XX. CONTRIBUTIONS TO THE BIOCHEMISTRY OF PATHOGENIC ANAEROBES. II. THE ACID PRODUCTION OF *BACILLUS WELCHII* (*B. PERFRINGENS*) AND *BACILLUS SPOROGENES* (METCHNIKOFF).

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In a recent paper, one of us with Harris [Wolf and Harris, 1917] has shown that, in the fermentation with *Bacillus Welchii* (*B. perfringens*), all activity ceases when the reaction of the medium reaches a certain hydrogen ion concentration. The cessation of growth appeared to be connected with the production of acids. We were able to confirm the statements of previous observers, who have worked on acid production, that normal butyric acid was one of the constituents of the fermentation liquid, by isolating the acid in a fairly pure condition and identifying it. Other acids were also present.

With the *B. sporogenes* of Metchnikoff, there was also a large amount of acid formed when the organisms acted on various media. In milk it was difficult to say from what component of the medium the acid arose.

Owing to the difficulties in titrating a complex medium, containing buffer salts and amphoteric nitrogenous compounds, it was impossible to ascertain what were the amounts of acids that these bacteria produced.

As the production of acids by these two organisms has been advanced to account for certain clinical features in gas gangrene infections [Wright, 1917], it appeared advisable to devote some attention to an investigation of this property.

For some years the only method of estimating quantitatively the volatile acid production of micro-organisms has been that of Duclaux [1900]. Owing

to certain inherent difficulties in the method it has not been very largely used by biochemists. Recently there appeared an article by Dyer [1917], in which he described a new method of dealing with the volatile fatty acids in mixtures and suggested that it might be employed in the study of acid production in bacteriological media. Briefly stated, this method consists of a determination of the distilling rate in steam of an acid solution when the latter is maintained at constant volume. The distillate is collected in portions and titrated. The number of cc. of alkali required to neutralise each portion is noted and the percentage of acid calculated. The amount of acid in a given fraction, the "Distilling Constant" for the fraction, is plotted against the corresponding amount of the distillate on logarithmic coordinate paper. Pure acids are graphically represented as straight lines. With mixtures of acids, curves are obtained, the nature of which depends on the acid components.

With a mixture of any two volatile acids, the first part of the curve which is obtained occupies a position intermediate to the lines representing the distilling rate of the higher and lower boiling acids. As the higher boiling acid is removed, the curve gradually becomes parallel with the line representing the lower boiling acid. This serves to identify the lower acid with certainty. Dyer showed that it was possible to ascertain the amount of each acid present when the distilling rate of the mixture had been determined, and the acids present were known. He also gave a number of chemical tests to enable one to identify the acids present in the distillates.

The method appeared to present obvious advantages for the work in which we were engaged, and we therefore made a certain number of preliminary experiments before using it on fermentation mixtures.

Our apparatus differed in one essential from that of Dyer, in that the heating of both flasks was done by gas instead of electricity. By using von Babo's flask guards underneath the distilling and steam generating flasks, and especially by insulating heavily all connecting tubes throughout the apparatus, it was possible to regulate the heating flame, so that the volume of the acid mixture kept constant through a long distillation.

Our first experiments were made with pure acids. The acids chosen were those that are known to be present in fermentation liquors, and ranged from formic to caproic acids.

The distilling rates of these acids were then graphically represented on a logarithmic chart, and the lines which were obtained were used to interpret the results of other distillation experiments. The distilling rates found were very similar to those obtained by Dyer.

Dyer used his method to identify the lower boiling acid in any given mixture by means of the parallelism of the curve representing the last portion of his distillate to a known curve. He identified the higher boiling acid by means of chemical tests. Working on artificial mixtures of known acids, this is satisfactory. With an unknown mixture of several acids resulting from a fermentation experiment, this does not apply. We were therefore compelled to modify Dyer's method in a manner which made it much more tedious, but ultimately allowed us to arrive at more satisfactory results.

The reasons for making this change were based on the following considerations. When a mixture of two acids is distilled the nature of the curve obtained will depend on the distilling rate of each component. With a mixture containing an acid high in the series, for example, caproic acid, the first part of the curve will approximate to the straight line on the chart which represents caproic acid, and the degree of this approximation will depend on the relative amount of that acid present.

The final part of the curve will become parallel to the straight line on the chart which represents the lower acid.

If present in small amount the higher acid can be concentrated and partially separated from the lower acid by fractionation so that the curve obtained on redistillation will approximate so closely to the acid line for caproic acid that it can thereby be identified.

