CONTRIBUTIONS TO THE BIOCHEMISTRY OF PATHOGENIC ANAEROBES.¹

IV.

THE BIOCHEMISTRY OF *BACILLUS HISTOLYTICUS*.

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IN the first paper of this series (1917⁶) we investigated the protein and carbohydrate metabolism of *Bacillus sporogenes* and *Bacillus welchii*. It was there shown that the former organism has quite an unusual capacity for degrading protein to amino-acids and ammonia. At the same time, the two organisms produce large quantities of organic acids, of which butyric acid is the chief component. Small quantities of acetic acid are formed, and, in the case of *B. sporogenes* growing on milk, acids higher than butyric acid are produced.

Among the organisms which are associated with "gas gangrene infections," the one appearing to have the most destructive effect on tissue is *B. histolyticus*, described by Weinberg and Séguin in October 1916 (1916⁵).

That this organism is an important one is shown by the clinical histories which Weinberg and Séguin give. It was isolated in eight cases out of thirty where it was sought, and it is worthy of remark that out of these eight cases, four underwent amputation and four died. It would appear, therefore, to be associated with "gas gangrene infections" of a virulent type.

Experiments by Weinberg and Séguin show that it possibly has a pronounced symbiotic-like effect, for doses of *B. perfringens* and *B. sporogenes*, which in themselves were not lethal, were made so by the simultaneous injection of a non-lethal dose of *B. histolyticus*. The lesions produced by this organism are, according to these authors, of the most violent character. A glucose-bouillon culture injected in a dose of 0.25 c.c. into the thigh of a guinea-pig produces so great

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tissue destruction, that in a short time all connection of the tibia with the femur is dissolved and spontaneous amputation occurs—the bones being completely denuded. There are no signs of putrefaction, nor is there formation of gas. The growth of this organism tends to confirm the belief that its protein-splitting powers are extensive, for, distributed among the particles of an old cooked-meat culture, one finds bundles of white needles, which remind one of tyrosine, and are obviously an insoluble amino-acid produced by proteolysis.

As the question of the latent period in "gas gangrene infections" appears to be connected in one of its phases with the amino-acid content of the substrate, it seemed to us important to devote some attention to an organism whose digestive action on protein is so pronounced. The strain which we used in the present investigation was procured from Captain H. Henry, R.A.M.C., and was originally isolated by M. Weinberg at the Institut Pasteur. It corresponds with the description originally given by Weinberg and Séguin, except that when grown for a long time on a large scale on cooked-meat medium, the odour of the growth is most nauseous. This is a late manifestation and is not found in young growths. In meat cultures hydrogen sulphide is produced, and eventually quite large quantities of gas.

The methods used in the investigation of this organism were substantially those employed in our previous papers. In consideration of the intense proteolytic action of this organism, more attention was paid to its action on cooked-meat medium, which seems to form an ideal substrate for its growth.

The following are the experimental results of the growth of this organism on various media.

B. histolyticus on Milk.

When inoculated into milk, *B. histolyticus* develops readily and causes the formation of considerable amounts of amino-acids and ammonia, together with acids and gas. For about eight days after inoculation there is very little obvious change in the milk. Then, as proteolysis proceeds, the milk becomes thinner and yellowish-brown in colour, till finally, after three to four weeks, all that is left is a brownish fluid on which floats a light fatty scum. Very rarely did we observe the formation of a clot. On one occasion we noticed a faint smell of hydrogen sulphide in a milk culture, but the presence of this gas could not be proved. The fermented milk does not possess an offensive smell.

The growth of *B. histolyticus* on milk has some of the characteristics of *B. sporogenes* on this medium. While the gas production is not so great as with the latter, one gets a certain similarity in the composition of the gas. There is, furthermore, the hydrolysis of

lactose, whereby the final concentration of sugar, as measured by the reducing power of the fermented liquid, is greater than that at the commencement. One also obtains similar ammonia values, but the quantities of amino-acids produced from the milk protein are much larger than one obtains with *B. sporogenes*.

