CONTRIBUTIONS TO THE BIOCHEMISTRY OF PATHOGENIC ANAEROBES.¹

VIII.

THE BIOCHEMICAL COMPARISON OF MICRO-ORGANISMS BY QUANTITATIVE METHODS.

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*Report to the Medical Research Committee from the Institute for the Study
of Animal Nutrition, School of Agriculture, Cambridge University.*

IN the following paper (which is in series with previous communications by the author and Captain C. G. L. Wolf) a comparison is made between two organisms which, judged by ordinary considerations of morphology and cultural reactions, are obviously closely related. The experimental work on which the comparison is based may be stated as follows :—

- (A) Investigation of the proteolytic and sugar-splitting properties of the organisms in a series of parallel fermentations on different media. For these experiments the methods described by Wolf and Harris (1917¹⁶) were used, with certain modifications.
- (B) Determination of the oxygen concentrations which permit or inhibit growth of these organisms. For this purpose a method has been developed which, it is hoped, will prove useful for routine investigations of this nature.

The comparison is made strictly from quantitative data. A study of the literature of bacteriology reveals the fact that only a comparatively small part of the information accumulated is of a quantitative nature. The majority of bacteriologists have been content to rely on cultural tests for purposes of comparison and differentiation. These cultural tests are qualitative, and since they

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are dependent on "naked-eye" changes, are limited in their usefulness. They undoubtedly have served for broad classifications, but when fine differences are to be detected they are of less value. This has been recognised for many years, and attempts have been made to improve matters. These attempts have taken the form of a search for quantitative data. Organisms which are of technical importance have naturally attracted a good deal of attention, and much of the pioneer work was done on the quantitative biochemical properties of yeasts. A comprehensive account of this work is given by Harden (1911⁷), who, with his collaborators, is responsible for much accurate quantitative information concerning the activities of yeasts.

Amongst the organisms associated with pathological conditions *B. coli* and members of the *B. typhosus* group claimed early attention. In 1901 Harden (1901⁷) published the results of a most convincing investigation of the action of *B. coli* and *B. typhosus* on carbohydrates and allied compounds. Using accurate methods of analysis he investigated the consumption of sugar, the formation of alcohol, and the production and nature of fixed and volatile acids. These experiments yielded important information with regard to the biochemical properties of *B. coli* and *B. typhosus*. Many other workers entered the field and a great deal of useful work was performed. Methods of analysis were improved, and increased attention was paid to the formation of gas as a measure of metabolism. The work of Pakes and Jollyman (1901¹³), and of Keyes and Gillespie (1912⁹), may be cited in this connection.

However, the application of quantitative methods was not widespread, and qualitative methods held the field with the ordinary worker, so that in 1912 we find the following words used by Kendall and Farmer (1912⁸):—

"In the past but little effort has been made to study bacterial metabolism quantitatively, at least from a comparative standpoint, yet it is largely from the comparative study of different types of bacterial metabolism that the fundamental principles can be elucidated. This lack of diligence cannot be explained wholly by the inadequacy of the older methods; it is rather attributable to rigid adherence to the narrow idea of morphology and differentiation of bacteria instead of the broader dynamical consideration of bacterial metabolism."

These remarks are quoted from one of the first of a series of papers on "Bacterial Metabolism" by Kendall (1912⁸) and his collaborators. During a period of five years these workers collected a vast amount of information regarding the proteolytic and sugar-fermenting properties of many widely different organisms. They determined the amount of ammonia produced as a measure of proteolysis. Acid formation was gauged by the method of titration. In an attempt to supplement the inadequacy of titration methods various indicators, sensitive to different concentrations of hydrogen-ion,

Table I.—SUMMARY OF RESULTS WITH

Experiment Number		1		2	
Medium fermented		PEPTONE WATER.		PEPTONE WATER.	
Organism		<i>Bacillus sporogenes.</i>	Reading Bacillus.	<i>Bacillus sporogenes.</i>	Reading Bacillus.
Initial Composition of Medium.	TOTAL NITROGEN Mgrm. per 100 c.c.	353	353	..	..
	AMINO-ACID NITROGEN " " "	78	78	..	..
	AMMONIA-NITROGEN " " "	18	18	..	..
	SUGAR CONTENT Per cent.	..	..	..	..
	REACTION PH (approx.)	7.0	7.0	..	..
Laboratory number of culture used as seed . . .		D 108	R 30	D 110	R 32
Age of seed when used (days, on milk)		21	21	23	23
Number of hours' fermentation		117	117	144	144
Final Composition and Fermentation Products.	AMINO-ACID NITROGEN { Final content Mgrm. per 100 c.c. Amount formed " " "	73 -5	71 -7		
	AMMONIA-NITROGEN { Final content " " " Amount formed " " "	107 89	104 86		
	SUGAR { Final content Per cent. Amount used Mgrm. per 100 c.c.				
	FINAL REACTION PH (approx.)	6.7	6.7	..	..
	VOLATILE ACIDS C.c. N/10 solution per 100 c.c.	50.6	48.5	..	..
	GAS FORMED C.c. per 100 c.c.	46	62	48	53
	PERCENTAGE OF CO ₂ IN GAS Per cent.	65	53	64	58