That is, we applied Dyer's method to determine the nature of fractionated portions of the fermentation liquid; and since the amount of acid in each of these portions was known, the calculation of the separate acids in the fermentation liquid could be made. Using caproic acid and butyric acid in a mixture, by means of this method of fractionation it was possible to obtain two portions of the mixture, one containing a relatively large amount of caproic acid and the other consisting chiefly of butyric acid. When distilled separately the curves obtained approximated to the lines for caproic acid and butyric acid on the chart.

When more than two acids are present the problem of identifying and estimating them becomes complicated. The difficulty is always with the acids of intermediate boiling point and appears in any method of separation of the acid series. In the Duclaux method this is emphasised in a recent criticism by Upson, Plum and Schott [1917]. In our opinion, Dyer's method affords no solution of this problem of dealing with the intermediate acids of a mixture. In conjunction with it we have had to resort to a process of fractionation which we describe below.

For example, in a mixture containing acetic, butyric, valeric and caproic acids, it is impossible without a number of refractionations, which is beyond the scope of ordinary technique, to separate these acids so that the colour tests for each will be distinctive. Moreover, the colour tests, which are undoubtedly helpful in determining what acids are present, are not specific; and unless the acid is present in sufficient quantity a positive result will not be obtained.

In making a quantitative analysis of a fermentation liquid we found it necessary to proceed in the following way:

- (1) To distil a portion exhaustively in order to find the total amount of volatile acid present.

- (2) To distil the fermented liquid as a simple acid mixture, according to Dyer's method, and graphically represent the distilling rate on a logarithmic chart. The curve obtained indicates the lowest acid and the probable nature of the highest acid in the liquid.

- (3) To collect fractionated portions of a series of separate steam distillations conducted under conditions identical with the above. The total amount of acid in these portions can be calculated from the previously ascertained distilling rate of the mixture.

The application of chemical tests to the concentrated distillates of these fractions affords useful information at this stage.

- (4) Finally, from consideration of the data so obtained to prepare an artificial mixture which will show a distilling rate sufficiently close to that of the fermentation liquid to warrant the assumption that they correspond in composition.

As will be seen from the above, the method is tedious in application, but it certainly gives important quantitative information regarding the acids produced by various organisms. At the same time it must be emphasised that the process above described will afford only approximate results.

After a large amount of preliminary work on mixtures of pure acids from which the limit of accuracy of the method was ascertained, we proceeded to examine some of the fermentation liquids yielded by *B. perfringens* and *B. sporogenes*.

The organisms were grown in vacuo in a Winchester quart bottle in various media.

The apparatus used for incubation was that employed by Wolf and Harris [1917]. Usually 1500 cc. of culture medium was employed, and samples taken from time to time. In all instances the production of gas was followed to correlate gas production with amount of acid produced.

FERMENTATION No. 1.

Bacillus sporogenes (Metchnikoff) on milk.

100 cc. of the sample of liquid were taken from the fermentation flask under aseptic precautions, placed in the distilling flask and rendered acid to Congo red paper by the addition of normal sulphuric acid.

The volume was then made up to 150 cc. and the flask connected to the condenser. The burner employed to heat the flask was placed in position, and at the first appearance of bubbles in the liquid, the gas jet was lowered so as to give a definite size of flame. The steam generating flask which contained water actively boiling was immediately connected to the distilling flask and the distillation begun. Fractions of 10 cc. were collected up to 50 cc. Then successively larger fractions of 50 cc., 100 cc., 200 cc., and 500 cc. were collected. It was usually found unnecessary to continue the distillation beyond 500 cc. in order to obtain sufficient data for the acid curve.

The total amount of acid present was obtained by exhaustively distilling another portion of the original liquid in a separate apparatus, and titrating the distillate with decinormal sodium hydrate. In all these experiments phenolphthalein was the indicator used.

TABLE I. *Fermentation at the end of 117 hours.*

100 cc. liquid = 47.6 cc. *N*/10 sodium hydrate.

Fraction of dist. in cc. ...	10	20	30	40	50	100	200	400	500
% acid in fraction ...	15.3	26.0	34.6	41.9	47.6	64.6	76.5	87.2	91.0

The figures in the lower column denoting the "Distilling Constants" were plotted on the chart against the corresponding fraction of the distillate.

From a consideration of the curve obtained (Fig. 1), it will be seen that the fermentation liquid contains a mixture of higher and lower volatile fatty acids, and that the curve tends to run parallel with the curve for acetic acid, after 75 % has distilled over.