The production of volatile acids is of about the same order as with *B. sporogenes*. In an experiment of Wolf and Telfer (1917⁷) with the latter organism, the total volatile acid production was equivalent to 47.6 c.c. of N/10 alkali per 100 c.c.

Table I. (EXPERIMENT 1).
B. HISTOLYTICUS ON MILK.

Time in Hours.	Gas. C.c. per Litre.	Content of Amino-Nitrogen. Mgrms. per 100 c.c.	Amount of Amino-Nitrogen formed. Mgrms. per 100 c.c.	Content of Ammonia-Nitrogen. Mgrms. per 100 c.c.	Amount of Ammonia-Nitrogen formed. Mgrms. per 100 c.c.
0	0	5	—	5	—
264	118	91	86	46	41
288	164	...	...	60	55
312	212	117	112	72	67
336	253	183	178	81	76
384	328	183	178	87	82

Experiment 1 (LABORATORY FERMENTATION, No. 51).

A quantity of milk was sterilised in the usual manner. After inoculation, determinations were made of the proteolysis and gas production.

For about eight days after inoculation there was no sign of growth. The milk maintained its ordinary appearance, and there was no indication that gas was being produced. At the end of this time, however, the milk changed in appearance and separation began. Gas was also evolved, the production being easily noticeable at the end of the eleventh day.

After sixteen days' fermentation, the liquid was examined for acids and bases. The acidified medium was steam-distilled exhaustively until all the volatile acids were over. When the acids had been removed, the liquid was made alkaline and the volatile base distilled off. This base was examined and found to be *ammonia*.

DATA.

1500 c.c. of milk; sterilised at 110° C. for forty-five minutes; inoculated with H 6; incubated at 37° C.

Initial Composition of Medium—

Amino-nitrogen	5 mgrms. per 100 c.c.
Ammonia-nitrogen	5 " " 100 "
Lactose	3.72 per cent.

Gas Production—

Volume of gas per litre of medium	328 c.c.
Carbon dioxide	78.5 per cent.
Hydrogen	21.5 "

Sugar (estimated gravimetrically, using Fehling's solutions)—

Apparent sugar content after 288 hours 3.84 per cent.

Apparent sugar content after 384 hours 3.90 „

There was probably a hydrolysis of lactose which masked the true value of the sugar.

Amino-acid Formation—

Final content of amino-nitrogen 183 mgrms. per 100 c.c.

Therefore 178 mgrms. of amino-nitrogen were formed per 100 c.c.

Ammonia Formation—

Final content of ammonia-nitrogen 87 mgrms. per 100 c.c.

Therefore 82 mgrms. of ammonia-nitrogen were formed per 100 c.c.

Final reaction of the milk $P_H = 6.25$.

The initial reaction of the milk was not measured, but the ordinary value for milk is sufficiently well known to enable one to see that the reaction of the milk had changed very little, if at all, during fermentation.

Volatile Acids—

The total volatile acid formed was equivalent to 42.8 c.c. of an N/10 solution per 100 c.c.

A rough examination of the acids was made by Dyer's method (1917²). There was not enough acid for very complete tests, but sufficient was done to show the presence of butyric and acetic acids.

Identification of Volatile Base—

Total base formed corresponded to 82 mgrms. of ammonia-nitrogen per 100 c.c.

The distillate containing the base was neutralised with hydrochloric acid and concentrated. The dirty brown residue was sublimed, and the white sublimate titrated against standard silver solution.

Weight of salt 0.6174 grms.

Made up to 100 c.c. with distilled water.

10 c.c. of solution required 11.15 c.c. of N/10 silver nitrate.

Chlorine calculated in ammonium chloride = 66.4 per cent.

Chlorine found in salt = 64.1 „

Experiment 2 (LABORATORY FERMENTATION, No. 52).

