

# NON-SPORULATING ANAEROBIC BACTERIA OF THE INTESTINAL TRACT<sup>1</sup>

## II. GROWTH-FACILITATING FACTORS

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The statements of Castellani and Chalmers (1919) and Bergey (1934) that the *Bacteroides* show good growth on ordinary laboratory media have been quite misleading, for difficulty of cultivation has been among the most trying problems encountered in working with the non-sporulating anaerobic bacteria. Harris (1901-1905) and Beaver, Henthorne and Macy (1934) have especially emphasized this point, and several other workers have considered survival of pure cultures for more than a month or two as worthy of special comment.

There has been little agreement among investigators concerning the conditions best adapted for the growth of these organisms, and little information is available as to which materials actually contain nutrients available for their use. On a more or less empirical basis, complex infusions, body fluids and tissues have been used in a great variety of formulae. Further inconsistencies have arisen from the use of numerous kinds of peptones, and of reactions varying from distinctly acid to markedly alkaline. The usefulness of most of these media has been limited by their lack of clarity, heat lability and complexity. Often growth appears to have been delicate, slow to appear, and difficult to observe.

<sup>1</sup> This paper covers in part, the Dissertation presented to the Graduate School of Yale University by the senior author as a requirement for the degree of Doctor of Philosophy (1939), and the thesis submitted to the Graduate School of the University of Nebraska by the second author as a requirement for the degree of Master of Arts (1938).

This investigation was designed to provide definite information regarding the relationships of temperature, gaseous environment, hydrogen ion concentration, and nutrients to optimum growth of the non-sporulating anaerobes of intestinal origin. Special emphasis was placed upon the development of an improved and simplified culture medium.

#### PROCEDURE

*Selection of cultures.* Strains were selected from nearly 400 stock cultures previously isolated from intestinal contents of man and rats, and from those supplied by other workers.<sup>2</sup> Although the number of strains used in any one experiment varied from 5 to 162, an attempt was always made to choose representative organisms. Checks on purity were made periodically throughout the investigation.

*Preparation of media.* Since the constituents of the media were varied widely for experimental purposes, their nature will be indicated with the results. An aseptic tubing method similar to that described by Riker and Riker (1936) was employed when addition of heat-labile substances required that sterilization precede dispensing into tubes. Following incubation of media thus prepared, usually none and never more than one per cent of the tubes showed contamination. When it was necessary to avoid heating, sterilization was effected by passage through Berkefeld W or N filters.

*Inoculation and incubation.* Four-day-old egg-meat cultures were employed as the source of inoculum. Anaerobic conditions were obtained either by the method of Weiss and Spaulding (1937) or the use of deep cultures in semisolid (0.3 per cent) agar. Incubation at 37°C. was maintained for at least two weeks, unless otherwise specified.

*Estimation of growth.* Growth was judged by (1) the relative rapidity and abundance of multiplication, (2) the degree of viability on transfer through four successive subcultures of a given

<sup>2</sup> Cultures were generously supplied by Dr. A. H. Eggerth, Long Island College of Medicine, Brooklyn, N. Y. and by Dr. J. E. Weiss, Brooklyn College, Brooklyn, N. Y.

medium, (3) the smallest amount of inoculum necessary to initiate development in a given time interval, or (4) plate counts.

*Temperature.* Growth temperature relationships of 162 strains were determined by incubating semisolid tomato agar (Weiss and Rettger, 1934) stab cultures containing 10 per cent sterile bovine serum at five degree intervals covering the range from 5° to 55°C. Observations were made through a period of 28 days.

### RESULTS

#### *Growth temperature relationships*

In table 1 are listed for each temperature the number of strains growing, average days of incubation preceding visible growth and the average distance below the surface at which growth appeared.

TABLE 1

*Growth temperature relationships of 162 strains of non-sporulating anaerobic bacteria*

TEMPERATURE OF INCUBATION	NO. OF STRAINS GROWING	AVERAGE INCUBATION BEFORE GROWTH APPEARED	AVERAGE HEIGHT OF MEDIUM ABOVE GROWTH
°C.		days	mm.
5	0		
10	4	14.3	24
15	143	6.0	13
20	148	3.5	12
25	159	3.5	11
30	162	1.2	5
35	162	1.0	6
40	162	1.0	6
45	88	1.3	6
50	6	3.5	12
55	0		

The optimum growth temperature was between 35° and 40°C., as judged by the average daily growth of all strains at both of these temperatures.

The minimum growth temperature for the majority of strains was between 15° and 20°C., although the four gram-negative strains previously described as type B-1 developed slowly at 10°C., while the gram-positive strains belonging to type A-1 were unable to multiply below 25° to 30°C.

