.
Intra-cerebral	50×10^6 .	50×10^6
Intra-nasal	25×10^6 .	25×10^8
Intra-peritoneal	15×10^8 .	25×10^8

The derived strain, although essentially Phase I or Smooth, must be regarded as having undergone slight degradation, the significance of which is not yet fully understood. It is subsequently referred to as "*Pertussis A.*" A large batch was immediately dried down by the method described by Stamp (1947), for use in growth experiments.

3. *Inoculation of media.*—The dried organisms were revived by growth for 3 or 4 days at 35° on a 10 per cent blood slope, which was then kept in the ice-chest to serve as inoculum-source. The inoculum itself was prepared from a 48 hours' growth on a blood slope inoculated from the first slope. The cells were scraped off with a wire and suspended in 2 ml. of tryptic meat broth to standard opacity (about 5×10^9 ml.), from which three 100-fold dilutions in broth were made. Actual inoculation was performed as soon as possible after dilution. A fresh ampoule of dried bacteria was used every fortnight. By these means it was hoped that stability of the culture would be maintained. As previously mentioned, there was no indication of any tendency to degradation, and attempts to train

the organism to grow in plain broth or on nutrient agar persistently failed, even with heavy inocula. Nevertheless, in all experiments, a plain broth or agar control was inoculated at the same time, in order to detect any possible sudden change. In experiments on solid media, 4 separate drop inocula (unspread), containing respectively 10^8, 10^6, 10^4 and 10^2 viable cells, were made on to each plate. All plates contained exactly 25 ml. of medium. The results were recorded as heaviness of confluent growth (judged roughly by eye) and diameter and numbers of colonies where discrete. In liquid media the inoculum was always about 100 cells inoculated into 5 ml. of medium in 50 ml. Erlenmeyer flasks and the growth was recorded in the usual way by numbers of plusses.

4. *Incubation.*—At 35° in air.

5. *Viable counts.*—On 10 per cent blood plates, using the method of Miles and Misra (1938).

6. *Protein content* of serum fractions was estimated by micro-Kjeldahl analysis of the N content.

RESULTS.

A. *Fractionation of blood.*

Because of the difficulty in determining growth in opaque liquid media, the initial experiments were all done on solid media, by the technique described. The results were clear cut and are not worth recording in full. It was found that the growth-promoting power of washed red cells, laked cells, serum or plasma

TABLE I.—*Growth of* Pertussis A *on Nutrient Agar Plates containing Various Fractions of Plasma.*

Medium	Dilutions of standard inoculum. 1 : 1	1 : 10^2	1 : 10^4	1 : 10^6
Control (tryptic meat agar alone)	Tr. +*	0	0	0
Plus—				
Dialysed plasma (2 per cent)	+++	+++	+++	100 (1 mm.)
Serum (2·2 per cent)	+++	+++	+++	100 ($\frac{1}{2}$ to 1 mm.)
Pure serum globulin (·07 per cent)	+*	0	0	0
Albumen residue (·07 per cent protein)	+++	+++	+++	100 ($\frac{1}{2}$ to 1 mm.)
Serum (2·2 per cent) + ·07 per cent additional globulin	+++	+++	++	100 ($\frac{1}{2}$ to 1 mm.)
Horse serum albumen (·07 per cent)	+++	+++	+++	100 (1 mm.)
Whole blood (10 per cent)	+++	+++	+++	100 (1 mm.)

Growth for 5 days at 35°. +++ = heavy confluent growth; ++ = moderate growth; +, tr. + = slight growth; 0 = No growth.
Where colonies are discrete, their approximate number and size (diameter) are recorded.
* Probably no true growth (see remarks in text).

was as great as that of whole blood. Further fractionation was confined to plasma. The activity resisted prolonged dialysis and treatment at 56° for 30 min., but was completely destroyed by 30 min. in a boiling water bath. The diffusate after dialysis was quite inactive. The activity was clearly, therefore, associated with protein. By half-saturation of serum with ammonium sulphate, and two further precipitations and washings of the fraction thus isolated, serum globulin was extracted and found to be quite inactive, though not inhibitory ; the albumen residue, however, was as active as whole plasma. Some twice crystallized horse serum albumen was found to be as active as the human serum albumen residue. This horse serum albumen was dissolved in water and dialysed for 3 days in the ice-chest against 4 changes of 10 times its volume of distilled water ; it was then clarified by filtration through a paper pulp and finally sterilized by Seitz filtration. Table I shows the results given by these fractions. Little comment is necessary. The traces of growth recorded with the heaviest inoculum on the control plate and that containing only globulin do not probably signify actual multiplication, but simply the opacity of the inoculum itself. As will be shown later, not only does *Pertussis A* fail to multiply in plain broth or on agar ; the cells actually die off fairly rapidly.