B. SPOROGENES AND THE READING BACILLUS.

3		4		5		6		7	
GLUCOSE PEPTONE.		MILK.		ALKALINE EGG.		COOKED MEAT.		COOKED MEAT.	
<i>Bacillus sporogenes.</i>	Reading Bacillus.	<i>Bacillus sporogenes.</i>	Reading Bacillus.	<i>Bacillus sporogenes.</i>	Reading Bacillus.	<i>Bacillus sporogenes.</i>	Reading Bacillus.	<i>Bacillus sporogenes.</i>	Reading Bacillus.
305	305	510	510	294	294	874	874	690	812
58	58	5	5	10	10	22	22	17	20
16	16	5	5	2	2	13	13	10	12
1·71	1·71	4·04	4·04	..	..	..	..	..	..
6·7	6·7	..	..	8·0	8·0	..	..	..	..
D 99	R 25	D 95	R 17	D 96	R 18	D 10	R 28	D 109	R 31
35	35	29	29	70	70	14	14	18	18
96	96	168	168	286	286	142	142	118	118
90	90	133	129	85	86	253	238	200	230
32	32	128	124	75	76	231	216	183	210
38	40	131	129	121	109	405	379	250	317
22	24	126	124	119	107	392	366	240	305
1·30	1·38	4·15?	4·26?	..	..	..	..	..	..
410	330	?	?	..	..	..	..	..	..
..	..	Acid	Acid	6·9	6·9	7·2	7·2	..	..
37	37	76	68	55·2	50·2	Lost	173	113	140
102 7	95	85	82	96	90	180	184	146	154
54·3	58·4	77	75	60·5	60·5	86	86	75	79

were used. Precautions were taken to obtain as far as possible similar conditions in order that the results might be strictly comparative. In addition to yielding much useful information regarding the properties of the individual organisms examined, this work showed how bacterial metabolism resembles animal metabolism, in that carbohydrate spares protein, or, in other words, "fermentation precedes putrefaction."

Improvements in the methods of measuring hydrogen-ion concentration led many investigators to use this factor for diagnostic purposes. Thus, recently, Clark and Lubs (1917²) published their "methyl-red" test, for differentiating the two main *B. coli* species, and correlated their results with those obtained from considerations of the gas ratio accurately determined. Besides establishing a beautiful method based on the most accurate quantitative considerations, these authors showed the futility of attempting to measure acid production by mere titration, as had been done so often previously.

In 1917, Wolf and Harris (1917¹⁶) described experimental methods by which the biochemical activities of anaerobic organisms could be investigated quantitatively. Using modern analytical methods they followed the changes which take place in various media when these are acted upon by different pathogenic anaerobes. Extensive use of the methods described by these authors have been made in the comparison experiments which are described later.

Turning to the question of bacterial growth in the presence of oxygen, one finds that a good deal of work has been done in determining the oxygen maxima and minima for various organisms. Some of the more prominent names connected with this class of work are Meyer (1905¹¹), Moore and Williams (1909¹², 1910¹²), and Adams (1912¹). Most of the methods employed, however, involve the use of large and complicated apparatus and are not suitable for routine work. I have not been able to find in the literature any method of determining the degree of oxygen tolerance of an organism which could be conveniently used as a routine test. It is therefore hoped that the method described later will meet a real need.

In the following experiments the two organisms compared are *B. sporogenes* (Metchnikoff) and the Reading bacillus.

B. sporogenes needs little description. Much work has been done on it recently, particularly during the war, and most bacteriologists are familiar with its properties. The particular strain used in these experiments was that employed by Wolf and Harris (1917¹⁶), and was originally obtained from Captain Henry, R.A.M.C. It had been extensively subcultured for laboratory purposes and appeared remarkably constant in its reactions.

The Reading bacillus was isolated at Reading in 1917 by Donaldson and Joyce (1917⁴) from a foul-smelling wound. These authors based a new method of wound treatment on its properties. The Reading bacillus possesses a remarkable similarity to *B. sporogenes* from the morphological and cultural point of view. This similarity has been further investigated by Donaldson (1918³, 1919³). He has shown that the resemblance extends to a similarity of action on tetanus toxin, on which the organisms have an effect not shown

by other closely related bacteria. From Donaldson's experiments it appears that what differences he has been able to detect between the two bacteria are merely of degree. The strain used for the present experiments was kindly supplied by Dr. Donaldson.

A. FERMENTATION EXPERIMENTS.

Fermentations were carried out with each of these organisms on five different media—peptone water, glucose peptone, milk, alkaline egg, and cooked meat. These media are described by Wolf and Harris (1917¹⁶) in the first paper of this series.

In order that results should be strictly comparable each organism was made to ferment the same medium under exactly similar conditions. As a preliminary measure steps were taken to obtain seed cultures of each organism that were, as nearly as possible, in the same state.

The general method of conducting the fermentations was as follows. Three flasks were sterilised, each containing the same amount of the medium under consideration.

On cooling, one flask was put aside for analysis; the other two were inoculated at the same time with equal amounts of similar cultures of *B. sporogenes* and the Reading bacillus respectively. The flasks were then arranged for anaerobic fermentation and incubated side by side.

Gas production was followed by the fall of mercury in mercury manometers. The period of incubation varied according to the medium employed, but was never less than four days. At the end of the incubation period, samples were taken of the fermentation gases, the flasks opened and the fermented liquids analysed.

For each fermentation determinations of the following were made:—

1. Volume and composition of gas produced.
2. Formation of ammonia.
3. Formation of amino-acid nitrogen.
4. Production of volatile acids, and a rough determination as to their nature.
5. Change in hydrogen-ion concentration.
6. Change in sugar content.

