XXI. CONTRIBUTIONS TO THE BIOCHEMISTRY OF PATHOGENIC ANAEROBES. III. THE EFFECT OF ACIDS ON THE GROWTH OF *BACILLUS WELCHII* (*B. PERFRINGENS*) AND *BACILLUS SPOROGENES* (METCHNIKOFF)

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In our first paper [1917] on the growth of pathogenic anaerobes we showed that growth of the bacillus of Welch ceased at a very definite hydrogen ion concentration when the organism was inoculated into milk. We also referred to the possibility of the inhibiting action of acids having a certain clinical interest in the treatment of infection by these bacteria.

The cessation of activity at a definite acidity is not a singular phenomenon. It is known that certain other bacteria, for example, the colon bacillus and *B. typhosus* and the allied *B. paratyphosus A* and *B*, cease growing at a fairly definite hydrogen ion concentration.

Clark and Lubs [1915, 1] found a certain correspondence in the final reaction and the type of colon bacillus with which they were working. Cole and Onslow [1916] claim to be able to differentiate the organisms of the typhosus group by the velocity with which they reach a certain acid reaction.

Previous to these observers, Brunn [1913] had shown that *B. coli* dies within twenty-four hours if exposed to an acid reaction of $P_H = 4.7$ but not of $P_H = 5.0$. Michaelis [1914] grew *B. coli* in lactose bouillon and found that the acidity always rose to but did not exceed $P_H = 5.0$; but the amount of literature bearing on the subject of the effect of reaction on the growth of micro-organisms is not extensive. Very recently Clark and Lubs [1917] have

published a series of very interesting papers in which will be found many references to this important problem and which should be consulted by those interested in its history.

Attempts have been made by many investigators to estimate the effect of acid solutions on bacteria by simply adding definite amounts of various acids to media containing bacteria, or by treating the media with acids before inoculation. So far this method has yielded very little useful information, as the experimenters have for the most part neglected to take into account the influence of a complex medium—containing many buffer substances—on the dissociation of the acids. Any investigation of the effect of acids on bacteria must be preceded by a study of the effects of the acids on the media which are being used.

So far as we are aware the only attempt to determine the effect of acids on culture media in altering their hydrogen ion concentration, where modern methods have been used, is that of Clark and Lubs [1915, 2]. Their conclusions regarding the futility of titrating media, as is done in most bacteriological laboratories, are of great importance and are fully borne out by the present work. The point at which their investigation touches our own is where they show that the effect of acids on media is not directly proportional to the quantity of acid added. In this connection it should be borne in mind that different acids affect media to different extents, depending on many factors. Amongst these one may mention the dilution, the degree of dissociation of the acids and the salts contained in the medium, and the combinations they may form with the complex substances of which bacteriological media are composed.

Clark and Lubs investigated the effect of adding lactic and hydrochloric acid to milk, peptone water, etc., and expressed the results by means of titration curves, in which the amounts of acid added were plotted against the absolute reactions resulting, as measured with the hydrogen electrode. These curves show clearly the buffer action of the media; how they absorb part of the hydrogen ions of the acid and cause the reaction to be far less acid than that which the same amount of acid would produce if added to a medium free from buffer substances. Thus, milk to which sufficient acid had been added to make the solution 0.023 *N* had a reaction of $P_H = 5.4$, which is the reaction corresponding roughly to that of a solution of hydrochloric acid only 0.000004 *N*.

Recently Taylor [1917] has been working on the effect of acids on the growth of bacteria and claims to have obtained certain specific effects with

certain acids. He has, however, made no attempt to show what effect is due to the anions of the acid and what is due to that cation which is common to them all—namely the hydrogen ion.

This matter is of importance, for in the work of Wolf and Telfer [1917] a study has been made of the acids involved in the fermentation of *B. perfringens* and *B. sporogenes*. Should any of the acids which are normally found as the fermentation products of these organisms have a specific effect we should be inclined to their use in combating local infection. This specific action of acids cannot be excluded without experimental evidence to the contrary, for we know that a large number of anions have a very decided inhibitory effect on the growth of bacteria.

In the following experiments it will be shown that *B. perfringens* fails to grow in a glucose peptone medium containing sufficient hydrochloric acid to give the solution a reaction of $P_H = 4.8$. This reaction corresponds to that of a solution of hydrochloric acid only 0.00016 *N* and is produced by adding to the medium containing buffer substances sufficient HCl to make it 0.02 *N* with regard to the acid itself. That this inhibitory effect is not due to the anion is proved by the fact that a 0.1 *N* solution of NaCl has no toxic action on bacteria, and may even have an accelerating effect on their growth.

On the other hand, acids like benzoic, salicylic or boric, distinctly inhibit bacterial growth, and this takes place whether they are present as free acids or as sodium salts.

We have therefore examined the effects of the more commonly occurring acids to see if any of them possess antiseptic properties outside of those conferred on them by their hydrogen ion.

The four main points to which attention has been directed in the present paper are the following:

1. What is the effect of equivalent amounts of acids of differing dissociation constants on the reaction of a standard medium?
2. Knowing this effect, what is the minimum amount of acid, and the corresponding hydrogen ion concentration, which exerts a lethal effect on the organisms chosen?
3. Is there in any of these acids a discoverable effect which cannot be set down to the hydrogen ion concentration?
4. Is the final hydrogen ion concentration, after the cessation of growth of the organisms, a so-called "physiological constant," or is it influenced by the initial reaction of the medium?

The question of the initial reaction of the medium is one of great interest

and may have an important bearing on the latent period of growth of these organisms. In a recent paper we showed that the latent period appears to have a connection with the amino-acid content of the medium, but there were many unexplained features, which we now think may have been due largely to the initial reaction of the medium. We found that the period of optimum growth on milk coincided with a very definite hydrogen ion concentration in the case of *B. perfringens* and suggested tentatively that this might be the most favourable reaction for growth. As the result of the present experiments we have been compelled to modify our views on this point.

If growth of *B. perfringens* and *B. sporogenes* ceases at a definite reaction, it is important to determine it, for in this knowledge we may have an additional weapon for the local treatment of infections of this type. As the hydrogen ion concentrations with which we are dealing are of the order of $1/50,000\ N$, it is obvious that one cannot employ highly dissociated acids of this strength, because the least trace of protein, amino-acid, or alkali from the walls of the containing vessel would at once transform this acid solution to a neutral one. It is necessary therefore to submit the organisms to these concentrations of hydrogen ions in media containing large concentrations of buffer substances, where the action of the glass or of the carbon dioxide of the air will have a much smaller or even negligible effect.

EXPERIMENTAL METHODS.

Preparation of Media. Titration Curves.