It was now possible to carry out satisfactory growth experiments in liquid media. It was found that an inoculum of 100 viable cells would grow well in broth containing 0·1 per cent albumen. The initial growth turbidity was usually visible after 3 days' incubation, and heavy growth of about 5×10^9/ml. cells obtained after 5 days. The lowest concentration of albumen that would support growth varied with the batch of broth used, but was usually about 0·04 per cent. It was found essential to maintain efficient oxygenation by tubing the medium in small (5 ml.) lots in 50 ml. Erlenmeyer flasks. With a very heavy inoculum (about 10^8 cells) growth was first apparent after only 36 hours.

B. *Effect of charcoal.*

It seemed unlikely that the albumen was actually metabolized by the organism, and attention was drawn to the mysterious growth-promoting effect of charcoal on meningococci and gonococci reported by Glass and Kennett (1939). These workers found that charcoal (heated to 1000° to destroy organic matter) could replace blood in the cultivation of these bacteria on nutrient agar. They drew no conclusions, but put forward a number of tentative suggestions, amongst which was the possibility of a catalytic function due to iron present in the charcoal, and the alternative possibility that charcoal absorbed some inhibitory substance from the broth.

Activated charcoal (B.D.H.), made up in a 10 per cent suspension in water and autoclaved, was added to nutrient agar to a final concentration of 0·2 per cent. It was found to support the growth of *Pertussis A* as well as 0·04 per cent. albumen or 10 per cent blood (see Table II and Plate I, Fig. 1(*a*), (*b*) and (*c*)). Charcoal treated twice with boiling 12 per cent HCl for 20 min. (to remove any traces of iron) and subsequently washed was found to be just as efficient in supporting growth. In liquid media charcoal was, if anything, better than albumen ; the optimum concentration was found to be 0·2 per cent, although quite good growth would occur with 0·04 per cent. The growth of *Pertussis A* in charcoal broth was characteristic : the first sign of growth was a stickiness or pseudo-agglutination

TABLE II.—*Growth of* Pertussis A *on Nutrient Agar Plates with Added Charcoal, Starch Albumen or Whole Blood.*

	Dilutions of standard inoculum.			
Medium	1 : 1	1 : 10^2	1 : 10^4	1 : 10^6
Control (tryptic meat agar alone)	tr.	0	0	0
Plus—				
10 per cent whole blood	+++	+++	+++	200 (1 mm.)
0·4 per cent starch	+++	+++	s–c	125 ($\frac{1}{2}$ mm.)
0·2 per cent albumen	+++	+++	+++	200 ($\frac{1}{2}$–1 mm.)
0·2 per cent charcoal	+++	+++	+++	200 (1 mm.)

Explanation as in Table I. s–c = heavy semi-confluent growth.

of the fine charcoal fragments; later the whole charcoal was glued together in a sticky, ropey mass which sank to the bottom, leaving a charcoal-free supernatant, turbid with growing organisms. On autoclaving, the glutinous material dissolved and the general appearance became that of the uninoculated medium. Glass and Kennett (1939) found that mere treatment of broth with charcoal, followed by its removal, did not produce a medium that would support growth of gonococci, and this phenomenon has been confirmed for *Pertussis A*. Moreover, charcoal-treated broth was found to support growth of *Pertussis A* if more charcoal was added, so that the failure of charcoal-treated broth alone cannot be ascribed to the previous absorption of essential growth factors as well. The charcoal must be present in the medium *during* growth.