The amounts of medium used for each fermentation were considerably less than those taken by Wolf and Harris. The size of the fermenting vessels was so chosen that there was just sufficient liquid available for the above determinations. Since no samples were required during the incubation period the apparatus was somewhat simpler than that employed by the above workers. Details of the apparatus and experimental methods are given below. For convenience of comparison, the experimental results of the two organisms for each medium are given side by side, and summarised in Table I.

· APPARATUS (see Fig. 1).—Three Kjeldahl flasks, of 500 c.c. capacity, were taken and into them were placed equal quantities of the same medium (250–300 c.c.). The flasks were well plugged with cotton wool and suitably sterilised. When cool, one flask was placed aside for analysis, whilst the other two were used for fermentations with *B. sporogenes* and the Reading bacillus.

Inoculation was carried out by removing the plug from the neck of the flask and introducing 1 c.c. of the seed culture by means of a sterile Pasteur pipette. The plug was then replaced, and pushed well down the neck of the

FIG. 1.

flask to make room for a well-fitting rubber stopper, provided with a glass delivery tube which just passed through it. The cork was made to fit tightly by painting with hot paraffin wax. Each flask was then placed in a water thermostat, regulated at 37° C. The delivery tube was connected through a mercury manometer (M) to an exhaust pump, and the air withdrawn till the medium boiled gently under the reduced pressure. The connection (P) from the pump was then closed by means of a screw clip and the flask left in connection with the manometer.

For the comparative experiments the two flasks were inoculated and incubated as nearly as possible at the same time. The time occupied for both was about fifteen minutes.

MEASUREMENTS OF PRODUCTS OF FERMENTATION.—The initial composition of the medium was determined from the liquid in the third flask set aside for the purpose.

1. *Gas production*.—The space not occupied by the liquid in each flask

was measured. The fall of mercury in the manometer served as a basis for the calculation of the amount of gas produced, corrections being introduced for the vapour pressure of the liquid, the temperature of the bath and the barometric height.¹

After incubation, readings of the gas production were taken at intervals, and from them curves were plotted showing the rate of gas formation. A few minutes before the level of the mercury was read, each flask was well shaken, in order to liberate the fermentation gases with which the medium tends to become super-saturated. When gas production had ceased, or fallen so as to be almost negligible, samples of the fermentation gas were removed at P, and analysed. The content in carbon dioxide alone was determined. The residual gas was found to be mainly hydrogen, but was not further examined.

2. *Products of proteolysis*.—The ammonia content was determined by Shaffer's (1903¹⁴) modification of Boussingault's method. An aliquot portion (25 c.c.) of the liquid was placed in a 750 c.c. distilling flask, made alkaline with a few c.c. of saturated sodium carbonate solution, and about 50 c.c. of rectified spirit added. The mixture was then distilled under reduced pressure on the water-bath at a temperature not exceeding 50° C. The distillate, containing the ammonia, was collected in a measured quantity of N/10 sulphuric acid. By titration of the excess of acid the amount of ammonia was determined.

The residue in the distillation flask was acidified with acetic acid, made to a definite volume (50 c.c.), and the amino-acid nitrogen determined by means of a Van Slyke (1913¹⁵) amino-nitrogen apparatus.

Results are expressed as milligrammes of ammonia-nitrogen and amino-acid nitrogen per 100 c.c. of media.

3. *Volatile acids*.—In order to determine the amount of volatile acids in the medium, an aliquot portion of the fermented liquid (usually 100 c.c.) was made acid to Congo-red with sulphuric acid and steam distilled. The distillation was carried out at a uniform rate for each experiment, and the same amount of distillate collected, 500 c.c. Except for acids of low volatility such as formic acid, this method would yield from 90 per cent. to 95 per cent. of the total acid present. The distillate was titrated with N/10 alkali, using phenol-phthalein as an indicator. Results are expressed as c.c. of N/10 acid solution per 100 c.c. of medium.

A rough comparison of the composition of the volatile acids was made as follows. The neutralised distillate from each fermentation was concentrated to a small bulk, acidified with sulphuric acid and distilled in steam by Dyer's (1917⁶) modification of Duclaux's method. The logarithmic distillation curve was then plotted. From a comparison of the shape of these curves the similarity in composition of the acid mixtures could be judged.

Portions of the concentrated acid distillates were also submitted to Dyer's qualitative colour tests, by which the presence of certain individual members of the volatile acid series could be detected.

4. *Reaction*.—The hydrogen-ion content was determined colorimetrically, using a set of phosphate standards containing phenol-sulphone-phthalein. Unfortunately many of the final reactions were outside the range of this indicator, but no other means were available where these experiments were performed.

¹ For most organisms, using a 500 c.c. flask and 250 c.c. of medium, there is usually sufficient free space in the flask to contain the evolved gases at atmospheric pressure. Should the mercury fall entirely, and gas still be formed, it may be collected over water, or mercury, in a cylinder. It is simpler, however, to arrange the size of the flask and the volume of medium in such a manner that the whole of the gas may remain in the fermentation vessel. As it is not desirable to decrease the volume of the medium below 250 c.c. (since this would not allow enough medium for the subsequent analysis) a larger flask is the best way out of the difficulty.

5. *Sugar content.*—When the medium contained sugar the changes in concentration due to fermentation were determined by a gravimetric modification of Fehling's method.

B. METHOD OF DETERMINING THE DEGREE OF OXYGEN TOLERANCE (FOR GROWTH) OF AN ORGANISM, AND ITS EXPRESSION AS THE "AEROBIC INDEX."