The maximum growth temperature was less easily determined, because it was often relatively close to the optimum temperature. Undoubtedly it fell slightly above or below 45°C. for most strains, since all of the 162 grew visibly at 40° in one day (average), 88 developed at 45° in 1.3 days, and only six showed growth at 50°C. after 3.5 days.

It was not clearly evident from these data whether the marked differences in nearness of growth to the surface of the medium represented alterations in oxygen tolerance with change in temperature, or were due to variations in the depths to which oxygen diffused into the medium during different periods of incubation.

#### *Gaseous environment*

Although atmospheric oxygen has been known to inhibit growth of these non-sporulating anaerobes completely, little information was available concerning the effect of the carbon dioxide and hydrogen used in the anaerobic jars.

A definite stimulating effect of carbon dioxide on the development of bacteria was recorded by Valley and Rettger (1927) and by others, but the earlier studies did not include non-sporulating anaerobes. Eggerth and Gagnon (1933) noted that some of their *Bacteroides* strains were favored by the addition of carbon dioxide, but presented no experimental evidence to support this observation.

To determine the influence of carbon dioxide on the growth of five strains, triplicate plate cultures, using glucose-cysteine agar (formula in section on nutrient requirements) or glucose-beef infusion agar plus 10 per cent potato extract, were prepared from quantitative serial dilutions of egg-meat cultures. One jar was freed from carbon dioxide by evacuating and flushing with hydrogen three times, then filling the jar with hydrogen. The second jar was evacuated and then filled with a mixture of 10 per cent carbon dioxide and 90 per cent hydrogen, as calculated from manometric readings. The third jar was freed from all gases except carbon dioxide by evacuating and filling with this gas three times; finally carbon dioxide was introduced into the evacuated jar to 10 per cent capacity.

Palladinized asbestos was placed in the first two jars to remove remaining traces of oxygen; pyrogallie acid and sodium hydroxide were used in the third jar for the same purpose.

After four days of incubation at 37°C. the colonies were counted. In table 2 are recorded the estimated numbers of organisms per milliliter of inoculum. When carbon dioxide was excluded, four strains failed to grow, and the fifth showed only very slight colony development. In contrast to this, counts ranging from about sixteen million to nearly three billion organisms were found for all strains when plates were incubated in jars containing a mixture of 10 per cent carbon dioxide and 90 per cent hydrogen. The somewhat lower counts obtained when 10

TABLE 2

*Influence of carbon dioxide on growth of five strains of non-sporulating anaerobic bacteria*

STRAIN NUMBER	ORGANISMS PER ML. OF INOCULUM AS INDICATED BY PLATE COUNTS					
	CO <sub>2</sub> 0	H <sub>2</sub> 100%	CO <sub>2</sub> 10%	H <sub>2</sub> 0*	CO <sub>2</sub> 10%	H <sub>2</sub> 90%
4		0	188,000,000		2,960,000,000	
30		0	412,000,000		529,000,000	
31		0	111,000,000		2,300,000,000	
186		0	51,200,000		51,200,000	
196		40,000	12,800,000		16,300,000	

\* Carbon dioxide to 10 per cent capacity of jar.

per cent carbon dioxide was used alone probably were due to less complete anaerobiosis secured by the use of pyrogallie acid and sodium hydroxide, or absorption of the gas by the alkali alone.

When plate cultures which had been kept in a carbon-dioxide-free atmosphere were incubated in the presence of 10 per cent carbon dioxide and 90 per cent hydrogen, normal colony development took place, thus showing that the absence of carbon dioxide was inhibitory rather than lethal in its effect.

Four repetitions of this experiment yielded similar results and adequately demonstrated that, although hydrogen had little effect on the development of the non-sporulating anaerobic bacteria, they did require carbon dioxide for initiation of growth.

*Hydrogen ion concentration*

Opinions of previous workers have varied widely regarding the optimum hydrogen ion concentration for growth of the non-sporulating anaerobic bacteria. Eggerth and Gagnon (1933) and Weiss and Rettger (1937) claimed that moderate alkalinity (pH 7.6 to 7.8) was most favorable for initiation of growth, but the latter workers also noted that slight acidity improved the growth of pure cultures of gram-negative species.