This experiment was carried out in a Kjeldahl flask. After inoculation, the flask was exhausted and incubated for thirty days. At the end of this time the fermented liquid was analysed and the gas measured.

DATA.

330 c.c. of milk; sterilised at 110° C. for forty-five minutes; inoculated with H 14; incubated at 37° C.

Initial Composition of Medium—

Total nitrogen 448 mgrms. per 100 c.c.

Amino-nitrogen 13 „ „ 100 „

Ammonia-nitrogen 5 „ „ 100 „

Proportion of total nitrogen originally present as ammonia and amino-acid nitrogen 4 per cent.

Initial reaction of milk $P_H = 6.16$

Gas Production—

Volume of gas per litre of medium 140 c.c.

Carbon dioxide 77 per cent.

Hydrogen 23 „

Amino-acid Formation—

Final content of amino-nitrogen 150 mgrms. per 100 c.c.

Therefore 137 mgrms. of amino-nitrogen were formed per 100 c.c.

Ammonia Formation—

Final content of ammonia-nitrogen 63 mgrms. per 100 c.c.
 Therefore 58 mgrms. of ammonia-nitrogen were formed per 100 c.c.
 Proportion of total nitrogen present as ammonia and
 amino-acids at the end of fermentation 47 per cent.
 Therefore 43 per cent. of the total nitrogen was broken down to
 ammonia and amino-acids during fermentation.

Experiment 3 (LABORATORY FERMENTATION, No. 53).

This experiment was similar to No. 2. The incubation, however, was continued for a longer period, namely, fifty-one days. An analysis of the gases at the end of fermentation showed oxygen to be present in appreciable quantities, from which it was assumed that there was either a slight leakage of air into the apparatus, or that there had been diffusion through the rubber connections, which were not of the heavy rubber usually employed.

After fifty-one days, the combined pressure of the air evolved gases, and water vapour was only 407 mm. of mercury, so that the entrance of air must have been taking place very slowly.

DATA.

330 c.c. of milk; sterilized at 110° C. for forty-five minutes; inoculated with H 14; incubated at 37° C.

Initial Composition of Medium—

As for Experiment 2.

Gas Production (after allowing for the air; approximate only)—

Volume of gas per litre of medium 282 c.c.

Amino-acid Formation—

Final content of amino-nitrogen 127 mgrms. per 100 c.c.
 Therefore 114 mgrms. of amino-acid nitrogen were formed per 100 c.c.

Ammonia Formation—

Final content of ammonia-nitrogen 67 mgrms. per 100 c.c.
 Therefore 62 mgrms. of ammonia-nitrogen were formed per 100 c.c.
 Proportion of total nitrogen present as amino-acid and
 ammonia at the end of fermentation 43 per cent.
 Therefore 39 per cent. of the total nitrogen was transformed
 into amino-acids and ammonia during the fermentation.

Volatile Acids—

The total volatile acids formed were equivalent to 40·8 c.c. of an N/10 solution.

B. histolyticus in Peptone Water.

The fermentation of peptone water by *B. histolyticus* is characteristic and shows considerable differences from that by *B. sporogenes*. The fermentation is delayed, and gas production is very small. The ammonia production of *B. sporogenes* is quite large, and takes place at the expense of the amino-acids, so that there is less concentration of these at the end of the experiment. *B. histolyticus* does not exhibit this excessive production of ammonia, and in consequence the concentration of amino-acids is greater at the end of the experiment. There is an increase in amino-acid nitrogen of 30 per cent. as the

result of the activity of this organism in a medium in which a notable amount of nitrogen was initially in this form.

The composition of the gas produced is the same as that from an experiment with *B. sporogenes*, when the percentage of carbon dioxide was 70 per cent.

Table II. (EXPERIMENT 4).