The method aims at determining approximately the limits of oxygen concentration in which an organism will grow.

Briefly, the method consists in inoculating a number of tubes of medium with the organism in question, removing the air from the tubes by means of a pump, and replacing it by oxygen at definite pressures, which can be easily measured and controlled.

The limits within which growth takes place are expressed as the "aerobic index."

APPARATUS REQUIRED (see Fig. 2).

MEDIUM.—Solid or liquid media may be used. The medium should be freshly prepared or else boiled just before using to remove dissolved oxygen.

FIG. 2.

Agar slopes, unless freshly prepared, should be remelted, kept in a water-bath at 100° C. for some time, sloped and then used as soon as they are set. As far as possible the medium should be free from sugars or other reducing bodies, and should be adjusted to a definite hydrogen-ion concentration, preferably $P_H = 7.2$.

CULTURE TUBES.—These should be of the ordinary size used for bacteriological experiments, *i.e.*, 6 inches by $\frac{5}{8}$ inch.

RUBBER STOPPERS.—The stoppers should fit the culture tubes tightly, and be provided with a single hole through which passes a small tube drawn out just above the entrance to the cork into a stout capillary (E).

PARAFFIN WAX, with which the stoppers may be painted to render them gas-tight.

AN EXHAUST PUMP (P), connected to a mercury manometer (M), arranged as in Fig. 2. An ordinary filter pump capable of maintaining a vacuum of about 20 mm. is all that is required.

OXYGEN GAS.—This is contained in a reservoir (R), which may be filled from a cylinder or any suitable generator. The oxygen should be as pure as possible, but a small percentage of nitrogen, such as is often present in the oxygen contained in cylinders will not affect appreciably the accuracy of the results.

STERILE PIPETTES OR PLATINUM WIRES FOR INOCULATION.

A number of tubes each containing 5 c.c. of freshly prepared medium are inoculated with the organism to be tested. (The number of tubes depends upon the degree of accuracy required. A suitable number is sixteen, as will be seen later.) Either two drops of a liquid culture may be introduced into each tube by means of a sterile pipette or small portions from an agar slope may be added. The first method is probably the simpler as the whole of the tubes can then be inoculated with one charge in the pipette. When the tubes have been inoculated, the cotton-wool plugs are pushed far enough into the tubes to permit the introduction of the rubber stoppers and delivery tubes. Hot paraffin wax is applied to the joints to render them gas-tight.

The tubes are now numbered 0, 1, 2, etc., up to 15, and are treated as follows:—

Tube 0.—This tube is to contain no oxygen at all. It is connected through the manometer (M) to the pump (P) by means of the rubber connection (K), and is evacuated.¹ Taps A and B are open; tap C, closed.

When the mercury in the manometer becomes stationary, tap A is closed. The reading is taken and noted for future reference. The height of the mercury registers the difference between the barometric height at the time of the experiment and the vapour pressure of the liquid medium. By means of a point flame (D) the tube is now sealed off at the constriction (E).

Tube 1.—This tube is similarly connected to the pump by means of K, and evacuated as with tube 0. Stopcock A is now closed, C carefully opened and oxygen introduced slowly till the mercury in the manometer falls 50 mm. below the level registered for tube 0. The tube is then sealed at the constriction (E). The partial pressure of oxygen in this tube is 50 mm.

Tube 2 is similarly treated, except that oxygen is introduced till the mercury falls 100 mm. below the level registered for tube 0. The tube is

¹ With liquid media the complete removal of oxygen is facilitated by the liquid boiling under reduced pressure and sweeping out with its vapours the last traces of air.

When solid media, such as agar slopes are employed, it is extremely difficult to remove all air even with the most efficient pump. It is therefore probably better to test for growth on solid media in the complete absence of oxygen by another method. One of the devices employed by McIntosh and Fildes (1916¹⁰) and involving the use of hydrogen and palladium is suggested.

sealed at the constriction E. The partial pressure of oxygen in this tube is 100 mm.

Proceeding in this way for the remaining tubes, and allowing a difference of 50 mm. of oxygen partial pressure in each, the following series is obtained :—

Tube	3.—	Partial pressure of oxygen = 150 mm.			
Tube	4.—	"	"	"	= 200 "
Tube	5.—	"	"	"	= 250 "
Tube	15.—	"	"	"	= 750 "

Tube 15 contains, approximately, oxygen at atmospheric pressure.

Sixteen different degrees of oxygen saturation are thus obtained, each degree differing by an equal amount, corresponding to 50 mm. of mercury.

The methods of preparing tubes 1 to 15 apply equally to liquid and solid media. (See note concerning Tube 0, on p. 39). The partial pressure of oxygen left in the mixture of water vapour and air which the pump fails to remove from above solid media will not affect appreciably the accuracy of the results for these tubes.

When all the tubes have been treated they should be gently shaken (in the case of liquids) so as to saturate the medium with oxygen at that pressure. If 5 c.c. only of medium is used, the depth of liquid is sufficiently small to allow thorough shaking and good exposure of the organism to the oxygen above.

The tubes are incubated and examined after a suitable period, according to the nature of the organism. Growth in the liquid media may usually be detected by turbidity.

For each organism a certain number of tubes will show development. With a strict anaerobe only tube 0 will show growth. Aerobic organisms may develop in tubes 3 to 15.

(Tube 3 represents approximately the oxygen concentration to which an organism is subjected when grown in air.)