c. *Effect of starch.*

Hornibrook (1939; 1940) and other workers have found the addition of starch necessary to semi-synthetic media in order to obtain growth of *H. pertussis*; and 0·1 per cent starch has been successfully substituted for the potato extract in the B-G medium for the growth of the original strain. With *Pertussis A* itself, soluble starch (Analar) in concentrations from 0·04 per cent to 0·4 per cent in solid media acted similarly, though not quite so efficiently or reliably (with small inocula) as charcoal or albumen (Table II). In liquid media (in concentrations from 0·1 to 0·5 per cent) and with small inocula of 100 cells growth was most unreliable.

d. *Activity through cellophane.*

Hornibrook (1939) has stated that starch or serum was necessary for the growth of *H. pertussis* in his semi-synthetic medium, and that the serum could exert its effect even though retained in a cellophane sac suspended in the medium. This of course might have been due to diffusible substances present in the serum, but Dubos (1946) has reported that serum albumen itself could exert its growth-promoting effect on the tubercle bacillus, although confined within a cellophane sac. Accordingly it was decided to find out whether albumen would support the growth of *Pertussis A* under similar conditions. Two ml. of 1·5 per cent crystalline horse serum albumen was suspended, within a cellophane sac, in 10 ml.

of broth which was inoculated (outside the sac) with 5×10^5 cells of *Pertussis A*. Growth was first visible after 5 days' incubation. The same amount of albumen added direct to the medium gave visible growth with the same inoculum in 2 days. This effect was confirmed by laying circles of sterile cellophane on the surface of charcoal-agar, albumen-agar and blood-agar plates and inoculating the centre of the exposed surface. Growth occurred almost as well as in the absence of cellophane, though slightly delayed, and single colonies developed satisfactorily (Fig. 2). It need hardly be pointed out that neither charcoal nor albumen could possibly pass through cellophane, and it is quite clear that these two substances exert part, at least, of their effect by absorbing some toxic material from the medium.

E. *Effect of fatty acids* on growth.*

The main question that remained to be decided was the nature of the toxic material thus removed from broth by charcoal and albumen. Davis and Dubos (1946) ascribed the stimulating effect of albumen for the tubercle bacillus to its power of combining with the minute quantities of oleic acid present in Dubos' "Tween 80" medium. Oleic acid, they found, was very highly inhibitory to the tubercle bacillus. Long chain fatty acids and their soaps, especially unsaturated acids, have long been known to be highly bactericidal to a variety of micro-organisms (Kodicek and Worden, 1945). It is also well known that their action is counteracted by serum (Lamar, 1911; Walker, 1924; Eggerth, 1927). Charcoal, moreover, is known to absorb fatty acids firmly (Klaesson, 1946); and the same is true of starch (Schoch and Williams, 1944). The inhibitory effect of soaps on pneumococci (Walker, 1924), haemolytic streptococci (Burtenshaw, 1942), meningococci and gonococci (Walker, 1926), *C. diphtheriae* (Walker, 1925; Burtenshaw, 1942), *Erysipelothrix rhusiopathiae* (Hutner, 1942; Kodicek and Worden, 1945) and other organisms has been described, but no information exists concerning *H. pertussis*.

DESCRIPTION OF PLATE.