B. HISTOLYTICUS ON PEPTONE WATER.

Time in Hours.	Gas. C.c. per Litre.	Content of Amino-Nitrogen. Mgrms. per 100 c.c.	Amount of Amino-Nitrogen formed. Mgrms. per 100 c.c.	Content of Ammonia-Nitrogen. Mgrms. per 100 c.c.	Amount of Ammonia-Nitrogen formed. Mgrms. per 100 c.c.
0	0	72	0	13	0
2	0	...	...	...	...
24	12	85	13	30	17
48	19	...	...	...	...
72	23	92	20	36	23
96	25	96	24	37	24

Experiment 4 (LABORATORY FERMENTATION, No. 54).

A solution of peptone water was prepared, using the tryptic broth of Cole and Onslow (1916¹). This was sterilised, inoculated, and incubated in the usual way.

DATA.

1500 c.c. of medium; sterilised at 110° C. for forty-five minutes; inoculated with H 14 (1); incubated at 37° C.

Initial Composition of Medium—

Amino-nitrogen	72 mgrms. per 100 c.c.
Ammonia-nitrogen	13 „ „ 100 „
Initial reaction	P _H =6.95.

Gas Production—

The amount of gas produced was extremely small, amounting to only 25 c.c. per litre.

In order to obtain a sample of the gas for analysis, it was necessary to place the fermentation flask under water, and allow the water to enter until the vacuum, which was almost complete, had dropped. A sample could then be taken. The figures are naturally only an approximation.

Volume of gas per litre of medium	25 c.c.
Carbon dioxide	69 per cent.
Hydrogen	31 „

Amino-acid Formation—

Final content of amino-nitrogen	96 mgrms. per 100 c.c.
Therefore 24 mgrms. of amino-nitrogen were formed per 100 c.c.	

Ammonia Formation—

Final content of ammonia-nitrogen	37 mgrms. per 100 c.c.
Therefore 24 mgrms. of ammonia-nitrogen were formed per 100 c.c.	

Volatile Acids—

The total volatile acids formed were equivalent to 15.9 c.c. of an N/10 solution. No examination as to the quality of the acids was made.

Absolute Reaction—

Final reaction of medium	P _H =7.05.
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B. histolyticus on Alkaline Egg-Medium (See Appendix).

On alkaline-egg medium this organism seems to develop with extreme difficulty. Only in one experiment out of the three about to be described was there a measurable amount of gas formed. In this case, after a prolonged incubation, there was a fair quantity of amino-acid and ammonia-nitrogen present in the fermented liquid, showing that the organism had managed to cause proteolysis in the medium after overcoming the initial difficulties in the way of its development.

This medium, even when a fair amount of proteolysis has taken place, shows very little change in appearance, merely becoming more opaque, and changing its bottle-green colour to a creamy-white. The fermented liquid does not smell.

Experiment 5 (LABORATORY FERMENTATION, No. 55).

This fermentation was carried out on 250 c.c. of medium contained in a Kjeldahl flask. After inoculation, the flask was evacuated and incubated in the usual way.

DATA.

250 c.c. of alkaline-egg medium; sterilised at 110° C. for forty-five minutes; inoculated with H 21; incubated at 37° C.

Initial Composition of Medium—

Amino-nitrogen	5 mgrms. per 100 c.c.
Ammonia-nitrogen	2 „ „ 100 „

Gas Production—

Traces of gas only.

Amino-acid Formation—

Final content of amino-nitrogen	11 mgrms. per 100 c.c.
Therefore 6 mgrms. of amino-nitrogen were formed per 100 c.c.	

Ammonia Formation—

Final content of ammonia-nitrogen	6 mgrms. per 100 c.c.
Therefore 4 mgrms. of ammonia-nitrogen were formed per 100 c.c.	

Experiment 6 (LABORATORY FERMENTATION, No. 56).

This experiment was similar to No. 5, except that incubation was continued for a longer period, seven weeks. Analysis of the fermented liquor showed that considerable proteolysis had taken place. There was also a formation of gas.

DATA.

250 c.c. of alkaline-egg medium; sterilised at 110° C. for forty-five minutes; inoculated with H 21; incubated at 37° C.