The "distilling constant" for the first 10 cc. fraction (15.3) is very high when the presence of acetic acid in the mixture is taken into account, and caproic acid or higher acids were therefore suspected.

On applying Dyer's colour tests to the first portions of the distillate the presence of caproic acid was confirmed. The process of fractionating the fermentation liquid was therefore next employed in the following manner.

Fractional distillation.

100 cc. of the liquid were acidified with decinormal sulphuric acid and the volume made up to 150 cc. This was then steam distilled under the usual conditions of constant volume.

The first 25 cc. were collected and labelled "A." The intermediate portion distilling over between 25 cc. and 75 cc. labelled "B"; and the third portion distilling over between 75 cc. and 175 cc. labelled "C."

Fig. 1

This was repeated with other three portions of 100 cc. of the fermentation liquid. The four "A" fractions of 25 cc. were then mixed, an aliquot part drawn off and titrated; and the remainder placed in the distilling flask and the volume made up to 150 cc. This was steam distilled. The results were as follows:

TABLE II. "*A*" portion = 31 % of total acid.

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	20.5	36.0	47.6	57.1	64.4	83.3	92.8	95.7

On examining this curve ("*A*" Fig. 1), it will be seen that it runs very nearly parallel with the line of *n*-butyric acid after 65 % of the total acid has distilled over.

The very high distilling constant for the first 10 cc. indicates that caproic acid was probably present, since it approximates to the "distilling constant" for that acid which is 22.6 %.

After concentrating the distillates caproic acid was definitely proved to be present by the colour tests. In this portion there must have been an almost complete absence of acids lower in the series than butyric acid, so that the 31 % of the acid of the fermentation liquid consisted almost entirely of acids high in the series and of these caproic and butyric acids were two.

The intermediate portion "*B*" was dealt with in a similar way, but since the mixed fraction constituting this portion amounted to 400 cc. it was evaporated on the water-bath after neutralisation with decinormal sodium hydrate and made up to 100 cc. exactly. 25 cc. of this solution were put in a separate apparatus, acidified with normal sulphuric acid to liberate the acids and exhaustively distilled. From the titration of this distillate the total amount of acid in the remaining 75 cc. was calculated.

The remaining 75 cc. were then acidified with normal sulphuric acid, made up to 150 cc. and steam distilled.

TABLE III. "*B*" portion = 32 % of total acid.

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	20.4	35.6	45.0	50.4	55.4	68.0	81.7	93.0

The very high "distilling constant" (20.4) obtained for the first fraction indicates that caproic or higher acids were also present in this fraction. The curve ("*B*" Fig. 1) takes a very decided bend near the point when 55 % of the acids have distilled over indicating that these are of a mixed nature. With ferric chloride and ether a test corresponding to valeric acid was given, but the colour test for caproic acid was not positive.

This fraction, amounting to 32 % of the total acid of the fermentation liquid, consisted then of valeric acid as well as of lower acids. The amount of caproic acid, which was undoubtedly present, was evidently too small to give a positive colour test.

The third portion "*C*" was dealt with in exactly the same way as the

preceding. It corresponded to 21 % of the total acid in the fermentation liquid.

The results of the distillation were as follows:

TABLE IV. "C" portion = 21 % of total acid.

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	14.9	24.0	31.6	37.3	42.5	59.9	76.0	89.8

When the distillates from this portion were concentrated and the ether-ferric chloride test applied none of the precipitated iron salt was found to be soluble in the ether solution, indicating the absence in quantity of acids higher than butyric in this portion. The last part of the curve ("C" Fig. 1) was almost parallel with the normal curve for acetic acid. Formic acid was never detected in any portion of the distillate.

This fraction, corresponding to 21 % of the total acid in the fermentation liquid, must have had butyric acid as its chief constituent along with a certain amount of acetic acid.

An analysis of the above results leads to the following conclusions:

(1) Caproic, valeric, butyric and acetic acids were present in the fermentation liquid since these have been demonstrated in the various fractions.

(2) From "A" portion (Table II), that 31 % of the acid of the fermentation liquid consisted almost wholly of butyric, valeric, caproic and higher acids.

(3) In the "B" portion (Table III), which corresponded to 32 % of the total acids in the fermentation liquid, about one-half consisted of butyric and valeric acids.

(4) Acetic acid was present in considerable quantity both in the "B" and "C" portion.

(5) Formic acid was absent.

It was impossible to estimate with any degree of accuracy the amount of these acids present. Nor could the presence or absence of propionic acid be definitely established either by colour tests or by consideration of the acid curves obtained.