To determine the effect of different hydrogen ion concentrations on viability, 16 strains, including three obtained from the above-mentioned authors, were inoculated into a series of glucose-cysteine-semisolid agar (formula in section on nutrient require-

TABLE 3

*Influence of hydrogen ion concentration on growth and survival of 16 strains of non-sporulating anaerobic bacteria*

pH	NUMBER OF STRAINS SURVIVING 4 TRANSFERS	AVERAGE DAYS OF INCUBATION PRECEDING GROWTH	PER CENT RECOVERY AFTER 30 DAYS
3.7	0		
5.3	4	4	40
6.0	15	3	67
7.0	16	1	81
7.8	11	3	82
8.4	9	3	100

ments) stab tubes which had been adjusted to pH 3.7, 5.3, 6.0, 7.0, 7.8 and 8.4. As soon as growth appeared in any culture tube a transfer was made to another agar tube having the same hydrogen ion concentration. This procedure was repeated until the strains either failed to grow or had been carried successfully through four serial transfers. After incubating the fourth transfers for one month, each tube was inoculated into a medium adjusted to pH 7.0, to determine the relative degree of survival on prolonged incubation in acid and in alkaline media.

Table 3 shows that, on the basis of the number of strains surviving the fourth transfer and the average period of incubation required for development, the optimum hydrogen ion concentra-

tion was between pH 6.0 and 7.0, with a slight balance of evidence in favor of the latter reaction.

The failure of all strains to multiply at pH 3.7, and the survival of only four strains at pH 5.3 indicated that the maximum acidity which permitted growth of the majority of strains lay near pH 5.0.

No medium sufficiently alkaline to prevent growth of all strains was employed; but the survival of only nine strains at pH 8.4, and the three-day incubation period required for their development, suggested that this reaction approached the maximum basicity permitting growth.

When the percentage of strains remaining viable after 30 days was used as the criterion of optimum hydrogen ion concentration, somewhat different conclusions were reached, for the proportion of viable strains steadily increased from 40 per cent at pH 5.3 to 100 per cent at pH 8.4, thus indicating that alkaline media favored continued viability to a greater degree than did neutral or acid media.

A second experiment, employing hydrogen ion concentrations of pH 6.1, 6.3, 6.8, 7.2 and 7.4, confirmed the above observations regarding the difference between optimum pH values for initiation of growth and for survival. It also indicated that near-optimum growth could be obtained over a range of pH 6.3 to 6.8.

In short, these data showed that neutral or slightly acid (pH 6.3 to 7.0) media were most favorable for initiation of growth, but that moderately alkaline (pH 7.2 to 8.4) media sustained viability over a longer period.

Perhaps the apparent differences between these findings and those of Eggerth and Gagnon (1933) and Weiss and Rettger (1937) may be due to the relatively long incubation periods (4 to 10 days) which preceded their observations.

#### *Nutrient requirements*

A complete review of the many experiments required to devise a suitable culture medium is inadvisable here. The information presented is a summary of the nature and scope of the investigation, supported by selected data to show points of major interest.

A survey of the nutrient qualities of a variety of previously used media, as well as individual constituents, was made to determine which substances, either alone or in various combinations, were actually responsible for growth. The materials used may be grouped as follows:

<b>Mineral salts</b>	<b>Carbohydrates and alcohol</b>	<b>Infusion</b>
Sodium chloride	Glucose	Beef
Di-sodium phosphate	Lactose	Liver
Salt mixture (Allison and Hoover, 1934)	Sucrose	Heart
	Mannitol	Veal
<b>Extracts</b>	<b>Protein-like substances</b>	
Beef	Gelatin	
Yeast	Sodium caseinate	
Savita	Egg albumin	
Potato	Serum	
Fecal	Peptonized milk	
Tomato	Peptone	

The substances which individually yielded the most promising results when tested in peptone agar were yeast extract, liver infusion, glucose and peptonized milk. Others, especially tomato juice, serum and veal infusion, were of some value when added to more complex mixtures, such as North gelatin agar (North, 1909; modified by Spray, 1929) or tomato agar (Kulp 1927; modified by Weiss and Rettger, 1934). Variation in nutrient requirements of individual strains indicated that more than one factor was responsible for growth, and that some of these substances were present in a number of the materials commonly employed in bacteriological media.

From these observations the possibility of modifying plain nutrient agar to support the development of the non-sporulating anaerobes was suggested. Table 4 shows the progress of this work, beginning with repeated failure to grow the organisms in ordinary nutrient agar containing 0.3 per cent Bacto beef extract and 0.5 per cent Bacto peptone (Medium A).

A concentrated nutrient agar (Medium B) containing five times the usual amount of beef extract and ten times that of ordinary peptone supported initial development of 10 strains in an average



period of three days, but only four strains survived in serial transfers.

A modified nutrient agar containing one per cent each of beef extract, peptone and tryptone (Medium C) was, in itself, no better than ordinary nutrient agar, for all strains failed to grow. However, the success of Spray (1936) in using tryptone for the cultivation of spore-forming anaerobes made it seem advisable to continue to employ this formula as a basal medium for the investigation of other materials.