Initial Composition of Medium—

As for Experiment 5.

Gas Production (approximate figures only)—

Volume of gas per litre of medium	400 c.c.
Carbon dioxide	25 per cent.
Hydrogen	75 „

Amino-acid Formation—

Final content of amino-acid nitrogen 56 mgrms. per 100 c.c.
 Therefore 51 mgrms. of amino-nitrogen were formed per 100 c.c.

Ammonia Formation—

Final content of ammonia-nitrogen 28 mgrms. per 100 c.c.
 Therefore 26 mgrms. of ammonia-nitrogen were formed per 100 c.c.

Experiment 7 (LABORATORY FERMENTATION, No. 57).

This experiment was similar to Experiments Nos. 5 and 6, except that the incubation period was five weeks. Traces of gas only were observed from the fermented liquid.

DATA.

250 c.c. of alkaline-egg medium ; sterilised at 110° C. for forty-five minutes ; inoculated with H 20 ; incubated at 37° C.

Initial Composition of Medium—

As for Experiments 5 and 6.

Gas Production—

Traces of gas only.

Amino-acid Formation—

Final content of amino-nitrogen 22 mgrms. per 100 c.c.
 Therefore 17 mgrms. of amino-acid nitrogen were formed per 100 c.c.

Ammonia Formation—

Final content of ammonia-nitrogen 6 mgrms. per 100 c.c.
 Therefore 4 mgrms. of ammonia-nitrogen were formed per 100 c.c.

B. histolyticus on Cooked-Meat Medium

(See Appendix).

By far the best growth with *B. histolyticus* is obtained on cooked-meat medium. The particular medium we used is a modification of one described by Miss Robertson (1916⁴). Instead of using the heart muscle of the bullock, we used that of the horse.

As prepared by us, the solid matters of the broth were so finely divided that, after stirring, they only settled out very slowly. They passed easily through a tube of 5 mm. bore. Thus it was possible to obtain good samples of the liquid during fermentation.

The bacillus quickly shows signs of growth in this medium. The supernatant liquid becomes cloudy, and within twenty-four hours small bubbles of gas may be observed. The fermentation will continue from two to three weeks, though the metabolic changes eventually take place so slowly as to be almost unmeasurable except at long intervals.

When the bacillus is grown in cooked meat, in ordinary culture tubes and without agitation, bundles of white, needle-shaped crystals separate out. We have assumed these to consist of tyrosine. In large-scale experiments, where agitation is necessary for the purpose of sampling, these crystals are not observed.

In addition to the formation of amino-acids and ammonia, the

fermentation of this medium is accompanied by the production of large quantities of volatile acids. Fixed acids are undoubtedly produced at the same time, but were not sought for in this investigation.

The absolute reaction of the cooked-meat medium changes very little during fermentation. Owing to the poisonous action of the liquid on the palladium-hydrogen electrodes (due, no doubt, to the sulphuretted hydrogen present), extensive experiments on the hydrogen-ion concentration were not performed, but we observed that the changes in reaction were only very slight.

Cooked-meat medium is not of a very definite composition; its properties seem to vary with the freshness of the horse's heart used. Thus, in one experiment, we obtained a very vigorous phase of gas production almost immediately after inoculation. In other experiments this initial phase was absent. We are inclined to attribute these differences to variations in the glycogen and glucose content of the heart muscle.

In the following experiments we used three different concentrations of nitrogenous matter. There are marked individual differences in the results of the experiments, but extensive proteolysis and large formation of volatile acids are common to them all.

Among the fermentation products, hydrogen sulphide is found. It is only present in small quantities, and is not easily noticed in fermentations carried out in culture tubes under paraffin, from which the gas can easily escape as formed. In closed-flask experiments, however, there is a strong smell of the gas, and a positive test can be easily obtained with lead acetate paper.

After about ten days' fermentation, the meat is so disintegrated by the action of the bacteria that the medium can be drawn into the capillary end of an ordinary 25 c.c. pipette.