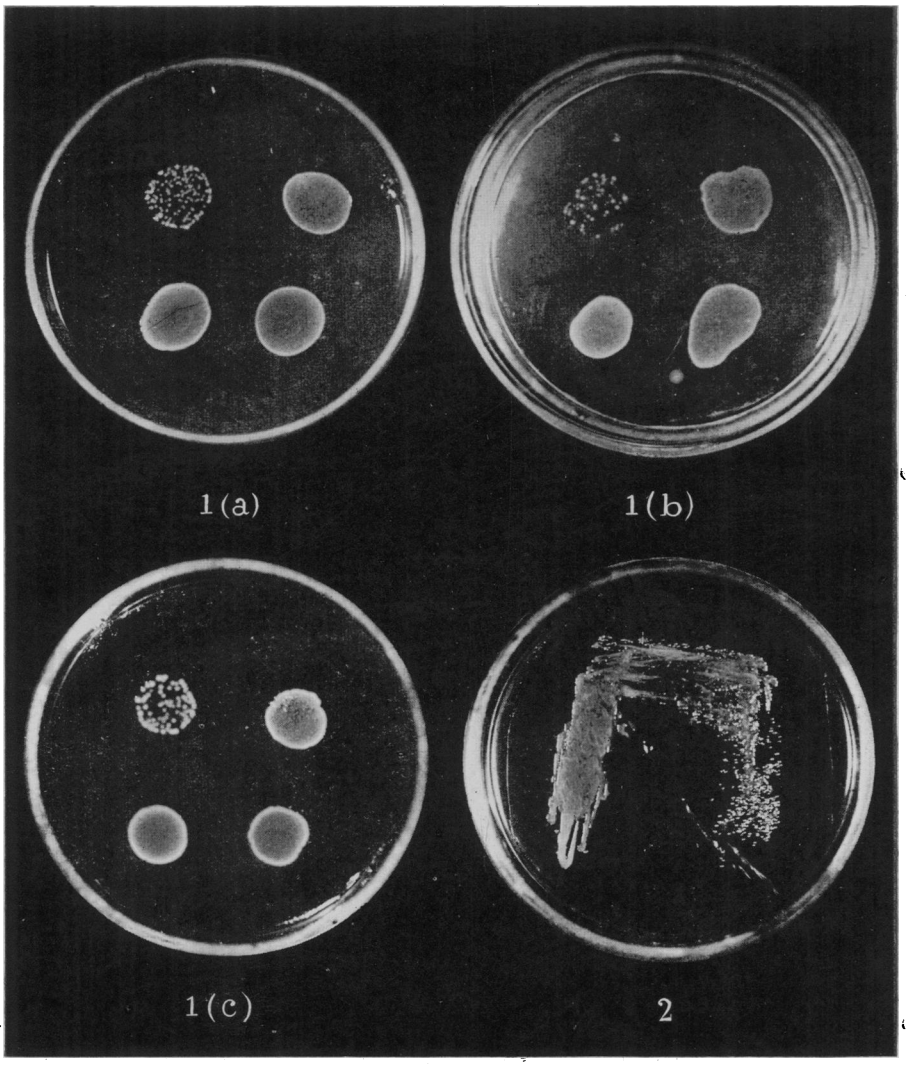
FIG. 1.—Comparison of the growth of *Pertussis A* on blood, albumen and charcoal in a basal medium of tryptic meat agar. 4 drop-inocula were made on to each plate. The drop with discrete colonies contained 100 cells, the others. 10^4, 10^6, 10^8 cells respectively, passing clockwise. (*a*) 10 per cent blood. (*b*) 0·04 per cent horse serum albumen. (*c*) 0·2 per cent charcoal.