Aerobic index.—In order to express the limits of oxygen pressure within which growth takes place for a particular organism it is suggested that they be given in the form of what may be called the "aerobic index." The aerobic index is composed of the partial pressures of oxygen in the extreme tubes showing growth, that of the tube containing the higher pressure of oxygen being written over that containing the lower pressure in the form of a fraction. Thus, if growth takes place in all the tubes, the aerobic index is 750/0. If growth takes place only in tubes 4, 5, 6, 7, and 8, the aerobic index is 400/200. For a strict anaerobe the aerobic index would be 0/0.

In order to show the degree of accuracy to which the determinations have been made, it is suggested that the value of the intervals between each tube should be written after the index. Thus in the present instance, where each tube differed in oxygen pressure by 50 mm. of mercury, the above indices would be written: 750/0 (50 mm.); 400/200 (50 mm.); and 0/0 (50 mm.). Thus any worker may choose his own intervals and produce results which may be strictly comparable with those of other investigators.

Experimental.

In order to prepare the seed for the comparison experiments two sets of

parallel cultures were made in which both organisms were subjected to the same conditions in long series of transfers.

Two tubes of freshly sterilised milk were taken. One was inoculated with *B. sporogenes*, and the other with the Reading bacillus. A layer of liquid paraffin was added to each. The two tubes were incubated side by side for seven days, and were then transferred to a second pair of milk tubes. After these had undergone seven days' incubation, two more transfers were made from them. This subculturing was continued in all six or seven times. The resulting cultures were ready for use as seed.

All subsequent seed cultures were prepared from them, transfers always being made at the same time and incubated for the same period.

For each pair of comparison experiments corresponding cultures were used as seed. These cultures were usually taken for seed purposes when 3 to 4 weeks old.

In the above method, a long incubation between the transfers was used in the preparatory stage, so that the organisms might have passed their period of rapid growth and be in the resting condition. One would expect any differences due to inequalities of seeding, etc., to be levelled out and the resulting cultures of the two organisms to be as nearly as possible in the same condition.

These parallel cultures showed in an interesting manner the similarity between *B. sporogenes* and the Reading bacillus. When grown in milk, both bacteria digest the casein, and, after four or five days, leave a clear yellowish-brown whey. The shade of colour varies from a pale yellow to a light chocolate brown, according, probably, to small differences in the composition of the milk.

When the two series of cultures prepared for seed purposes were examined it was seen that many shades of brown had been produced. It was observed, however, that corresponding cultures of *B. sporogenes* and the Reading bacillus, that is to say, cultures prepared at the same time and incubated for the same period, always exactly matched each other in the tint produced.

Experiment No. 1.

PARALLEL FERMENTATIONS ON PEPTONE WATER.

250 c.c. peptone water were fermented by each organism. Sterilisation was effected by heating at 100° C. for thirty minutes on three successive days. Incubation was continued for 117 hours.

Initial Composition of Medium—

		<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Total nitrogen . . .	Mgrms. per 100 c.c. . .	353	353
Initial amino-nitrogen . . .	„ „ „ . .	78	78
Initial ammonia-nitrogen . . .	„ „ „ . .	18	18
Initial reaction . . .	P _H = . . .	7.0	7.0
Culture used as seed . . .		<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Age of seed culture . . .	3 weeks	3 weeks	3 weeks

Products of Fermentation—

		<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Gas produced . . .	C.c. per 100 c.c. . .	46	62
Carbon dioxide in gas . . .	Per cent. . .	65	53
Final amino-nitrogen . . .	Mgrms. per 100 c.c. . .	73	71
Loss in amino-nitrogen . . .	„ „ „ . .	5	7
Final ammonia-nitrogen . . .	„ „ „ . .	107	104
Gain in ammonia-nitrogen . . .	„ „ „ . .	89	86
Volatile acids formed . . .	C.c. N/10 sol. per 100 c.c. . .	50.6	48.5
Final reaction . . .	P _H = . . .	6.7	6.7

It will be observed that, with the exception of the gas, there is a very close agreement in the amounts of products formed by the two organisms.

Examination of the volatile acids by Dyer's method yielded logarithmic distillation curves which were very similar. The acid distillates were submitted to certain of Dyer's qualitative colour tests whereby *caproic acid* was shown to be present in the acids from each organism.

The forms of the curves showing gas production agreed closely. It was thought that the differences in the gas data might be due to experimental inaccuracies. The fermentations on peptone water were therefore repeated in Experiment 2.

Experiment No. 2.

PARALLEL FERMENTATIONS ON PEPTONE WATER.

In this experiment attention only was paid to the formation and composition of the gas. 250 c.c. of medium were used in each case. Incubation was continued for 144 hours.

		<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Age of seed culture		23 days	23 days
Gas produced	C.c. per 100 c.c.	48 "	53 "
Carbon dioxide in gas	Per cent.	64 "	58 "

It is seen that the data for gas production and composition show a closer agreement than in Experiment 1.

Experiment No. 3.

PARALLEL FERMENTATIONS ON GLUCOSE-PEPTONE.

The medium was made by adding 2 per cent. of glucose to the peptone water used above. 300 c.c. of medium were taken in each case. Sterilisation was effected as in Experiment 1.

Incubation was continued for ninety-six hours.