When an acid is added to a medium such as glucose peptone water, the resulting reaction depends on several factors. Firstly, there is the buffer action of the solution itself, due to the presence of amino-acids, phosphates, etc. The degree of dissociation of the acid has also to be considered and that of the salts it may form. As a preliminary measure, therefore, we investigated the changes of reaction of the medium to which known amounts of various acids had been added.

The medium employed was a solution of peptone made by the tryptic digestion of caseinogen. This medium is quite definite in its properties and work that we have done has shown it to be very suitable for the development of *B. perfringens* and *B. sporogenes*, with or without the addition of glucose. As the presence of glucose causes a more vigorous fermentation with each organism, the solutions used in these experiments were prepared so as to contain 2 % of this sugar.

The effect of the following acids was tried: hydrochloric, lactic, formic, butyric, acetic, malic, and succinic¹.

For each acid a titration curve was drawn (Fig. 1), showing the relationship between the amount of acid contained in the medium and the absolute reaction resulting from its presence.

The following procedure—that for hydrochloric acid—was followed for each acid used, with slight modifications as to the amounts added. A double strength solution of glucose peptone water was prepared, containing 4 % of glucose and 4 % of peptone. Eight portions of 25 cc. of this were taken and pipetted into 50 cc. graduated measuring flasks, and the amounts of *N*/10 acid and water shown in Table I were added.

This gave eight lots of 2 % glucose peptone water, differing only in the amount of acid contained per 50 cc. Each lot was divided amongst five tubes containing Durham tubes, plugged and sterilised at 110° for forty-five minutes. The tubes were of hard glass and were specially selected so as to be uniform. Their dimensions were 1.5 cm. × 16.0 cm.

After sterilisation, the reaction of each lot of tubed medium was determined at 20° electrometrically, using the hydrogen electrode, the value of P_H being calculated from the E.M.F. obtained experimentally.

In later experiments only one tube from each lot was used for determining the reaction, as it was found that there was little or no difference between corresponding tubes. The remaining tubes were used for the fermentation experiments described later.

When the reactions had been measured they were plotted against the amount of acid added. The amount of acid was recorded as cc. of *N*/10 acid contained in 10 cc. of the 2 % broth. The results for hydrochloric acid and for the other acids employed are given in Table I and Fig. 1. It will be seen from the curves that the amounts of different acids required to produce the same reaction vary considerably. Highly dissociated acids, such as hydrochloric and lactic, produce a given reaction when added in much smaller quantities than weaker acids such as acetic and butyric. These latter acids, too, soon arrive at a value of P_H where large additions of acid cause only slight changes in the value of P_H ; that is to say, they are very highly influenced by the action of the buffer, the peptone solution.

¹ An attempt was made to obtain a titration curve for trichloroacetic acid, and also to observe the effect of this acid on the growth of organisms. As might have been foreseen, however, the acid decomposed during the sterilisation, yielding chloroform and carbon dioxide. These latter passed off as vapour and the media remaining possessed the reaction of ordinary glucose peptone water to which no acid had been added. On inoculation with *B. perfringens* the whole of the tubes fermented vigorously.

TABLE I. *Data for Titration Curves.**Hydrochloric Acid.*

Tube No.	50 cc. of broth made up from 25 cc. double strength broth and		Amount of N/10 acid contained in 10 cc. of broth cc.	Reaction, P _H
	cc. of N/10 acid	water cc.		
1	0	25.0	0	6.63
2	3.0	22.0	0.6	6.03
3	4.5	20.5	0.9	5.78
4	6.0	19.0	1.2	5.52
5	7.5	17.5	1.5	5.30
6	9.0	16.0	1.8	4.99
7	10.5	14.5	2.1	4.75
8	12.0	13.0	2.4	4.60

Lactic Acid.

1	0	25.0	0	6.63
2	2.05	22.95	0.41	6.20
3	4.10	20.95	0.82	5.78
4	6.15	18.85	1.23	5.46
5	8.20	16.80	1.64	5.05
6	10.30	14.70	2.06	4.70
7	12.35	12.65	2.47	4.56
8	14.40	10.60	2.88	4.38
9	16.45	8.55	3.29	4.28

Formic Acid.

1	0	25.0	0	6.44
2	2.0	23.0	0.4	6.10
3	4.0	21.0	0.8	5.74
4	6.0	19.0	1.2	5.42
5	8.0	17.0	1.6	5.06
6	10.0	15.0	2.0	4.77
7	12.0	13.0	2.4	4.60
8	14.0	11.0	2.8	4.44
9	16.0	9.0	3.2	4.30

Malic Acid.

1	0	25.0	0	6.52
2	2.5	22.5	0.5	6.03
3	5.0	20.0	1.0	5.65
4	7.5	17.5	1.5	5.34
5	10.0	15.0	2.0	5.10
6	12.5	12.5	2.5	4.89
7	15.0	10.0	3.0	4.71
8	17.5	7.5	3.5	4.61

TABLE I. *Data for Titration Curves.—Continued.*

Tube No.	50 c.c. of broth made up from 25 c.c. double strength broth and		Amount of N/10 acid contained in 10 cc. of broth cc.	Reaction P_H
	cc. of N/10 acid	water c.c.		
1	0	25.0	0	6.52
2	3.0	22.0	0.6	5.98
3	6.0	19.0	1.2	5.56
4	9.0	16.0	1.8	5.21
5	12.0	13.0	2.4	4.95
6	15.0	10.0	3.0	4.81
7	17.5	7.5	3.5	4.75
8	20.0	5.0	4.0	4.65
9	22.5	2.5	4.5	4.60

Butyric Acid.

1	0	25.0	0	6.63
2	2.5	22.5	0.5	6.20
3	5.0	20.0	1.0	5.80
4	7.5	17.5	1.5	5.47
5	10.0	15.0	2.0	5.26
6	12.5	12.5	2.5	5.02
7	15.0	10.0	3.0	4.90
8	17.5	7.5	3.5	4.80
9	20.0	5.0	4.0	4.76

Succinic Acid.

1	0	25.0	0	6.19
2	2.5	22.5	0.5	5.84
3	5.0	20.0	1.0	5.55
4	7.5	17.5	1.5	5.36
5	10.0	15.0	2.0	5.17
6	12.5	12.5	2.5	5.06
7	15.0	10.0	3.0	4.93
8	17.5	7.5	3.5	4.83

Reaction Series. Inoculation and Fermentation.

Following the methods outlined above, various lots of media at different reactions were prepared, using the above-mentioned acids. After sterilisation, one tube from each lot of medium was opened and its reaction determined. After a few preliminary experiments we were usually able to obtain media for each acid with reactions fairly evenly distributed between $P_H = 4.0$ and $P_H = 7.0$.

The remaining tubes were arranged into four similar "series," these latter consisting of one tube from each lot of medium. The series were called Series A, Series B, etc. The least acid member of the series was numbered 1, the others being numbered consecutively with increase of acidity.