Long continued fermentation produces a most nauseous smelling liquid.

Experiment 8 (LABORATORY FERMENTATION, No. 58).

1600 c.c. of cooked-meat medium were prepared from horse's heart muscle. The broth was sterilised in a large fermentation flask in the ordinary way, and inoculated with a culture of *B. histolyticus*. A vacuum was made in the flask, which was then incubated. After inoculation, the gas production was observed, and samples of the liquid withdrawn for analysis from time to time.

The fermentation began almost immediately. Four and a half hours after seeding, the 1600 c.c. of medium had produced 785 c.c. of gas. Analysis of the fermented liquid at the end of this vigorous period showed that practically no proteolysis had taken place. On the other hand, the reaction of the liquid had become decidedly acid, having changed from $P_H=6.46$ to $P_H=5.95$. Thus it would appear that this opening phase of gas production is accompanied by the formation of acids, probably at the expense of the glycogen or glucose content of the meat.

After this initial vigorous phase, there was a falling off in the production

of gas for a period of twenty-nine hours. During this time, as shown by Table III., active proteolysis was taking place. Twenty-four hours after seeding, a second evolution of gas began, which continued with steadily decreasing vigour throughout the experiment. Gas readings were not taken after the seventy-second hour, but gas continued to be formed in small quantities. The amount of amino-acid nitrogen in the liquid reached its height about this time, and then fell off slightly. Ammonia continued to be formed, reaching the very high figure of 594 mgrms. per 100 c.c. of liquid after 191 hours. Sulphuretted hydrogen was present in the liquid twenty-four hours after inoculation.

During the fermentation the reaction of the liquid only altered very slightly after the first change towards acidity during the first four hours. The tendency was for the medium to remain in the region of neutrality; this neutrality being maintained by the simultaneous formation of acids and bases.

When the fermentation had ended, a portion of the fermented liquid was made frankly acid to Congo-red with sulphuric acid, and exhaustively steam-distilled. The distillate was exactly neutralised with normal soda, and then concentrated to a small volume. On acidification of the concentrated solution

Table III. (EXPERIMENT 8).

B. *HISTOLYTICUS* ON COOKED MEAT.

Time in Hours.	Gas. C.c. per Litre.	Content of Amino-Nitrogen. Mgrms. per 100 c.c.	Amount of Amino-Nitrogen formed. Mgrms. per 100 c.c.	Content of Ammonia-Nitrogen. Mgrms. per 100 c.c.	Amount of Ammonia-Nitrogen formed. Mgrms. per 100 c.c.	Absolute Reaction P _H .
0	0	32	0	16	0	6.46
4.5	490	35	3	18	2	5.95
23	665	149	117	115	99	6.75
31	874	...	...	...	...	...
47	1058	200	168	210	194	6.73
71	1132	...	...	...	...	...
191	...	181	149	594	578	...

with sulphuric acid, there was an immediate separation of the volatile acids into a layer. These acids were removed by extraction with ether in a liquid extractor for twenty-four hours. The ethereal extract was dried over sodium sulphate, and the free acids obtained after removal of the ether by evaporation. About 10 c.c. of the pure acids were obtained from 500 c.c. of the fermentation liquid.

The acids were carefully examined by Dyer's method (1917²). A preliminary distillation showed the presence of acetic and butyric acids, and maybe traces of other acids difficult to identify in such a mixture.

The proportions indicated by the logarithmic curves were 2 parts of butyric acid to 1 of acetic. These figures were confirmed by carrying out two distillations on equal parts of the acid solution by Dyer's method, and then adding corresponding fractions, neutralising, concentrating, and redistilling in the manner suggested by Wolf and Telfer (*loc. cit.*). This confirmatory experiment was useful in showing that only very small traces of other acids could be present.

After the acids had been removed from the liquor, the latter was made alkaline with soda and distilled to remove the volatile base. This was finally purified in the form of the hydrochloride, and found by simple quantitative tests to be *ammonia*.