Initial Composition of Medium—

		<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Total nitrogen	Mgrams. per 100 c.c.	305	305
Initial amino-nitrogen	" " " "	58	58
Initial ammonia-nitrogen	" " " "	16	16
Initial glucose content	Per cent.	1.71	1.71
Initial reaction	$P_H =$	6.7	6.7
Age of seed culture		5 weeks	5 weeks

Products of Fermentation—

		<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Gas produced	C.c. per 100 c.c.	102	95
Carbon dioxide in gas	Per cent.	54.3	58.4
Final amino-nitrogen	Mgrams. per 100 c.c.	90	90
Gain in amino-nitrogen	" " " "	32	32
Final ammonia-nitrogen	" " " "	38	40
Gain in ammonia-nitrogen	" " " "	22	24
Final glucose content	Per cent.	1.30	1.38
Amount of glucose used	Mgrams. per 100 c.c.	410	330
Volatile acids formed	C.c. N/10 sol. per 100 c.c.	37	37
Final reaction		acid	acid

From the above data, a close agreement between the products is at once seen. What differences there are may reasonably be considered within the limits of experimental error. The curves for gas production were similar. No curves were plotted for the distillation values of the acids.

Experiment No. 4.

PARALLEL FERMENTATIONS ON MILK.

Ordinary milk was used as purchased at a French dairy.

300 c.c. were taken for each fermentation. Sterilisation was effected by heating at 110° C. for forty-five minutes.

Incubation was continued for 168 hours. There was rather a long period between inoculation and the formation of gas. This latent period was about thirty hours for *B. sporogenes* and fifty hours for the Reading bacillus.

<i>Initial Composition of Medium—</i>				<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Total nitrogen content . .	Mgrms. per 100 c.c. . .			510	510
Initial amino-nitrogen . .	" " " " . .			5	5
Initial ammonia-nitrogen . .	" " " " . .			5	5
Initial lactose content . .	Per cent.			4.04	4.04
Age of seed culture				29 days	29 days
<i>Products of Fermentation—</i>				<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Gas produced	C.c. per 100 c.c.			85	82
Carbon dioxide in gas . .	Per cent.			77	75
Final amino-nitrogen . .	Mgrms. per 100 c.c.			133	129
Gain in amino-nitrogen . .	" " " "			128	124
Final ammonia-nitrogen . .	" " " "			131	129
Gain in ammonia-nitrogen . .	" " " "			126	124
Final lactose content . .	Per cent.			4.15?	4.26?
Volatile acids formed . .	C.c. N/10 sol. per 100 c.c. . .			76	68

There are several points to be noted in the above data. In the first place there is an apparent gain in sugar content. Wolf and Harris have already pointed out the occurrence of this phenomenon with *B. sporogenes* and have ascribed it to hydrolysis of the lactose, whereby sugars are obtained with a higher reducing value towards Fehling's solution.

The agreement between the final products is striking in these fermentations. Except for the longer latent period shown by the Reading bacillus, the curves showing the rate of gas formation were similar in form.

The logarithmic curves of the two lots of volatile acids obtained showed that these acids were very closely related.

Experiment No. 5.

PARALLEL FERMENTATIONS ON ALKALINE EGG.

237 c.c. of alkaline egg medium were used in each fermentation. Sterilisation was effected by heating at 110° C. for forty-five minutes. Incubation was continued for 286 hours. With this medium as long as forty-eight hours may elapse before gas formation is noticeable.

<i>Initial Composition of Medium—</i>				<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Total nitrogen	Mgrms. per 100 c.c.			294	294
Initial amino-nitrogen . .	" " " "			10	10
Initial ammonia-nitrogen . .	" " " "			2	2
Initial reaction	P _H =			8.0	8.0
Age of culture used as seed . .				10 weeks	10 weeks
<i>Products of Fermentation—</i>				<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Gas produced	C.c. per 100 c.c.			96	90
Carbon dioxide in gas . .	Per cent.			60.5	60.5
Final amino-nitrogen . .	Mgrms. per 100 c.c.			85	86
Gain in amino-nitrogen . .	" " " "			75	76
Final ammonia-nitrogen . .	" " " "			121	109
Gain in ammonia-nitrogen . .	" " " "			119	107
Volatile acids formed . .	C.c. N/10 sol. per 100 c.c. . .			55.2	50.2
Final reaction	P _H =			6.9	6.9

The curves showing gas formation were similar in form, as also were the distillation curves of the volatile acids. Qualitative colour tests revealed the presence of traces of caproic acid in each distillate.

There was no noteworthy difference between any of the products in these fermentations.

Experiment 6.

PARALLEL FERMENTATIONS ON COOKED MEAT.

The two organisms have a very profound action on cooked-meat medium, which is well suited to their growth. In spite of the formation of large amounts of volatile acids the medium does not become acid in reaction owing to the formation of correspondingly large amounts of ammonia.

232 c.c. of medium were used for each fermentation. Sterilisation was effected by heating at 110° C. for forty-five minutes. Incubation was continued for 142 hours.

<i>Initial Composition of Medium—</i>				<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Total nitrogen	.	.	Mgrms. per 100 c.c.	874	874
Initial amino-nitrogen	.	.	" " "	22	22
Initial ammonia-nitrogen	.	.	" " "	13	13
Initial reaction	.	.	P _H =	7.2	7.2
<i>Products of Fermentation—</i>				<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Gas produced	.	.	C.c. per 100 c.c.	180	184
Carbon dioxide in gas	.	.	Per cent.	86	86
Final amino-nitrogen	.	.	Mgrms. per 100 c.c.	253	238
Gain in amino-nitrogen	.	.	" " "	231	216
Final ammonia-nitrogen	.	.	" " "	405	379
Gain in ammonia-nitrogen	.	.	" " "	392	366
Volatile acids formed	.	.	C.c. N/10 sol. per 100 c.c.	Lost	173
Final reaction	.	.	P _H =	7.18	7.18

The agreement between the products of the two organisms is close, despite the large amounts involved. There is rather more divergence than usual in the amino-nitrogen and ammonia. This may be accounted for as follows. When cooked-meat medium is prepared it is somewhat difficult to distribute and sample evenly. In the above experiments the meat was made very fine, but it is doubtful whether the flasks contained the same concentration of solid matter. Therefore the estimations of initial total nitrogen may not be absolutely reliable. Determinations made at the end of fermentation are not accompanied by this difficulty as the solid meat particles are by then considerably reduced in quantity and size by the digestion that has taken place. The results given for the final products may therefore be taken as correct.