The inoculation was made with a Pasteur pipette, each tube receiving three drops of the seed culture. As far as possible, a different culture was used for each series, so that representative results could be obtained. We were careful to use cultures, which, in our opinion, based on previous work, were vigorous.

After inoculation, the contents of each tube were covered aseptically with a layer of liquid paraffin previously sterilised, about 0.5 cm. in depth, to ensure anaerobic conditions. The necessity of this we have since shown to be open to serious doubt, but as our first experiments were conducted with the use of paraffin we continued to use it.

The inoculated tubes were now incubated at 37°. *Perfringens* cultures were allowed to remain in the incubator for three days before examination. It is advisable to give *B. sporogenes* four days to complete fermentation.

At the end of the incubation period, the series were examined and those tubes which had fermented were noted. Samples were then taken from each tube and the reaction of the fermented liquid determined, using the hydrogen electrode.

Reaction Resultants.

These final reactions proved to be of great interest and it will be well at this point to describe our method of plotting them and obtaining what we have named *reaction resultants*.

Fig. 2 shows the reaction resultant for *B. perfringens* grown on glucose peptone water acidified with varying amounts of hydrochloric acid.

On ordinary squared paper a vertical line is taken for one axis and a scale of ordinates is chosen to represent the range of reactions over which we are working. Then at an angle of 120° to the vertical a line *AB* is drawn. This becomes the second axis. The only scale actually employed in plotting is the vertical one, as it will be seen from simple geometrical considerations that *AB* is conveniently divided into a scale by the parallel horizontal lines of the squared paper passing through the ordinates. On *AB*, the point corresponding to the initial reaction of the particular medium is marked off. Then, on a line through this point and parallel to the vertical axis, the point corresponding to the final reaction of the medium is marked. For each initial reaction marked on the line *AB*, a point for the final reaction is found in a similar manner, and

through the series of points thus obtained, a smoothed curve is drawn. This curve we have named the *reaction resultant*.

In employing this method of plotting results, what we have actually done is to make use of a pair of axes at an angle of 120° to each other, instead of being at right angles as axes usually are. The choice of the angle between the axes is only governed by considerations of general convenience. Where comparative results are required, the angle must necessarily be definitely fixed.

In Fig. 2, the reaction resultant is derived from large numbers of fermentations in which *B. perfringens* has acted on glucose peptone water containing various added amounts of hydrochloric acid. In the figure, only fifteen points are shown owing to lack of space, but we have many other confirmatory points from additional experiments.

It will be observed that the points recording the final values of P_H lie very close to the smooth curve that has been drawn to represent them, and establish quite definitely the form of this line.

Fig. 2

BACILLUS PERFRINGENS.

Data are given below for experiments carried out with *B. perfringens* on glucose peptone water in the presence of various acids.

The tables show the initial and final reactions of the media in which fermentation took place. In cases where the bacillus was able to ferment the medium a positive sign (+) is placed under the heading "Result"; where no fermentation took place there is a negative sign (-).

It was found that in tubes where there was no sign of fermentation—as evidenced by the absence of gas or turbidity in the medium—the reaction remained unchanged at the end of the incubation period.

The final reactions were plotted against the initial reactions by the method previously described. When two or three similar series of tubes were used, the mean value of the final P_H for each lot of medium was taken for the purpose of plotting the reaction resultant.

Special interest is attached to the particular value of initial P_H at which the bacillus loses its power to ferment, and this has been noted in the following experimental data.

It should be noted that the reactions of the media were determined at 20°, whereas the fermentations took place in the incubator at 37°. At the higher temperature, the media would have a higher acidity than at 20°. The difference is not considerable but cannot be overlooked.

We have not made experiments touching the change in reaction of the media with change in temperature, but if we may be allowed to use Michaelis' figures for sodium acetate—acetic acid mixtures which will probably serve the purpose, 0.8 millivolt may be subtracted for each degree above 20°.

This would give, for a rise of 17°, a decrease in E.M.F. of 13.6 mv. This correction on a P_H of 4.67 would give a final corrected value of $P_H = 4.45$.

Experiment No. 1 (Laboratory Experiment R. S. 1). Hydrochloric acid and sodium hydroxide.

In this preliminary experiment not only was hydrochloric acid added to the peptone water, but also sodium hydroxide to some of it in order to give media with a reaction above $P_H = 7.0$. There was every sign of caramelisation of these alkaline media during the sterilisation, and the experiments were discontinued¹.

Data (see also Fig. 3).

Series A. Inoculated with K 71 (thirteen hours old on milk).

¹ The effect of sterilisation on the reaction of media was investigated by the older methods by Hesse [1904]. He observed an increase in titratable acidity after heating. Clark and Lubs [1917] have investigated the change using the hydrogen electrode and have found an increase in P_H ranging from 0.01 with Witte's peptone to 0.44 with skimmed milk. The change is partly dependent on the carbohydrate content of the medium and the hydrogen ion concentration of the solution. By oxidation organic acids are formed. The caramelisation is due to melanines, formed by interaction of amino-acids and glucose [Maillard, 1913].

Series B. Inoculated with K 72 (thirteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 3, Series A, with value of $P_H = 4.89$.

The next set of tubes, with $P_H = 4.3$, failed to ferment.

TABLE II. (*Exp. 1.*)

No. of tube	Initial re-action, P_H	Series A		Series B	
		Result	Final re-action, P_H	Result	Final re-action, P_H
1	3.9	—	3.87	—	3.92
2	4.3	—	4.33	—	4.33
3	4.89	+	4.28	—	4.88
4	5.78	+	4.40	+	4.38
5	6.77	+	4.59	+	4.59
6	7.37	+	4.75	+	4.83
7	7.73	+	4.83	+	4.80
8	8.1	+	4.80	+	4.76

Experiment No. 2 (Laboratory Experiment R. S. 2). Hydrochloric acid.

Data (see also Fig. 3).

Series A. Inoculated with K 80 (fifteen hours old on milk).

Series B. Inoculated with K 78 (fifteen hours old on milk).

Series C. Inoculated with K 79 (fifteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 5, Series A, with value of $P_H = 4.86$.

The next set of tubes, with $P_H = 4.60$, failed to ferment.

TABLE III. (*Exp. 2.*)

No. of tube	Initial re-action, P_H	Series A		Series B		Series C	
		Result	Final re-action, P_H	Result	Final re-action, P_H	Result	Final re-action, P_H
1	6.22	+	4.54	+	4.52	+	4.53
2	5.82	+	4.40	+	4.44	+	4.37
3	5.40	+	4.33	+	4.33	+	4.35
4	5.13	+	4.34	+	4.32	—	...
5	4.86	+	4.34	—	...	—	...
6	4.60	—	...	—	...	—	...
7	4.36	—	...	—	...	—	...
8	4.19	—	...	—	...	—	...
9	3.78	—	...	—	...	—	...