TABLE 4

*Influence of various nutrient media on the growth of 11 strains of non-sporulating anaerobic bacteria*

MEDIA	NUMBER OF STRAINS SHOWING GROWTH	AVERAGE INCUBATION BEFORE GROWTH APPEARED
		<i>days</i>
Nutrient agar (A).....	0	
Concentrated nutrient agar (B).....	10	3
Nutrient tryptone agar (C).....	0	
Filtered glucose + (C).....	7	7
Autoclaved glucose + (C).....	11	3
Cysteine* + (C).....	11	2
Cysteine* and glucose + (C).....	11	1

(A) Bacto beef extract, 0.3 per cent; Bacto peptone, 0.5 per cent.

(B) Bacto beef extract, 1.5 per cent; Bacto peptone, 5.0 per cent.

(C) Bacto beef extract, 1.0 per cent; Bacto peptone, 1.0 per cent; Bacto tryptone, 1.0 per cent.

\* As hydrochloride.

Glucose, when sterilized by filtration and then added to medium C in one per cent concentration, allowed seven strains to grow sparsely in seven days (average). Heat sterilization of the glucose in the medium brought about some change which further improved its growth-promoting properties to the extent that the entire group of 11 strains developed in three days. Whether this involved decomposition of the glucose to more readily available compounds, or a combination of the glucose with other substances in the medium, was not determined.

The use of cysteine hydrochloride was suggested by the work

of Valley (1929), Spaulding and Rettger (1937) and others who had successfully employed it for growing various anaerobic bacteria. When 0.05 per cent cysteine hydrochloride was added to medium C it encouraged all strains to develop in approximately two days.

Addition of both glucose and cysteine hydrochloride stimulated development still further, all strains showing visible growth in about one day.

Further studies on favorable concentrations of the various constituents employed in medium C plus glucose and cysteine showed that the original amounts were at or near optimum, but that some variation in proportions of the constituents, especially glucose, could be made without markedly affecting growth. Comparison of Bacto peptone, Fairchild peptone, neopeptone, proteose peptone, Parke Davis peptone and tryptone revealed that the tryptone supported more rapid and abundant growth than the other peptones employed.

On the basis of these results a medium designated as the *glucose-cysteine medium* was prepared as follows:

Tryptone (Bacto).....	20	gm.
Beef extract (Bacto).....	10	gm.
Glucose.....	5-10	gm.
Cysteine hydrochloride.....	0.5	gm.
Di-sodium phosphate (hydrate).....	4	gm.
Distilled water.....	1,000	ml.

Dissolve all ingredients and adjust to pH 7.4. For solid medium add agar. Sterilize in the autoclave at 120°C. for 20 minutes.

Adjustment to pH 7.4 was necessary to give the desired final hydrogen ion concentration, namely pH 6.3 to 7.0, after heating. Addition of di-sodium phosphate provided a buffer against the acidity resulting from the fermentation of glucose.

Successful cultivation of more than one hundred of our own isolations, as well as related organisms of the *Bacteroides*, *Lactobacillus* and *Bacterium* types obtained from other workers, supplied adequate evidence that the glucose-cysteine medium was suitable for growing pure cultures of the non-sporulating anaerobic bacteria of intestinal origin.

Since all strains used had been maintained on laboratory media for more than a year, a further check on the value of this formula was made by comparing the ease with which these anaerobes could be isolated from feces when plated on beef infusion-glucose-blood agar (Eggerth and Gagnon, 1933) and on the adopted glucose-cysteine agar. Fifty-eight per cent of 100-odd colonies originating from three human fecal samples proved to be non-sporulating anaerobes regardless of which medium was employed, thus indicating that the two formulae were of equal value for isolation purposes. When the glucose-cysteine medium was enriched with liver infusion, yeast extract and tomato juice,<sup>3</sup> the proportion of non-sporulating anaerobes to the total recovered rose to 70 per cent.

In short, the glucose-cysteine formula may be recommended as an improved and simplified medium for the cultivation of the non-sporulating anaerobic bacteria of intestinal origin; it supports growth adequately, is comparatively simple in composition, easily prepared, adaptable to a variety of laboratory manipulations, stable at temperatures of the autoclave, and is practically free from turbidity. Furthermore, it is composed of readily available and fairly inexpensive materials.

#### SUMMARY

Growth of the non-sporulating anaerobic bacteria of intestinal origin is favored by:

1. An incubation temperature of 35 to 40°C.
2. The presence of 10 per cent carbon dioxide.
3. A hydrogen ion concentration of pH 6.3 to 7.0.
4. The improved and simplified glucose-cysteine agar medium described in this paper.

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<sup>3</sup> Liver infusion from 75 grams Difco dehydrated liver, 500 cc. per liter of medium. Juice from canned tomatoes, 200 cc. per liter of medium. Yeast extract (Difco desiccated), 10 grams per liter of medium.

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