The remaining quantity of acid which had not been dealt with as a fraction was equivalent to 16 % of the total acids in the fermentation liquid. It must have consisted chiefly of lower acids, probably acetic acid.

The problem then was to determine in what proportion the various acids were present. After some consideration it was decided that an attempt might be made to arrive at a solution by distilling artificial mixtures of caproic, valeric, butyric and acetic acids the result of which would give a close approximation to the figures obtained in distilling the fermentation liquid.

In making up such a mixture the calculation of the relative quantities of the components to be chosen was based on the rough estimate of the composition of the fermentation liquid which has just been made.

After several trials the following mixture was distilled and the results tabulated:

Acetic acid	25.2 %
Butyric „	17.2
Valeric „	16.2
Caproic „	41.4

TABLE V.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	14.9	25.6	34.1	41.4	47.6	65.2	77.5	87.7	90.0

The distilling rate of this mixture is an approximation to that of the fermentation liquid, as will be seen when the two tables are compared. It is clear however that the composition of the artificial mixture cannot be regarded as any more than this. No account has been taken of the presence or absence of propionic acid, or of small quantities of acid higher in the series than caproic acid; and it was found that the amount of intermediate acids could be varied considerably without appreciably changing the distilling rate of the mixture.

FERMENTATION No. 2.

B. perfringens on milk.

The fermentation liquid obtained by the growth of *B. perfringens* on milk under strict anaerobic conditions was subjected to a similar examination. As before, the sample was drawn off at the end of the fermentation when no further gas was evolved, and a quantity distilled exhaustively to determine the total amount of volatile acids present. 100 cc. of the liquid were then steam distilled under the usual conditions, the distilling rate of the acid mixture determined, and the results plotted on the logarithmic chart.

TABLE VI.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	8.4	15.8	22.3	28.2	33.3	52.3	71.3	86.6	91.3

From a consideration of the curve (Fig. 2) it will be seen that 52.3 % of the whole distills over in 100 cc., suggesting that butyric acid and some lower acids were present in the mixture. The presence of valeric acid and caproic acid could not be detected by colour tests when these were made, using the

first portion of the distillate, and these acids could only have been present in small quantities.

Fig. 2

Fractional distillation.

Three portions of 100 cc. each of the fermentation liquid were acidified with normal sulphuric acid and the volumes made up to 150 cc. and steam distilled. The first fractions of 25 cc. were collected and mixed. After titration of an aliquot part the remainder was steam distilled. The quantity of acid used represented 20 % of the total acid present in the fermentation liquid.

TABLE VII.

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	12.3	21.7	29.6	36.8	43.3	61.6	79.6	97.4

Acids higher in the series than butyric could not be detected by colour tests in this portion which probably consisted of butyric and lower acids. This is evident from the nature of the curve which finally becomes parallel to that of acetic acid. Also the "distilling constant" for the first 10 cc. (12.3) is considerably higher than that of propionic acid. Since valeric and higher acids are not present in any great quantity, butyric acid only could account for this fairly high figure. It was unnecessary to consider further fractionation since the assumption was that butyric and acetic acids constituted this mixture.

Assuming that the fermentation liquid consisted chiefly of butyric acid and acetic acid, it should be possible to find out what amount of each was present from its "distilling constant" by means of a simple calculation.

The "distilling constant" for 50 cc. of the fermentation

liquid containing the mixed acids	...	...	...	= 33.3 %
"Distilling constant" of butyric acid	...	...	...	= 45.6
"Distilling constant" of acetic acid	...	...	...	= 16.5

So that such a mixture would correspond approximately to 42 % acetic acid and 58 % butyric acid.

A mixture containing 40 % acetic acid and 60 % butyric acid was distilled under the usual conditions with the following results:

TABLE VIII. *Total acid = 63.9 cc. N/10 sodium hydrate.*

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	8.6	15.8	22.4	28.5	33.8	53.5	73.9	89.0	92.5

When this table is compared with Table VI it will be seen that the fermentation liquid obtained from the action of *B. perfringens* on milk consisted approximately of the volatile fatty acids, butyric and acetic, in the proportions distilled.

It is of course open to objection that no account has been taken of the possible presence of propionic acid or of the acids higher in the series.

The colour test for propionic acid which is given by Dyer is perhaps the least definite of all; and since this acid occupies a position intermediate to butyric and acetic acids an attempt at isolation would entail a long and careful fractional distillation.