As the acid distillate from *B. sporogenes* was lost the volatile acids could not be compared. There was a strong resemblance between the gas curves.

Experiment 7.

PARALLEL FERMENTATIONS ON COOKED MEAT.

In these fermentations the total nitrogen was determined on the digested medium, when sampling is easily performed. A sample from each flask was taken after fermentation. As is seen from the data, the nitrogen concentrations showed a considerable difference. The initial concentrations of amino-acid nitrogen and ammonia were calculated from the total nitrogen content by comparison with other determinations. As there are only small quantities of these substances present in the unfermented meat, small errors in their determination would not affect the results appreciably.

268 c.c. of medium were used for each fermentation. Sterilisation was effected as in Experiment 6. Incubation was continued for 118 hours.

<i>Initial Composition of Medium—</i>				<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Total nitrogen	.	.	Mgrms. per 100 c.c.	690	812
Initial amino-nitrogen	.	.	" " "	17	20
Initial ammonia-nitrogen	.	.	" " "	10	12

<i>Products of Fermentation—</i>				<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Gas produced	.	C.c. per 100 c.c.	.	146	154
Carbon dioxide in gas	.	Per cent.	.	75	79
Final amino-nitrogen	.	Mgms. per 100 c.c.	.	200	230
Gain in amino-nitrogen	.	" "	.	183	210
Final ammonia-nitrogen	.	" "	.	250	317
Gain in ammonia-nitrogen	.	" "	.	240	305
Volatile acids formed	.	C.c. N/10 sol. per 100 c.c.	.	113	140

Owing to the differences in initial composition of the media, these results cannot be considered strictly comparable. There is, however, a marked proportionality between the products formed in the two fermentations.

The gas curves showed fair resemblance. From the distillation curves of the volatile acids, it appears that there was a slightly larger amount of high members of the volatile fatty acid series produced by the Reading bacillus than by *B. sporogenes*. Qualitative colour tests on the distillates showed caproic acid to be produced by both organisms in this medium.

DETERMINATION OF THE AEROBIC INDICES OF *B. SPOROGENES* AND THE READING BACILLUS.

The aerobic indices of these organisms were determined both in peptone water and on agar. Values were found for spores (4 weeks old) and young bacteria (24 hours old). Readings were taken after incubation had been continued for seven days.

RESULTS.

(a) In Peptone Water—

	<i>B. sporogenes.</i>	<i>Reading Bacillus.</i>
Aerobic index of 4 weeks old organisms	600/0 (50 mm.)	0/0 (50 mm.)
Aerobic index of 24 hours old organisms	600/0 (50 mm.)	0/0 (50 mm.)

(b) On Agar Slopes—

Aerobic index of 4 weeks old organisms	0/0 (50 mm.)	0/0 (50 mm.)
Aerobic index of 24 hours old organisms	0/0 (50 mm.)	0/0 (50 mm.)

The above data reveals an unexpected difference between *B. sporogenes* and the Reading bacillus.

It is seen that the Reading bacillus behaves as a strict anaerobe both in peptone water and on agar.

B. sporogenes, on the other hand, can tolerate quite large amounts of oxygen, nearly up to saturation value at atmospheric pressure. On agar, however, *B. sporogenes* only develops in complete absence of oxygen.

The above data represent the results of several determinations.

DISCUSSION OF RESULTS.

From the experimental results detailed above it is obvious that the two organisms compared are practically identical in their bio-chemical reactions, with the exception of their behaviour towards oxygen.

When made to ferment the same medium under similar conditions they form products which are almost quantitatively equal within the limits of experimental error.

It is possible that greater refinements—in the preparation of seed, for example—might eliminate the small differences obtained. On the

other hand, these differences may be fundamental and associated with the divergences displayed in peptone water, in behaviour towards oxygen. This latter distinction is certainly striking, but it may be capable of simple explanation. The strain of *B. sporogenes* used had been extensively sub-cultivated in artificial media during a long series of experiments. It is possible that during this treatment it had acquired the property of tolerating oxygen. Unfortunately, no data are available concerning the early history of the particular strain from this point of view, though it was always assumed to be a strict anaerobe. However, the question of anaerobiosis in liquids is an extremely difficult one. As shown by Douglas, Fleming, and Colebrook (1917⁵), the presence of small particles of porous substances in a liquid medium permits the development of so-called anaerobes, whilst Wolf and Harris (1917¹⁶) showed that *B. welchii* could find sufficient shelter from oxygen at the bottom of a comparatively shallow layer of medium contained in an open test tube. On the other hand, according to Pakes and Jollyman (1901¹³), the presence of a nitrate renders an anaerobic medium aerobic.

In the present instance it is obvious that a fine shade of difference is revealed on the liquid medium. On agar, curiously enough, both organisms behave similarly; that is, they require the removal of all traces of oxygen before growth takes place.