Experiment No. 3 (Laboratory Experiment R. S. 5). Lactic acid.

Data (see also Fig. 3).

Series A. Inoculated with K 107 (twelve hours old on milk).

Series B. Inoculated with K 109 (twelve hours old on milk).

Series C. Inoculated with K 108 (twelve hours old on milk).

TABLE IV. (Exp. 3.)

No. of tube	Initial re-action, P _H	Series A		Series B		Series C	
		Result	Final re-action, P _H	Result	Final re-action, P _H	Result	Final re-action, P _H
1	6.19	+	4.60	+	4.57	+	4.58
2	5.46	+	4.49	+	4.51	+	4.49
3	5.15	+	4.51	+	4.51	+	4.51
4	4.85	+	4.80	-	...	-	...
5	4.61	-	...	-	...	-	...
6	4.42	-	...	-	...	-	...
7	4.26	-	...	-	...	-	...

B. PERFRINGENS ON 2% GLUCOSE-PEPTONE.

Fig. 3

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 4, Series A, with value of $P_H = 4.85$.

The fermentation was only very slight.

The next set of tubes, with $P_H = 4.61$, failed to ferment.

TABLE V. (*Exp. 4.*)

No. of tube	Initial re-action, P_H	Series A		Series B	
		Result	Final re-action, P_H	Result	Final re-action, P_H
1	6.44	+	4.50	+	4.50
2	6.10	+	4.39	+	4.39
3	5.74	+	4.39	+	4.41
4	5.42	+	4.40	+	4.44
5	5.06	+	4.60	?	4.81
6	4.77	-	...	-	...
7	4.60	-	...	-	...
8	4.44	-	...	-	...
9	4.30	-	...	-	...

Experiment No. 4 (Laboratory Experiment R. S. 12). Formic acid.

Data (see also Fig. 4).

Series A. Inoculated with K 127 (twenty-four hours old on cooked meat).

Series B. Inoculated with K 123 (ninety-six hours old on cooked meat).

The tubes were examined seventy-two hours after inoculation.

The most acid tubes to ferment were Nos. 5, Series A and B, with value of $P_H = 5.06$. The fermentations were very slight in each case.

The next set of tubes, with $P_H = 4.77$, failed to ferment.

TABLE VI. (*Exp. 5.*)

No. of tube	Initial re-action, P_H	Series A		Series B		Series C	
		Result	Final re-action, P_H	Result	Final re-action, P_H	Result	Final re-action, P_H
1	6.52	+	4.52	+	4.51	+	4.49
2	6.03	+	4.46	+	4.46	+	4.45
3	5.65	+	4.38	+	4.38	+	4.39
4	5.34	+	4.38	+	4.38	+	4.39
5	5.10	+	4.37	+	4.39	+	4.37
6	4.89	-	...	+	4.37	+	4.38
7	4.71	-	...	+	4.38	+	4.36
8	4.60	-	...	-	...	-	...

Experiment No. 5 (Laboratory Experiment R. S. 8). Malic acid.

Data (see also Fig. 4).

Series A. Inoculated with K 118 (thirteen hours old on milk).

Series B. Inoculated with K 119 (thirteen hours old on milk).

Series C. Inoculated with K 119 (thirteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tubes to ferment were Nos. 7, Series B and C, with value of $P_H = 4.71$.

The next set of tubes, with $P_H = 4.60$, failed to ferment.

REACTION RESULTANTS.
B. PERFRINGENS ON 2% GLUCOSE-PEPTONE.

Fig. 4

Experiment No. 6 (Laboratory Experiment R. S. 13). Acetic acid.

Data (see also Fig. 4).

Series A. Inoculated with K 120 (twenty-four hours old on cooked meat).

Series B. Inoculated with K 121 (twenty-four hours old on cooked meat).

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 5, Series B, with value of $P_H = 4.95$.
The next set of tubes, with $P_H = 4.81$, failed to ferment.

TABLE VII. (*Exp.* 6.)

No. of tube	Initial re-action, P_H	Series A		Series B	
		Result	Final re-action, P_H	Result	Final re-action, P_H
1	6.52	+	4.38	+	4.41
2	5.98	+	4.43	+	4.41
3	5.56	+	4.39	+	4.39
4	5.21	+	4.43	+	4.44
5	4.95	-	...	+	4.47
6	4.81	-	...	-	...
7	4.75	-	...	-	...
8	4.65	-	...	-	...
9	4.60	-	...	-	...

Experiment No. 7 (Laboratory Experiment R. S. 4). Butyric acid.

Data (see also Fig. 5).

Series A. Inoculated with K 102 (fifteen hours old on milk).

Series B. Inoculated with K 103 (fifteen hours old on milk).

Series C. Inoculated with K 101 (fifteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tubes to ferment were Nos. 5, Series A, B and C, with value of $P_H = 5.03$.

The next set of tubes, with $P_H = 4.96$, failed to ferment.

TABLE VIII. (*Exp.* 7.)

No. of tube	Initial re-action, P_H	Series A		Series B		Series C	
		Result	Final re-action, P_H	Result	Final re-action, P_H	Result	Final re-action, P_H
1	6.33	+	4.52	+	4.50	+	4.52
2	5.62	+	4.48	+	4.48	+	4.48
3	5.39	+	4.64	+	4.60	+	4.60
4	5.17	+	4.61	+	4.64	+	4.57
5	5.03	+	4.67	+	4.67	+	4.65
6	4.96	-	...	-	...	-	...
7	4.83	-	...	-	...	-	...
8	4.80	-	...	-	...	-	...
9	4.68	-	...	-	...	-	...

Experiment No. 8 (Laboratory Experiment R. S. 6). Succinic acid.

Data (see also Fig. 5).

Series A. Inoculated with K 113 (fourteen hours old on milk).

Series B. Inoculated with K 114 (fourteen hours old on milk).

Series C. Inoculated with K 115 (fourteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

TABLE IX. (Exp. 8.)

No. of tube	Initial re-action, P _H	Series A		Series B		Series C	
		Result	Final re-action, P _H	Result	Final re-action, P _H	Result	Final re-action, P _H
1	6.19	+	4.52	+	4.52	+	4.51
2	5.84	+	4.50	+	4.49	+	4.48
3	5.55	+	4.51	+	4.52	+	4.52
4	5.36	+	4.54	+	4.54	+	4.53
5	5.17	+	4.56	+	4.56	+	4.56
6	5.06	+	4.58	+	4.57	+	4.57
7	4.93	+	4.57	+	4.56	-	...
8	4.83	-	...	-	...	-	...

The most acid tubes to ferment were Nos. 7, Series A and B, with value of $P_H = 4.93$.

The next set of tubes, with $P_H = 4.83$, failed to ferment.