The artificial mixture of butyric and acetic acids can therefore only be regarded as an approximation to the composition of the fermentation liquid.

FERMENTATION No. 3.

B. sporogenes on 2 % glucose peptone.

100 cc. of the liquid at the end of fermentation was placed in the distilling flask, rendered acid to Congo red with normal sulphuric acid and the volume made up to 150 cc.

This was steam distilled under constant volume and the fractions collected and titrated as before.

The results given in Table IX were plotted on the chart. The total amount of acid present was obtained by distilling a separate 100 cc. exhaustively.

TABLE IX.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	7.2	13.2	18.7	23.8	28.5	44.9	65.5	86.1	92.4

From a consideration of the curve obtained it was seen that the final part tended to become parallel with the line for acetic acid. The "distilling constant" for the first 10 cc. fraction is 7.2, that for the first 10 cc. of a solution of pure propionic acid is 7.03. But since acetic acid is present in the mixture, the "distilling constant" of which is 3.5, an acid with a much higher distilling rate than propionic acid must be present.

Colour tests were applied to the collected distillates and also to fractions which contained the higher acids in greater proportion. In none of these was the presence of valeric acid or acids higher in the series shown.

The acid associated with acetic acid in the fermentation liquid must then have been butyric acid, the "distilling constant" for the first 10 cc. of which is 11.47 %.

In order to confirm the presence of this acid the fermentation liquid was subjected to a fractional distillation as before, the higher acids being obtained in more concentrated form. The following table shows the results obtained from the redistillation.

TABLE X. *Fraction = 15 % of total acid.*

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	10.7	19.8	27.7	34.4	39.9	58.9	77.2	91.5	95.4

The first 10 cc. gives a "distilling constant" of 10.7 %. This indicates conclusively the presence of butyric acid in the fermentation liquid.

Assuming that butyric and acetic acids alone were present, a mixture containing these in pure form was made up and redistilled. The proportion in which they were mixed was obtained, as in the previous case, by a calcula-

tion based on the distilling rates of the fermentation liquid, pure acetic acid and pure butyric acid.

The "distilling constant" for the first 50 cc. of the

fermentation liquid = 28.5 %

"Distilling constant" of butyric acid = 45.6

"Distilling constant" of acetic acid = 16.5

To obtain a mixture of butyric and acetic acids in which the first 50 cc. shall contain 28.5 % of the total acid, these acids must be present in the following proportion:

Butyric acid = 41 %

Acetic „ = 59

Such a mixture when distilled gave the following results:

TABLE XI.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	7.0	13.3	18.9	23.8	28.3	45.5	64.6	84.6	90.9

Comparing this table with the results obtained from the distillation of the fermentation liquid shown in Table IX it will be observed that the latter must be similar in composition to the artificial mixture.

As before, no account has been taken of the presence or absence of propionic acid in the fermentation liquid, because it was impossible by colour tests or a consideration of the curve to determine its presence or absence. The close approximation of the curve of the above artificial mixture to that of the fermentation liquid cannot be taken as proof that the fermentation liquid contained no propionic acid. By the introduction of a certain quantity of propionic acid into a mixture of butyric and acetic acids a curve almost identical with that of acetic and butyric acids could be obtained.

The following example will illustrate this:

A mixture of butyric and acetic acids in almost equal parts was distilled under the usual conditions, and the distilling rate obtained.

The same mixture of these two acids was taken and 20 % of propionic acid added. This second combination was distilled under the same conditions as the first.

The results showing distilling rates of the two mixtures are tabulated for comparison.

TABLE XII.

Fraction in cc.	10	20	30	40	50	100	200	400	500
Butyric and acetic acid	8.0	14.9	21.1	26.6	31.7	51.2	73.4	91.0	95.0
Butyric and acetic acid with 20 % propionic	7.7	14.5	20.5	26.1	31.2	51.0	73.1	90.3	94.1

Here we have two acid mixtures differing widely in composition yet giving almost identical distilling rates.

This experiment could be repeated with a combination of any three other consecutive acids in the series, so as to demonstrate generally that the introduction of the "intermediate" acid in moderate amount has little effect on the distilling rates of the combination of the other two.

There appears to be no way out of this difficulty of dealing with the "intermediate" acid except by a long and tedious fractionation.

FERMENTATION No. 4.

B. perfringens on 2 % glucose peptone.

100 cc. of the sample at the end of fermentation was taken, acidified with normal sulphuric acid till acid to Congo red, the volume made up to 150 cc. and distilled as before.