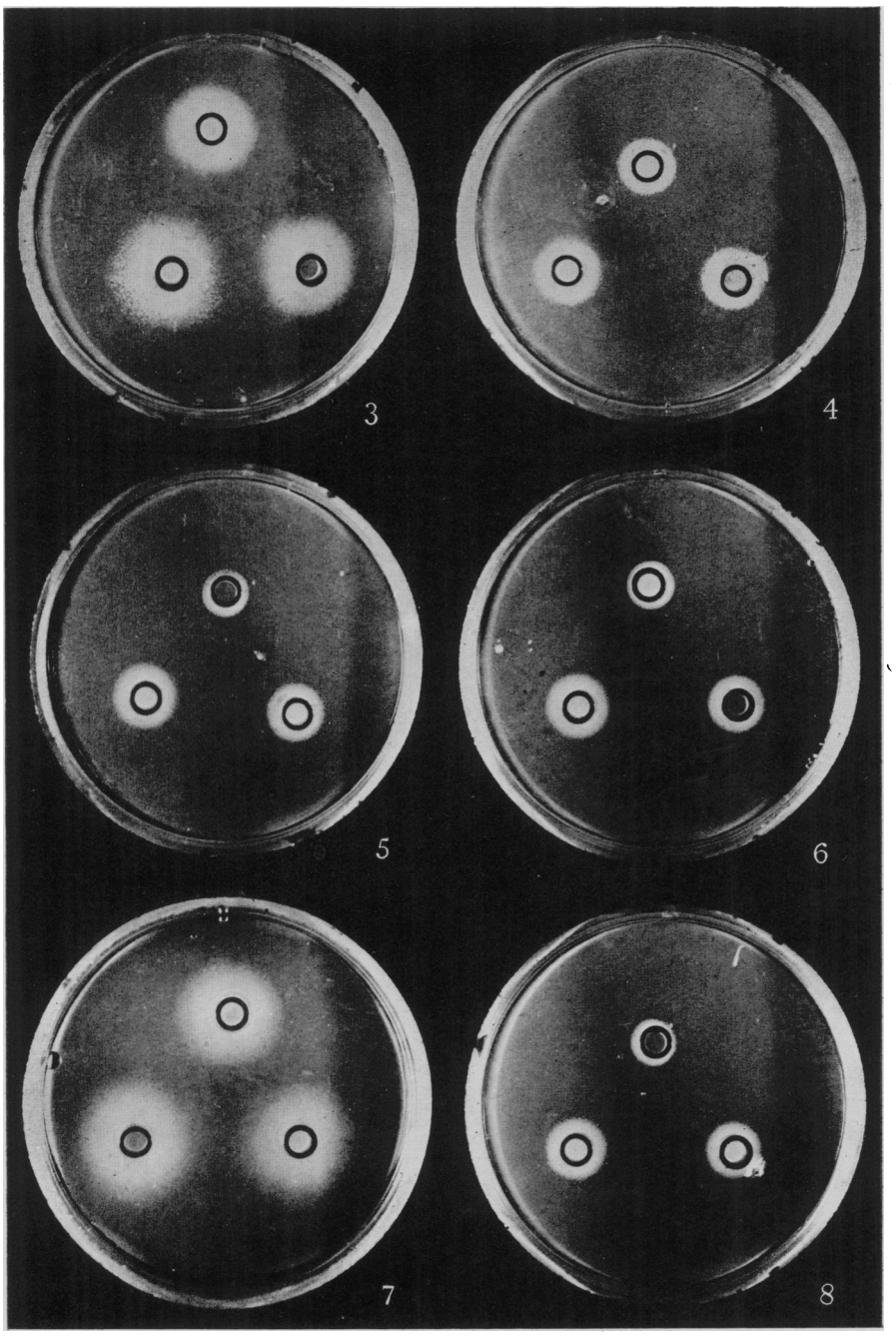
FIG. 2.—Growth of *Pertussis A* (from inoculum spread by wire) on the surface of cellophane lying on 0·2 per cent charcoal in nutrient agar.

Effect of certain inhibitory substances on the growth of *Pertussis A*, as demonstrated by the "Albumen Cup" method (Text and Table III).

FIG. 3.—Control.
FIG. 4.—4 μg./ml. oleate.
FIG. 5.—4 μg./ml. linoleate.
FIG. 6.—4 μg./ml. linolenate.
FIG. 7.—4 μg./ml. palmitate.
FIG. 8.—2 mg./ml. extract from charcoal used for growth of *Pertussis A* in broth.

* Throughout this work the words "fatty acid" and "soap" have been used interchangeably. All experiments have been done in relatively well buffered tryptic meat media of pH 7·6. At this pH the proportion of free fatty acid will be very small, and in any case, at the low concentrations used, will not be affected by whether it is added as such or in the form of a soap.


1(a)
1(b)
1(c)
2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A systematic investigation on the susceptibility of *Pertussis A* to soaps was undertaken with pure samples of oleic, linoleic, linolenic, palmitic and stearic acids (used as their sodium salts).

On the hypothesis that the toxic action of fatty acids is antagonized by albumen, their inhibitory effect might be expected to be masked unless the concentration of albumen was kept sufficiently low. With too little albumen, on the other hand, there would be no growth at all, even without the addition of fatty acid. The balance might therefore be expected to be rather critical. To obviate the complication of having to titrate many different concentrations, it was decided to do all preliminary work on solid media by means of a special technique.