Part of the gas produced was lost by accident, but a portion, collected over water, was analysed and found to contain 35 per cent. of carbon dioxide.

DATA.

533 grms. of horse's heart muscle used to make 1600 c.c. of medium; sterilised at 110° C. for forty-five minutes; inoculated with H 25 (1); incubated at 37° C.

Initial Composition of Medium—

Total nitrogen	1056 mgrms. per 100 c.c.
(This corresponds roughly to a protein content of 6·5 per cent.)	
Amino-nitrogen	32 mgrms. „ 100 „
Ammonia-nitrogen	16 „ „ 100 „
Proportion of total nitrogen originally present as ammonia and amino-acid nitrogen	
	4·5 per cent.
Volatile acids	3 c.c. N/10 solution per 100 c.c.
Initial reaction	P _H =6·46.

Gas Production (up to the seventy-first hour only)—

Volume of gas per litre of medium	1132 c.c.
Carbon dioxide ¹	35 per cent.
Hydrogen ¹	65 „

Amino-acid Formation—

Final content of amino-nitrogen	181 mgrms. per 100 c.c.
Therefore 149 mgrms. of amino-nitrogen were formed per 100 c.c.	

Ammonia Formation—

Final content of ammonia-nitrogen	594 mgrms. per 100 c.c.
Therefore 578 mgrms. of ammonia-nitrogen were formed per 100 c.c.	
Proportion of total nitrogen present as amino-acids and ammonia at the end of fermentation	
	73·5 per cent.
Therefore 69 per cent. of the total nitrogen was transformed into amino-acid and ammonia-nitrogen during fermentation.	

Volatile Acids—

The total volatile acids formed were equivalent to 257 c.c. of N/10 solution per 100 c.c.
 The acids were mainly butyric and acetic acids, present in the proportion of two molecules of butyric to one of acetic.
 Thus there were produced per litre of medium—
 15·2 grms. of butyric acid.
 5·2 grms. of acetic acid.

Identification of the Volatile Base—

The total volatile base formed corresponded to 578 mgrms. of nitrogen per 100 c.c.
 A portion of the hydrochloride of the base was first titrated against standard silver nitrate solution.
 Weight of hydrochloride 0·5316 grms.
 Made up to 100 c.c. with distilled water.
 10 c.c. of this solution required 9·65 c.c. N/10 silver solution.
 Chlorine found in salt 64·4 per cent.
 Chlorine calculated for ammonium-chloride 66·4 „
 The nitrogen content of the salt was determined by distilling 20 c.c. of the above solution, after the addition of an excess of soda, into 25 c.c. of N/10 sulphuric acid.
 20 c.c. of solution required 19·5 c.c. N/10 acid.
 Nitrogen found in salt 25·6 per cent.
 Nitrogen calculated for ammonium chloride 26·1 „
 Therefore the volatile base is ammonia.
 From 1 litre of medium there were produced 8·1 grms. of ammonia.

¹ On 63 per cent. of the gas only; it could not be considered a fair sample of the whole gas.

Experiment 9 (LABORATORY FERMENTATION, No. 59).

This experiment was carried out in a cooked-meat medium similar to that used for Experiment 8.

The fermentation was much less vigorous; gas production was not so intense; and, further, the initial period of gas formation was absent. Analyses of the liquid were only made before inoculation, and after fermentation had proceeded for 216 hours. The gas production was only observed up to the fifty-ninth hour.

The volatile acids were removed by distillation and examined by Dyer's method. They were found to consist in the main of acetic acid, with only the smallest traces of other acids. In this respect the fermentation differed from Experiment 8, where large amounts of butyric acid were produced in addition to acetic acid.

DATA.

500 grms. of horse's heart muscle to make 1500 c.c. of medium; sterilised at 110° C. for forty-five minutes; inoculated with H 27 (1); incubated at 37° C.