The following results were obtained :

TABLE XIII.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	7.4	13.3	18.1	22.5	26.7	43.7	65.0	87.6	93.5

Comparing this with the results of the previous experiment, it will be seen that the volatile fatty acids produced by the action of *B. perfringens* and *B. sporogenes* on 2 % glucose peptone are almost identical. Also the total quantity of volatile acid formed by each organism was found to be the same. This fact is interesting when the great difference in the nature of the acid production by the growth of the organisms on milk is considered. Large quantities of valeric and caproic acids result from the action of *B. sporogenes* on the latter medium and this distinguishes this organism at once from *B. perfringens* which apparently produces very small amounts of these higher acids. A single distillation of a fermented milk would always serve to differentiate the two, but this would be impossible if the medium were glucose peptone.

On reviewing the above experiments we concluded that Dyer's method may be employed with limitations in the study of volatile acid production of fermented media.

It is impossible by a single distillation alone to determine from the distilling rates of the mixture the nature of the acids present. Partial isolation of the constituents is necessary to establish both the nature and the amount of each acid present. Dyer's colour tests are only of value when the acid exists in an almost pure state.

By employing the method of partial fractionation which has been described, an approximate analysis of the volatile acids present may be made. As we have shown, exact confirmation of such an analysis cannot be obtained by identifying the distilling rate of the fermentation liquid with that of an artificial mixture. As a means of obtaining comparative results the scheme of analysis which has been outlined above is decidedly useful.

When the distilling rates of a fairly large number of artificial mixtures have been graphically represented on a chart, the approximate composition of a fermented medium can at once be obtained simply by identifying the mixture with which its distilling rate coincides.

Non-volatile acid production.

The acidity of these fermentation liquors was found to be due partly to the formation of a relatively large quantity of non-volatile acids. The nature of these acids is not discussed in this paper. Their estimation was made solely to demonstrate that they cannot be neglected in any consideration of the effects of acid production. The following method was employed for their determination.

100 cc. of the fermentation liquid was acidified with normal sulphuric acid and exhaustively steam distilled. The distillate was titrated with decinormal sodium hydrate, using phenolphthalein as an indicator, and the total amount of volatile acids present ascertained.

The residue in the distilling flask, containing the non-volatile portion, was transferred to a liquid extraction apparatus and the acids dissolved out with ether. This operation was a continuous one, and extended over a period of forty-eight hours.

The ethereal solution was evaporated to dryness and the residue dissolved in water. The solution containing the acids was then filtered and titrated with decinormal sodium hydrate.

The following table shows the results obtained in the examination of the fermentation liquors which were produced by the action of *B. sporogenes* and *B. perfringens* on milk and 2 % glucose peptone.

Organism	Medium	Relative vol. acidity	Relative non-vol. acidity
<i>B. sporogenes</i>	{ Milk	69 %	31 %
	{ 2 % glucose peptone	55	45
<i>B. perfringens</i>	{ Milk	68.5	31.5
	{ 2 % glucose peptone	50	50

SUMMARY.

The investigation of the acid production of our fermentations of *Bacillus perfringens* and *Bacillus sporogenes* shows that large quantities of volatile acids are produced. Of these butyric acid may be said to be a constant component, but it is worthy of note that in the action of *B. sporogenes* on milk, acids higher in the series than butyric are formed in considerable quantity. The action of *B. sporogenes* on milk results in the production of caproic and valeric acids. The presence of these higher acids is not however a specific quality of *B. sporogenes*, for the fermentation of glucose peptone by this organism produces only acetic and butyric acids. We have been unable to detect propionic acid in any of the fermentation liquids, but its presence is not excluded. Formic acid was not present.

In addition to volatile acids, there is a notable proportion of non-volatile acids, amounting roughly to 40 % of the total acid production. What the nature of these acids is has not yet been ascertained.

An experimental critique of the Dyer method of estimating volatile fatty acids has been made. This method, while perfectly satisfactory in the form stated by its author in dealing with a mixture of two volatile acids, the nature of which is known, fails when a mixture of unknown acids has to be analysed. The colour tests proposed by him are satisfactory when dealing with pure acids, but are not as positive as could be desired for the identification of an acid in the mixture. The separation of the acids is necessary before any reliance can be placed on these colour tests.

By refractionation clearer results are obtained; but when a mixture of more than two acids is dealt with, the components of which are consecutive in the acid series, the task of identifying and estimating them is a problem of extreme difficulty.

To what extent Dyer's method is applicable for this purpose has been indicated in the foregoing paper.

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