One of the first things noticed in these experiments was that increasing the initial acidity of a medium had an effect on the latent period. Thus, when a series of tubes was inoculated with the same seed at the same time, the least acid member of the series was the first to ferment. The more acid the reaction of the medium the longer the latent period, showing that whatever may be the effect of the acidity on the subsequent fermentation it has a large influence in determining the latent period.

With *B. perfringens*, a glucose peptone solution begins to ferment in from four to five hours after inoculation when the reaction of the medium is from $P_H = 7.0$ to $P_H = 6.2$. As more acid media are employed, the latent period lengthens, until, for a medium with an initial reaction of 4.9, the latent period may last about forty-eight hours.

In reviewing the data given above it will be observed that the values of P_H at which the bacillus just manages to grow all lie close together in the neighbourhood of $P_H = 4.8$. In the following table are given the most acid values at which growth takes place and those which just prevent it.

TABLE X.

Experiment No.	Acid	Most acid value of P_H which allows growth	Least acid value of P_H which inhibits growth	Mean value of P_H . Critical P_H
2	Hydrochloric	4.86	4.60	4.78
3	Lactic	4.85	4.61	4.68
4	Formic	5.06	4.77	4.91
5	Malic	4.71	4.60	4.66
6	Acetic	4.95	4.81	4.88
7	Butyric	5.03	4.96	4.99
8	Succinic	4.93	4.83	4.88

Mean value of critical $P_H = 4.82$

With a large number of experiments it might be possible to find the exact value of P_H for each acid which would just permit growth, the slightest excess in acidity above this value preventing growth. In our experiments the differences between the "permitting reaction" and the "inhibiting reaction" are not great in any case, and probably the mean of the two values found experimentally is not far from this *critical* P_H . These mean values are given in the fifth column of the above table, and it will be observed that they are quite close together.

The following table shows the amount of each acid that must be present in the glucose peptone water to give these critical reactions. The amounts are determined from the titration curves (Fig. 1).

TABLE XI.

Acid	Critical P_H	cc. of $N/10$ acid contained in 10 cc. of broth	Acidity of medium in terms of N acid	Molar strength of medium in acid	G. of acid per litre of medium
Hydrochloric	4.78	2.05	0.0205 N	0.0205 M	0.748
Lactic	4.68	2.10	0.0210 N	0.0210 M	1.910
Formic	4.91	1.80	0.0180 N	0.0180 M	0.828
Malic	4.66	3.50	0.0350 N	0.0175 M	2.335
Acetic	4.88	2.70	0.0270 N	0.0270 M	1.620
Butyric	4.99	2.60	0.0260 N	0.0260 M	2.288
Succinic	4.88	3.20	0.0320 N	0.0160 M	1.888

The titration curves in Fig. 1 show the comparative strengths of the acids in presence of glucose peptone water. Thus, lactic, hydrochloric and formic acids behave as strong acids compared with acetic, butyric and succinic acids. It will be seen from the above table that the strong acids are more effective in inhibiting growth than the weaker acids when present in equivalent concentrations. Thus, when hydrochloric is present in sufficient quantity to make the medium 0.0205 N the critical reaction for *B. perfringens* is reached. On the other hand enough succinic acid must be present to give a 0.032 N solution before the critical reaction is attained.

These facts lead one to think that the dominant factor in the development of *B. perfringens* is not so much the amount of acid present as the acidity that the acid produces.

It seems that at about $P_H = 4.8$ there is a point where the reaction just permits the organism to develop. A slight excess in hydrogen ion concentration over this value prevents growth. Each acid may have some slight effect of its own, due to its molecular structure. Thus, malic acid is to some extent an anomaly, allowing growth to take place when the value of P_H is 4.71. Butyric acid on the other hand has a high value for its critical P_H (4.99), and seems to have a slight inhibiting effect in addition to that produced by its hydrogen ions.

On the whole it appears that the reaction due to the acid is more important than the actual amount or molecular quality of the acid in determining the growth or non-development of *B. perfringens*.

We now come to a consideration of the final reactions of the media after fermentation by *B. perfringens*. These final reactions are given in the tables and represented graphically in the reaction resultants.

These results show that the final reaction at which growth ceases is by no means a "physiological constant." On the contrary, it varies not only with the reaction of the medium before inoculation but also with the acid used to produce this particular reaction.

With hydrochloric acid (Fig. 2), when the initial reaction is $P_H = 6.77$, growth stops at the point where the bacillus has produced sufficient acid to give the medium a reaction of $P_H = 4.59$. For an initial reaction of $P_H = 4.86$, however, the final reaction is $P_H = 4.34$. For initial reactions between the two, final reactions are obtained which take up orderly positions between $P_H = 4.59$ and $P_H = 4.34$, and give the curve shown in the figure. There is therefore a very definite relation between the initial and final reactions.

Recently, in connection with *B. coli*, attempts at diagnosis have been made in which the final reaction of a particular medium when inoculated with the organism was determined. In view of the above results it would seem that great care must be exercised in using such a method. In order to use the final reaction of a fermentation as a criterion for a given organism, absolute uniformity of the medium is essential. This, applies especially to the hydrogen ion concentration, on which, as will be seen, the final reaction depends.

In examining the curves for the other acids used, one finds that the question of the final reaction becomes further complicated. Here, for the first time in these experiments, one sees signs of specificity in the action of the acids. The reaction resultants for hydrochloric, lactic and malic acids show that the final reactions of the media become definitely more acid as the initial hydrogen ion concentration increases. On the other hand, with butyric, succinic and acetic acids, the reverse rule holds good. The experiments appear to bring out the important point that all acids, in the presence of which *B. perfringens* can develop, probably give characteristic reaction resultants, as do the seven acids with which we have worked. Considerable interest is attached to the point *X* (Fig. 2), obtained by the intersection of the reaction resultant produced and the line *AB*. The reaction corresponding to this point might be considered theoretically as that which definitely stops the growth of *B. perfringens*. It should be remarked, however, that we have never been able to cause a medium having this reaction to ferment, no doubt on account of inhibiting influences other than the reaction. The value obtained at the point *X* differs for each individual acid. This, in itself, is an indication of specific action.

It is necessary to search closely for the factors responsible for these resultants. When *B. perfringens* ferments glucose peptone water, it forms,

as Wolf and Telfer [1917] have shown, a mixture of lactic acid, butyric acid and small amounts of other low members of the fatty acid series.

How far the *quality* of these acids is affected by the nature of the acid added to obtain the required initial reaction one is unable to say at present. The reaction resultants, however, will give a fair idea of the *quantity* of acids produced, provided one has some notion of the factors which influence them. To this end it will be necessary to take a specific example.

Let it be assumed that a series of flasks of glucose peptone water are prepared differing from one another in their initial hydrogen ion concentration, this effect being produced by the addition of increasing amounts of butyric acid to the medium. These flasks are then inoculated with an organism producing butyric acid exclusively. The quantity of butyric acid produced is also assumed to be independent of the initial reaction of the medium and is a constant amount. At the end of the fermentation the reaction resultants are plotted.