The substance to be tested was incorporated in the agar plates (final vol. 25 ml.). One ml. of a suspension of *Pertussis A* (containing approx. 10^9 organisms /ml.) was flooded over the plate and the excess pipetted away. Three porcelain cups (as used and described by Heatley (1944) for penicillin assay) were sealed on the surface, and into these cups were added, respectively, 1, 2 and 3 standard drops of horse serum albumen solution and the plates incubated for 4 days. The albumen diffused slowly out into the medium, producing a gradient with a limiting concentration necessary for growth of *Pertussis A* at a distance from the centre of the cups depending, amongst other factors, upon the degree of inhibition by the substance under test. Growth plaques thus developed around the cups (Fig. 3), the diameter of which depended only (other conditions being equal) upon the amount of albumen in the cup and the degree of anti-bacterial action of the test material. As might have been expected, different batches of nutrient agar gave very different sized growth plaques, even with identical inocula. This emphasized the necessity of using the same batch of basal medium throughout any one experiment, and suggested that tryptic meat medium, as ordinarily prepared, contained very varying quantities of the toxic material removable by albumen or charcoal.

The method is, in fact, the ordinary penicillin cup test in reverse, with the inhibitor in the medium instead of within the cups. In practice, it was found to give approximate but reliable and reproducible results. The main advantages were :

(*a*) Ability to demonstrate the inhibitory effect of a fatty acid or similar substance without the need to control the albumen concentration.

(*b*) A reduction to a minimum (in solid media) of diffusion effects.

(*c*) Suitability for the estimation of the antibacterial action of unknown toxic substances in crude extracts.

(*d*) The rapid and simple technique, saving much time and material.

A reduction in size of the growth plaque is presumably due to a combination of (1) the anti-albumen effect of the substance tested, so that there is less "free" albumen at the periphery to exert its activity, and (2) a direct lethal action of the substance on the cells before the albumen reaches them, accelerating the death rate so that even if there is enough "free" albumen present to exert its effect no growth results. The factors concerned are no doubt complex, but the net result is that a general antibacterial effect is detected with considerable sensitivity.

The results, in which recorded figures are the diameters of growth plaques (in mm.) developing around the cups after 4 days' incubation, are summarized

in Tables III and IV and illustrated in Fig. 3 to 8. Results with the unsaturated fatty acids were confirmed in experiments with liquid media (Table V).

TABLE III.—*Effect of Soaps on the Growth of* Pertussis A *on Nutrient Agar Plates. "Albumen-cup" Method (see text).*

Soap.	μg./ml.	Diameter of growth plaques (mm.) around cups containing the following quantities of albumen: 1 drop.	2 drops.	3 drops.
—	0	15	18	20
Sod. oleate	8	12	12·5	13·5
Sod. linoleate	8	(8)	10	11
Sod. linolenate	8	(8)	(8)	10·5
Sod. palmitate	8	16	20	19
Sod. palmitate	40	25	21	25
Sod. stearate	8	18	21	24
Sod. stearate	40	27	27	35

The diameter of the cups is 8 mm., so that a growth plaque of less than 9 mm. is not measurable and is recorded as (8).

TABLE IV.—*Titration of the Inhibitory Effect of Different Concentrations of Sod. Oleate on the Growth of* Pertussis A *on Nutrient Agar. "Albumen-cup" Method.*

Conc. (μg./ml.) of sod. oleate in nutrient agar.	Diameter of growth plaques (mm.) around cups, containing the following quantities of albumen: 1 drop.	2 drops.	3 drops.
0	15	17	19
0·8	15	17	19
2·0	13	15	16
4·0	13	15	16
10	10	13	14
20	10	10	12
40	(8)	(8)	9

For full explanation see text and footnote to Table III.

It is clear that—

(*a*) The unsaturated fatty acids (oleic, linoleic, and linolenic) are highly inhibitory at a concentration as low as 4 μg./ml. The two saturated fatty acids (palmitic and stearic), on the other hand, have no action at 8 μg./ml.; at the higher concentration of 40 μg./ml. they actually appear to stimulate growth (Table III).

(*b*) The inhibitory effect of the unsaturated soaps is completely counteracted by albumen. For example, in the presence of 8 μg./ml. oleate no growth occurred at an albumen concentration of 0·06 per cent., but with 0·24 per cent albumen growth was practically as good as without any oleate at all (Table V).