With regard to the identity of the Reading bacillus and *B. sporogenes*, perhaps the safest conclusion from the above experiments is that these two organisms are of the same race, having the same biochemical properties to a remarkable degree, but showing certain small differences (possibly acquired) such as one might expect to find in different strains of the same organism.

These findings agree well with the results of Donaldson's comparison (1918³).

Notes on the Methods.—It is thought that the experimental methods described above may prove useful in further investigations of this nature, and in determining the biochemical reactions of organisms generally. It seems important that the biochemical properties of all prominent organisms should be determined. This could be conveniently carried out in apparatus similar to that described and on the same scale. The quantity of medium required for each fermentation is not large; nevertheless, it allows ample material for the various analytical determinations. Further, as will be shown below, the methods may be easily adapted for use with aerobic bacteria.

Rigid comparison such as that made between *B. sporogenes* and the Reading bacillus would only be necessary where one is dealing with closely allied organisms, that is, difficultly distinguishable members of the same group. For most purposes it would suffice to determine quantitatively the products formed by the particular organism on one or two suitable media. In this way data would be obtained from

which bacteria might be classified according to their biochemical properties with far greater accuracy than is obtained by the qualitative methods now used.

In order that such data should be comparative, strict attention would need to be paid to the preparation of media, seed cultures, and fermentation details generally. The media should be prepared, as far as possible, according to standard methods, and the initial reactions carefully adjusted. With regard to the seed cultures, organisms in the "spored," or resting, condition would be most suitable. At this stage they are probably more constant in their reactions than in their early period of rapid growth. Cultures for use as seed should be grown, where possible, on media which are highly buffered, and in which free acids do not accumulate. For many bacteria, such a medium is cooked meat. It contains a large amount of nitrogenous substances, which come into play as buffers in the presence of free acids, and contains very little carbohydrate.

The period of incubation is of importance. After the first vigorous action has abated, there is, with many organisms, a slow, continued action, which may in time double the quantity of products formed in the first rapid stage. Therefore, in stating results, the length of the incubation period should be noted. Generally speaking, seven days' incubation would serve for most organisms, and comparisons might be made on this basis.

Amongst the estimations described above the one that is the most time-consuming is the determination of the distillation curves of the volatile acids. A knowledge of the nature of these acids is, however, of great importance as it throws light on the metabolic changes involved in their formation. In the comparison between *B. sporogenes* and the Reading bacillus, a similarity between the distillation curves was all that was sought, but in many cases a more detailed investigation would be found of great use. It was a comparison of the acids produced by *B. typhosus* and *B. coli* from glucose that led to an explanation of the production of gas by the latter organism (by the decomposition of formic acid into carbon dioxide and hydrogen), thus distinguishing its action from that of *B. typhosus* which does not attack formates (Pakes and Jollyman, 1901¹³; Harden, 1901⁷).

It has been stated above that the experimental methods may be adapted for aerobic organisms. A simple way of doing this is as follows:—The fermentation flask is fitted up in the usual manner and connected with the manometer. When the flask has been evacuated, a certain amount of oxygen is introduced, as in the determination of the aerobic indices. It is recommended that oxygen be allowed to enter till the mercury falls 150 mm. below the level registered for complete removal of air. By this means a partial pressure of oxygen approximately equal to that in the atmosphere is obtained. This partial pressure may be varied with different organisms, but it should

always be stated for purposes of comparison. The production of gas is followed in the usual manner by the fall of the mercury in the manometer. After fermentation the gases in the flask are analysed, and after making allowances for the residual oxygen, the volume of the formed gas is calculated. It should be borne in mind that some bacterial cultures absorb oxygen during fermentation so that the actual amount of oxygen present at the end of fermentation must always be determined by analysis before the volume and composition of the evolved gases are calculated. The author has used this method with aerobic organisms with success, and has been able to determine the volume and composition of gases produced by fermentation as well as to note absorption of oxygen.

In conclusion, it is hoped that the methods described above will be found useful by other investigators. From the results of the comparison experiments it is seen how cultural and morphological resemblance may be confirmed by quantitative experiments. At the same time there is no doubt in the mind of the writer that many differences at present only roughly indicated by qualitative experiments will be fully revealed by the more accurate methods of quantitative investigation.

Finally, new light will undoubtedly be shed on many perplexing problems of bacterial metabolism.

SUMMARY.

1. Experimental methods are described for carrying out a comparison of the biochemical reactions of two closely allied organisms.

2. Details are given of the apparatus used for fermentation experiments, and of the methods for obtaining values for gas production, ammonia and amino-acid formation, production of volatile acids, and changes in hydrogen-ion concentration and sugar content.

3. A simple method is described for determining the degree of oxygen toleration of organisms for routine purposes; it is suggested that results should be expressed in the form of the "aerobic index," which is defined.

4. A comparison is made of two anaerobes—*B. sporogenes* and the Reading bacillus—which, morphologically and in cultural reactions, are closely related. The results are given of fermentations on five different media, and of determinations of the aerobic indices both of spores and young organisms on liquid and solid media.

5. From the experimental results it is concluded that these two organisms are of the same race, but show small differences possibly acquired. In their biochemical behaviour towards the five media used they are remarkably similar, but they show a somewhat striking difference in their powers of growing in the presence of oxygen.

6. The use of methods, such as those described, for investigations

of the biochemical properties of bacteria in general is discussed, and a means is suggested for using these methods with aerobic organisms.

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