We have already determined the changes in reaction of glucose peptone caused by the addition of various amounts of butyric acid. These results can be expressed graphically in a titration curve, which will aid us to construct the reaction resultants for the above hypothetical fermentation. All that it is necessary to do is to select on the titration curve a number of points and consider the reactions for these points as the initial reactions of the media. To the quantity of acid corresponding to each point a definite amount of acid is added, and the reactions corresponding to the total amounts noted. These reactions will be the final reactions for the fermentation, and when plotted in the usual way against the initial reactions will give the reaction resultant. Thus, one can construct the reaction resultants for the production of any amount of acid providing one has a suitable titration curve. This has been done for four hypothetical fermentations (Fig. 6, I).

In the first case the fermentation is supposed to produce butyric acid equivalent to 3 cc. of $N/10$ acid per 10 cc. of broth; in the second, 5 cc.; in the third, 7 cc.; in the fourth, 9 cc. It is seen that the reaction resultants have a very definite form. They rise steadily from right to left on the paper, and the four curves tend to meet at a point on AB at which an addition of butyric acid will not make the reaction more acid. Different acids will, of course, give different curves, the general form of the reaction resultant, however, will be the same, providing the amount of acid produced does not vary. They will all rise from right to left of the paper towards the value of P_H where the acidity is not increased by further addition of the acid. It is clear also

that the resultant arising from the added acid must always approach the line AB tangentially, when a constant amount of acid is being formed.

Now let it be imagined that the accumulation of acids hinders the bacteria and causes their production of acid to fall off steadily in proportion to the concentration of acid present. The reaction resultants will then take the form

Fig. 6

shown in the second diagram (Fig. 6, II). We give the curves for three cases. In curve No. 1 the inhibiting influence is made slight, in No. 2 more marked, and in No. 3 greater still.

From a consideration of this imaginary fermentation one can now see how, from the reaction resultants, it can be estimated to what extent the acids have

affected fermentation. If the curve rises from right to left and tends to approach *AB* tangentially it means that the amount of acid produced is not falling off very greatly as the initial hydrogen ion concentration increases. If, on the other hand, the concentration of acids is causing a decrease in fermenting power, the reaction curves will tend to fall from right to left, according to the magnitude of the inhibiting influences. Where the bacteria cease activity as soon as a definite *concentration* of acid is reached, the reaction resultant will be a horizontal line (Fig. 6, II, 2).

From our experiments we have obtained various reaction resultants. The one most nearly approaching the case where the fermentation is not hindered by the accumulation of acids is with hydrochloric acid.

The following series of fermentations will show to what extent the foregoing considerations are borne out experimentally, and how far one may rely on conclusions as to acid formation and intensity of metabolism drawn from the reaction resultants.

Experiment No. 9 (Laboratory Fermentations Nos. 42-47).

Into each of six 500 cc. Kjeldahl flasks were placed 250 cc. of 2 % glucose peptone medium, as used in the foregoing reaction experiments. During preparation varying amounts of hydrochloric acid had been added to each lot of medium so that we had six broths exactly similar, except in so far as the amounts of contained acid, and consequently the reactions, were concerned. The flasks were plugged and sterilised in the ordinary way, afterwards being inoculated each with 1 cc. of the same culture of *B. perfringens*. The plugs were then pushed lower into the necks of the flasks and well fitting rubber stoppers, each provided with a short delivery tube, inserted. By means of a Fleuss pump the flasks were now evacuated by way of the delivery tubes, which were then closed off by means of a piece of pressure tubing and a screw clip. All joints were made tight with hot paraffin wax. The flasks were incubated for four days. At the end of this time the gas production was measured and a determination made of the amount of acid produced. To measure the gas production, the flasks were connected to an evacuated mercury manometer, the clip unscrewed, the pressure of the gas determined, and hence its volume calculated.

A sample of the gas was in each case analysed. From each flask also a portion of the liquid was taken and the final P_H measured electrometrically.

The amounts of volatile and fixed acids were determined by Captain Telfer, R.A.M.C., to whom our acknowledgements are due. The total volatile acids

were determined by exhaustive steam distillation and the fixed acids, by ethereal extraction of the residue in a liquid extractor. As the final stages of the distillation are accompanied to some extent by volatilisation of the fixed acids, and as also the final traces of lower members of the fatty acid series are only removed with difficulty, we have, in tabulating our results, added together the amounts of volatile and fixed acids, and taken their sum to represent the total amount of acids produced.

TABLE XII. (*Exp. 9.*)

No. of Fermentation	Initial P _H	Gas production			Final P _H	Acid production cc. of <i>N/10</i> acid per litre of broth		
		cc. per litre	CO ₂	H ₂		Volatile acids	Fixed acids	Total acids
			%	%		cc.	cc.	cc.
<i>F 42</i>	6.31	1036	42.3	57.7	4.63	232	171	403
<i>F 43</i>	6.08	1096	40.5	59.5	4.59	259	196	455
<i>F 44</i>	5.74	1000	41.6	58.4	4.51	200	213	413
<i>F 45</i>	5.49	888	41.3	58.7	4.42	156	231	387
<i>F 46</i>	5.23	916	41.0	59.0	4.40	229	205	434
<i>F 47</i>	4.88	940	...	...	4.33	181	241	422

It will be seen from the above table that in the final reactions alone can we trace any sign of progressive relation between the final measurements on the different media. These final reactions have been plotted against the initial reactions (see Fig. 5), and give an ordinary reaction resultant such as has been found characteristic for hydrochloric acid.

The gas analyses indicate that the products of fermentation are very similar, differing as they do to only the smallest degree. One sees too that the final amounts of gas produced are so close that they may be considered equal, as may also the total amounts of acid. What differences we have obtained in the individual experiments may fairly be considered as due to experimental errors.

These results show that the fermentation of *B. perfringens* is very little affected by the addition of hydrochloric acid, up to a quantity sufficient to give the critical reaction.

We hope to carry out similar experiments with the other acids involved, but such experiments are long and laborious and for the moment one can obtain a large amount of information merely from the reaction resultants.

Lactic and malic acids, which act as strong acids, apparently play a less passive role than hydrochloric acid. The final products, judging from the resultants, fall off somewhat as the initial acidity rises. With the weaker acids, succinic and butyric (and it should be noted that the absolute strength

of these acids in the presence of peptone water can be accurately judged from the titration curves), there is a greater inhibiting effect shown. Acetic acid, which behaves more or less as an intermediate acid from the point of view of strength, has a corresponding intermediate resultant.

Formic acid behaves slightly anomalously, apparently having a rather larger inhibiting effect than acids of similar strength. The aldehyde grouping of the acid may be a partial explanation of this fact.