TABLE V.—*Effect of Certain Soaps and Albumen on the Growth of* Pertussis A *in Broth. Inoculum* : 100 *Organisms.*

Soap.	ml. 1·5 per cent albumen (in 5 ml.).	Growth. 3 days.	Growth. 5 days.
Nil	0	—	—
	0·1	—	++
	0·2	—	+++
	0·4	—	+++
	0·8	tr.	+++±
Oleate	0	—	—
	0·1	—	—
4 μg./ml.	0·2	—	+
	0·4	—	+++
	0·8	tr.	+++
	0	—	—
	0·1	—	—
8 μg./ml.	0·2	—	—
	0·4	—	++±
	0·8	—	+++
Linoleate	0	—	—
	0·1	—	—
4 μg./ml.	0·2	—	+++±
	0·4	tr.	+++±
	0·8	+±	++++
	0	—	—
	0·1	—	—
8 mg./ml.	0·2	—	—
	0·4	tr. +	++++
	0·8	++	++++
Linolenate	0	—	—
	0·1	—	—
4 μg./ml.	0·2	—	++±
	0·4	tr.	+++
	0·8	tr. +	+++±
	0	—	—
	0·1	—	—
8 μg./ml.	0·2	—	tr.
	0·4	—	+++
	0·8	tr.	++++

The sensitivity of *Pertussis A* to unsaturated fatty acids and the known property of charcoal and albumen in absorbing these substances supports the hypothesis that they, or related compounds, might be the main components of the toxic material developed in meat broth. It is highly probable that both

saturated and unsaturated soaps occur in most " natural " media, though their content is likely to be very variable.

F. *Extraction of toxic material from broth.*

It remained to demonstrate directly the presence of a toxic substance in tryptic meat broth. 500 ml. of broth containing 1 g. of charcoal were distributed in 10 Roux flasks, and inoculated with *Pertussis A*. After 6 days' incubation at 35° the bottles were autoclaved, and the charcoal was filtered off and extracted, without washing, with 100 ml. of methanol in a Soxhlet for 3 hours. The extract was evaporated down *in vacuo*, leaving 2.03 g. of a yellow fatty material which dissolved easily in 20 ml. of broth to form a stable cloudy emulsion. It was tested for anti-*Pertussis* action by the albumen cup method and found to give marked inhibition at a concentration of 1 in 5000, corresponding very approximately to 1/100 of the activity of pure oleate (Table VI).

TABLE VI.—*Titration of the Inhibitory Effect of Toxic Material Extracted from Charcoal Used for Promoting Growth of* Pertussis A *in Broth. "Albumen-cup" Method.*

Conc. of extract in nutrient agar (μg./ml.).	Diameter of growth plaques (in mm.) around cups containing the following quantities of albumen :		
	1 drop.	2 drops.	3 drops.
Nil (control) . .	10·5	13	14
40 . . .	(8)	11·5	12·5
200 . . .	(8)	(8)	11
600 . . .	(8)	(8)	(8)

For full explanation of method see text and footnote in Table III.

These results confirm conclusively that charcoal removes from broth cultures material that is highly toxic to *Pertussis A*.

G. *Viability of* Pertussis A *in plain broth.*

It seemed important at this stage to obtain some idea of the survival time of the organism in plain broth and on agar without the addition of albumen and charcoal.

Ten ml. of broth were inoculated with approximately 25,000 cells and incubated at 35°, samples being removed at intervals for viable counts (Table (VII (*a*)). It is clear that all organisms were dead within 20 hours. This was confirmed by a similar experiment with an inoculum of only 100 cells in a series of flasks ; instead of attempting viable counts, 0·5 ml. of 2 per cent albumen was added after intervals to each flask in order to determine at what stage growth failed to occur. Table VII (*b*) shows that some cells were still viable after 22 hours, but after 30 hours all were dead.

With heavy inocula on the surface of agar plates it can be shown, using the albumen cup technique already described (modified only in that the same quantity of albumen was added to each cup in succession at different intervals after inoculation) that even a large inoculum does not survive longer than 46 hours without albumen (Table VII (*c*)).

TABLE VII.—*Viability of* Pertussis A *in Plain Broth and on Nutrient Agar, without Albumen.*

(a) *Viable counts in broth.*

	Hours after inoculation.					
	0	2	4½	7	23	31
Viable cells/ml. . .	1820	1330	1270	780	0	0

Inoculum : Approx. 25,000 cells into 10 ml.

(b) *Effect of adding albumen to broth at different periods after inoculation.*

Time of adding albumen, hours after inoculation.	Subsequent growth after 7 days' incubation.
0	+
2	+
4	+
6	+
8	+
22	+ (delayed)
30	0

Inoculum : 100 cells into 4·5 ml. of broth.
Albumen added : 0·5 ml. of 1·5 per cent solution.

(c) *Effect on size of growth plaque on agar (in the albumen-cup technique) of adding albumen to the cups at varying intervals after inoculation.*

Time of adding 3 drops of 2 per cent albumen to cups hours after inoculation.	Diameter (in mm.) of growth plaque after 7 days' incubation.
0	22·5
6	18
22	16
30	13·5
46	0

It can therefore be concluded that cells of *Pertussis A* are killed fairly rapidly if incubated in broth or on agar without the addition of some suitable protective colloid.

DISCUSSION.

It has been shown that charcoal, albumen, probably starch and presumably blood owe their growth-promoting effect in broth, in part at least, to their power of absorbing a substance or substances demonstrably toxic to *Pertussis A*. The evidence so far suggests that these toxic substances are unsaturated fatty acids or nearly related compounds. The chemical nature of the toxic extract from charcoal is being further investigated, and it is hoped that the active fraction will be purified and identified. It is, however, far from certain (*a*) whether the whole of the growth-promoting power of charcoal or albumen can be ascribed to

this property of fatty acid absorption, and (*b*) whether all the toxic substances are originally present in the broth or are formed, to some extent at least, during the growth of the organism. The fact that mere treatment of broth with charcoal (followed by its removal) was not sufficient to promote growth suggests that a fraction of the toxic material may result from the metabolism of the cells themselves.

It is likely that these findings will prove to have a wider significance and apply to other genera of bacteria, especially to those that are known both to need the addition of serum to ordinary media for good growth, and to be susceptible to fatty acids. Both meningococci and gonococci are very sensitive to fatty acids (Walker, 1926), and the growth-promoting effect of charcoal discovered by Glass and Kennett (1939) is, in the light of the present work, almost certainly due largely to its property of combining with toxic fatty acids or similar substances in the culture, as with *Pertussis A*. That some such process is responsible is also suggested by the work of Gould, Kane, and Mueller (1943), who found that starch was necessary for the growth of gonococci on a solid synthetic medium, and concluded that the function of the starch was to neutralize the inhibitory effect of some batches of agar. Later, Ley and Mueller (1946) isolated from agar, by methanol extraction, a product similar to the higher fatty acids, which was inhibitory to gonococci and was neutralized by starch. In liquid synthetic media, however, starch was found to have no differential effect.

The extreme susceptibility of pneumococci to unsaturated fatty acids may well have some connection with the growth-promoting power of serum for this organism ; and the same principles may apply to streptococci, corynebacteria and other organisms. It has, in fact, been somewhat of a mystery why so many micro-organisms need, or thrive on, plasma or serum. The tacit assumption has usually been that such organisms require some growth factor present in plasma that is absent from broth. The alternative explanation—that broth may contain too much of something, rather than too little—has received small attention.

SUMMARY.

1. Some of the growth requirements of a derived strain of *H. pertussis* that would grow on 10 per cent blood agar, but not on plain tryptic meat agar, have been investigated.

2. The active fraction in blood was found to be albumen. Charcoal could also completely replace blood.

3. The growth-promoting effects of albumen and charcoal could be demonstrated through cellophane.

4. If incubated in broth without charcoal or albumen, the organisms died rapidly.

5. The inhibitory effect of higher unsaturated fatty acids upon *H. pertussis* was demonstrated, and the hypothesis put forward that the growth-promoting power of both albumen and charcoal is attributable, in part at least, to their ability to combine with toxic fatty acids (or similar compounds) present in the culture.

6. By methanol extraction of charcoal used for the growth of *H. pertussis* in broth, a material has been obtained which is highly toxic to the organism.

7. The implications and wider significance of these facts are discussed, with

particular reference to the cultivation of organisms already known to need serum for growth and to be susceptible to the toxic effects of fatty acids.

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