Generally speaking, then, we find little specificity of action in the acids as regards the initial P_H , but a greater difference in effect towards the final reaction. To what can this be ascribed? The best explanation seems to be that the inhibiting action of these acids, as shown by slackened fermentations, is due to an effect of *mass*. It should be remembered that we have been working throughout over the same range of initial reactions; also that to produce these initial reactions a larger amount of a weak acid is necessary than of a strong acid. Therefore, if we assume that all of these acids, considered merely as foreign substances, have an effect in concentrated solutions independent of their P_H but proportional to their mass, we can see why in the case where a weak acid is used to give the initial reaction the fermentations are partially inhibited. On the other hand, a strong acid, such as hydrochloric acid, only present in small molecular quantities, has little effect.

Summing up therefore these results, it would seem that the seven acids under consideration have a definite effect on *B. perfringens* proportional to the concentration of their common cation—hydrogen ion. Their anions, on the other hand, and the undissociated acids merely behave as foreign bodies, possessing a certain inhibiting effect proportional to their molecular concentration.

At some future date we hope to show the effect of adding various amounts of neutral salts of these acids to peptone water. We can obtain a constant initial reaction by adding hydrochloric acid which we have shown to have little effect on the fermentations. By this method we shall be able to estimate accurately the effect of the anions of the acids.

BACILLUS SPOROGENES.

Experiments similar to those described above with *B. perfringens* were carried out with *B. sporogenes*. Glucose peptone water was again used as the fermentation medium, and the effect was tried of the following acids: hydrochloric, formic, lactic, acetic and butyric.

With the *B. sporogenes* the fermentation is accompanied by the formation

of a base, ammonia, which, although large amounts of acid are produced, causes the final reaction to be less acid than those resulting from *B. perfringens*.

The experimental details were similar. Readings were taken of the final reactions and the reaction resultants plotted.

Fig. 7

B. sporogenes acts rather slower than *B. perfringens* and it was found necessary to continue the incubation for a fourth day, making ninety-six hours in all, before the final reactions were determined.

Experiment No. 10 (Laboratory Experiment R. S. 9). Hydrochloric acid.
Data (see also Fig. 7).

Series A. Inoculated with D 74 (forty-eight hours old on cooked meat).

Series B. Inoculated with D 75 (1) (forty-eight hours old on milk).

Series C. Inoculated with D 75 (2) (forty-eight hours old on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tubes to ferment were Nos. 6, Series B and C, with value of $P_H = 4.99$.

The next set of tubes, with $P_H = 4.75$, failed to ferment.

TABLE XIII. (*Exp. 10.*)

No. of tube	Initial re-action, P_H	Series A		Series B		Series C	
		Result	Final re-action, P_H	Result	Final re-action, P_H	Result	Final re-action, P_H
1	6.63	+	5.51	+	5.45	+	5.52
2	6.03	+	5.39	+	5.34	+	5.35
3	5.78	+	5.37	+	5.31	+	5.31
4	5.52	+	5.22	+	5.20	+	5.23
5	5.30	+	5.07	+	5.10	+	5.08
6	4.99	-	...	+	4.91	+	4.90
7	4.75	-	...	-	...	-	...
8	4.60	-	...	-	...	-	...

Experiment No. 11 (Laboratory Experiment R. S. 11). Lactic acid.

Data (see also Fig. 7).

Series A. Inoculated with D 74 (forty-eight hours on cooked meat).

Series B. Inoculated with D 75 (1) (forty-eight hours on milk).

Series C. Inoculated with D 75 (2) (forty-eight hours on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tube to ferment was No. 5, Series C, with value of $P_H = 5.05$.

The next set of tubes, with $P_H = 4.70$, failed to ferment.

TABLE XIV. (*Exp. 11.*)

No. of tube	Initial re-action, P_H	Series A		Series B		Series C	
		Result	Final re-action, P_H	Result	Final re-action, P_H	Result	Final re-action, P_H
1	6.63	+	5.51	+	5.45	+	5.52
2	6.20	+	5.49	+	5.34	+	5.37
3	5.78	+	5.32	+	5.25	+	lost
4	5.46	+	5.20	+	5.17	+	5.17
5	5.05	-	...	-	...	+	5.10
6	4.70	-	...	-	...	-	...
7	4.56	-	...	-	...	-	...
8	4.38	-	...	-	...	-	...
9	4.28	-	...	-	...	-	...

Experiment No. 12 (Laboratory Experiment R. S. 15). Formic acid.

Data (see also Fig. 7).

Series A. Inoculated with D 82 (ninety-six hours old on milk).

Series B. Inoculated with D 83 (ninety-six hours old on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tube to ferment was No. 5, Series B, with value of $P_H = 5.06$.

The next set of tubes, with $P_H = 4.77$, failed to ferment.

TABLE XV. (*Exp. 12.*)

No. of tube	Initial re-action, P_H	Series A		Series B	
		Result	Final re-action, P_H	Result	Final re-action, P_H
1	6.44	+	5.45	+	5.45
2	6.10	+	5.43	+	5.53
3	5.74	+	5.39	+	5.33
4	5.42	+	5.23	+	5.22
5	5.06	-	...	+	5.15
6	4.77	-	...	-	...
7	4.60	-	...	-	...
8	4.44	-	...	-	...

Experiment No. 13 (Laboratory Experiment R. S. 14). Acetic acid.

Data (see also Fig. 8).

Series A. Inoculated with D 81 (forty-eight hours old on milk).

Series B. Inoculated with D 80 (forty-eight hours old on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tubes to ferment were Nos. 4, Series A and B, with value of $P_H = 5.21$.

The next set of tubes, with $P_H = 4.95$, failed to ferment.

TABLE XVI. (*Exp. 13.*)

No. of tube	Initial re-action, P_H	Series A		Series B	
		Result	Final re-action, P_H	Result	Final re-action, P_H
1	6.52	+	5.39	+	5.40
2	5.98	+	5.37	+	5.35
3	5.56	+	5.30	+	5.29
4	5.21	+	5.29	+	5.10
5	4.95	-	...	-	...
6	4.81	-	...	-	...
7	4.75	-	...	-	...
8	4.65	-	...	-	...
9	4.60	-	...	-	...

Experiment No. 14 (Laboratory Experiment R. S. 10). Butyric acid.

Data (see also Fig. 8).

Series A. Inoculated with D 74 (forty-eight hours old on cooked meat).

Series B. Inoculated with D 75 (1) (forty-eight hours old on milk).

Series C. Inoculated with D 75 (2) (forty-eight hours old on milk).

The tubes were examined ninety-six hours after inoculation.

TABLE XVII. (*Exp. 14.*)

No. of tube	Initial re-action, P_H	Series A		Series B		Series C	
		Result	Final re-action, P_H	Result	Final re-action, P_H	Result	Final re-action, P_H
1	6.63	+	5.51	+	5.45	+	5.52
2	6.20	+	5.46	+	5.37	+	lost
3	5.80	+	5.49	+	5.39	+	5.48
4	5.47	+	5.42	+	5.35	+	5.23
5	5.26	+	5.17	+	5.18	+	5.34
6	5.02	+	5.15	+	5.10	+	5.08
7	4.90	-	...	-	...	-	...
8	4.80	-	...	-	...	-	...
9	4.76	-	...	-	...	-	...

The most acid tubes to ferment were Nos. 6, Series A, B and C with value of $P_H = 5.02$.

The next set of tubes, with $P_H = 4.90$, did not ferment.

When inoculated into glucose peptone media at different reactions *B. sporogenes* behaves similarly to *B. perfringens*. In the first place the higher the acidity of the reaction the longer is the latent period before obvious signs of growth are seen. There also seems to be a fairly well defined critical reaction at which growth is just permitted, as can be seen from the table below.

TABLE XVIII.

Experiment No.	Acid	Most acid value of P_H which allows growth	Least acid value of P_H which inhibits growth	Mean value of P_H . Critical P_H
10	Hydrochloric	4.99	4.75	4.87
11	Lactic	5.05	4.70	4.87
12	Formic	5.06	4.77	4.91
13	Acetic	5.21	4.95	5.08
14	Butyric	5.02	4.90	4.96

Mean value of critical $P_H = 4.94$

This critical reaction, $P_H = 4.94$, is very close to the value obtained for *B. perfringens*. These two organisms, *B. sporogenes* and *B. perfringens*, are widely different in character and in chemical behaviour, yet they are both equally affected by the initial hydrogen ion concentration of the medium into which they are implanted.

When the final reactions of *B. sporogenes* are considered, the matter is found to be complicated by the formation of both acids and bases. In a previous paper we have shown that fermentation by *B. sporogenes* is accompanied by the production of large amounts of ammonia in addition to acids. This naturally affects the final reaction so that one is unable to draw such definite conclusions from the reaction resultants as could be done in the case of *B. perfringens*.

The reaction resultants, however, show a similar orderly relation between the initial and final reactions. This is the more surprising when one considers the complicated character of the fermentations. In spite of the formation of several distinct acids, large amounts of amino-acids and ammonia, all having a great influence on reaction either as sources of H-ions or OH-ions, or merely as buffers, this orderly reaction persists. Obviously the various phases of fermentation must be very definitely inter-related in order that this nicely balanced state of affairs should exist.

It will be observed that the reaction resultants in most cases cross the line *AB*, the hydroxyl ions of the ammonia actually causing a decrease in the acidity of the medium after a certain stage in the fermentation is reached. To this is no doubt due the ability of *B. sporogenes* to ferment for a very long period. The cessation of activity is probably caused almost entirely by the accumulation of the products of metabolism, and very little by the inhibiting influence of hydrogen ions.

SUMMARY.

An investigation has been made on the effect of acids on the growth of *Bacillus perfringens* and *Bacillus sporogenes*. This was preceded by an examination of the behaviour of the acids used with nutrient media.

The addition of acids to liquids containing large amounts of buffer substances—to which category all efficient nutrient media belong—produces a complex effect on the true reaction of the media. This effect is partly determined by the type of acid employed and partly by the nature and content of buffer substances in the mixture. With the less highly dissociated acids a point is soon reached where successive amounts of added acid affect the reaction but slightly.

The action of acids on the growth of the bacteria in question can be divided into three parts.

The first effect noticed is on the latent period of growth. The more highly acid the medium, the greater time elapses before obvious signs of growth are observed.

The second effect is on the final reaction at which growth ceases. While the limits for the cessation of growth with all acids are comparatively narrow, the differences observed are distinct and obviously governed by definite laws. They are quite wide enough apart to negative the idea of a "physiological constant." The final reaction is undoubtedly dependent on the initial reaction. With a given acid, and varying initial concentrations, a series of final reactions is obtained which can be expressed by a curve. Each acid shows a curve which is individual and to this extent we may speak of specificity of acid effect. The difference between the acids is not large.

The third feature of the action of acids on these bacteria is the total inhibition of growth.

This varies for *Bacillus perfringens* between $P_H = 4.99$ using butyric acid and $P_H = 4.66$ using malic acid, and has an average value of $P_H = 4.82$,

which we have called the critical reaction. This, for practical purposes, may be considered the inhibiting reaction.

It will be observed that for *B. perfringens* the hydrogen ion concentration for inhibition may be lower than that reached by the medium at the end of fermentation. That is to say, after overcoming the inhibiting influence of a certain hydrogen ion concentration (as shown by the lengthened latent period) the bacillus causes a rise in concentration of hydrogen ion by producing acids. During fermentation a secondary toxic effect is observed. This is due to the accumulation of metabolic products, and to the influence of the anions and the undissociated molecules of the acid initially present. Fermentation therefore ceases when different amounts of acid have been formed. The final hydrogen ion concentration also varies. When a highly dissociated acid has been used to produce the initial reaction, and the high concentration of hydrogen ion has been produced by the addition of a small quantity of acid, the acid and gas production is little affected. This is shown in Experiment 9, where hydrochloric acid was used for this purpose. With feebly dissociated acids, much larger amounts are necessary to produce the same hydrogen ion concentration, and the acid exerts an effect which is not only due to the cation. The mass of the acid molecule inhibits fermentation, and smaller amounts of acid fermentation products are formed. Hence the toxic action of a weak organic acid is twofold.

With *B. sporogenes*, inhibition takes place at an average value of $P_H = 4.94$. This organism differs from *B. perfringens* in that the hydrogen ion concentration of the fermented medium may be lower than that of the medium before inoculation, although large amounts of acid have been formed. Obvious signs of growth may cease to be observed at a concentration of hydrogen ions which is less than the critical reaction. This is to be explained by the complex character of the katabolism of this organism whereby large amounts of basic and amphoteric substances are produced and neutralise the acids, as well as acting as buffers.

Stoppage of growth is probably due almost entirely to the accumulation of metabolites. The tendency of *B. sporogenes* to produce a constant or even lower hydrogen ion concentration than that observed at the time of inoculation may account for the fact that fermentations with *B. sporogenes* may continue for very long periods.

In conclusion one may say that the fermentation of both these organisms is inhibited by a rise in hydrogen ion concentration. The acidity may merely delay growth, or it may stop it entirely. The effect of all the acids we have

used in the course of these experiments is alike. Specific qualities are almost entirely absent.

These experiments seem to show that by submitting the organisms involved in gas gangrene infections to solutions of a hydrogen ion concentration slightly above the critical point growth may be inhibited. They afford confirmation of the views expressed in our first paper that the treatment of local infections of this type by means of acid solutions, highly buffered, is worthy of trial.

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