Initial Composition of Medium—

Total nitrogen	1039 mgrms. per 100 c.c.
(This corresponds roughly to a protein content of 6.4 per cent.)	
Amino-nitrogen	31 mgrms. per 100 c.c.
Ammonia-nitrogen	18 „ „ 100 „
Proportion of total nitrogen originally present as ammonia and amino-acid nitrogen	4.7 per cent.
Volatile acids	3 c.c. of an N/10 solution per 100 c.c.
Absolute reaction	$P_N = 6.67$.

Gas Production (up to fifty-ninth hour only)—

Volume of gas per litre of medium	560 c.c.
Carbon dioxide	96.2 per cent.
Hydrogen	3.8 „

Amino-acid Formation—

Final content of amino-nitrogen	339 mgrms. per 100 c.c.
Therefore 308 mgrms. of amino-nitrogen were produced per 100 c.c.	

Ammonia Formation—

Final content of ammonia-nitrogen	309 mgrms. per 100 c.c.
Therefore 291 mgrms. of ammonia-nitrogen were produced per 100 c.c.	
Proportion of total nitrogen present as ammonia and amino-acid nitrogen at the end of fermentation	62.4 per cent.
Therefore 57.5 per cent. of the total nitrogen was transformed into ammonia and amino-acid nitrogen during fermentation.	

Volatile Acids—

The total volatile acids formed were equivalent to 199 c.c. of an N/10 solution per 100 c.c.

Thus there would be *per litre of medium—*
12.12 grms. of acetic acid.

Experiment 10 (LABORATORY FERMENTATION, No. 60).

For this experiment the medium was prepared so as to be comparatively dilute. Samples were taken from the fermenting liquid at intervals and analysed. The gas production was measured.

The volatile acids were examined by Dyer's method, and found to consist mainly of acetic acid, with small traces of other members of the fatty acid series, not identified.

DATA.

425 grms. of horse's heart muscle to make 1700 c.c. of medium; sterilised at 110° C. for forty-five minutes; inoculated with H 32 (2); incubated at 37° C.

Initial Composition of Medium—

Total nitrogen 783 mgrms. per 100 c.c.
 (This corresponds roughly to a protein content of 4.9 per cent.)
 Amino-nitrogen 26 mgrms. per 100 c.c.
 Ammonia-nitrogen 12 „ „ 100 „
 Proportion of total nitrogen originally present as amino-
 acid nitrogen and ammonia 4.8 per cent.
 Volatile acids 2 c.c. of N/10 solution per 100 c.c.

Gas Production—

Volume of gas per litre of medium 654 c.c.
 Carbon dioxide 96 per cent.
 Hydrogen 4 „

Table IV. (EXPERIMENT 10).

B. HISTOLYTICUS ON COOKED MEAT.

Time in Hours.	Gas. C.c. per Litre.	Content of Amino-Nitrogen. Mgrms. per 100 c.c.	Amount of Amino-Nitrogen formed. Mgrms. per 100 c.c.	Content of Ammonia-Nitrogen. Mgrms. per 100 c.c.	Amount of Ammonia-Nitrogen formed. Mgrms. per 100 c.c.
0	0	26	0	12	0
6	15	...	...	...	...
23	134	123	97	84	72
48	282	194	168	150	138
72	383	220	194	178	166
96	465	...	...	...	...
120	512	244	218	203	191
144	557	...	...	...	...
264	654	323	297	216	204

Amino-acid Formation—

Final content of amino-nitrogen 323 mgrms. per 100 c.c.
 Therefore 297 mgrms. of amino-nitrogen were produced per 100 c.c.

Ammonia Formation—

Final content of ammonia-nitrogen 216 mgrms. per 100 c.c.
 Therefore 204 mgrms. of ammonia-nitrogen were formed per 100 c.c.
 Proportion of total nitrogen present as amino-acid and ammonia-nitrogen at the end of fermentation 68.8 per cent.
 Therefore 64 per cent. of the total nitrogen was transformed into amino-acid and ammonia-nitrogen during fermentation.

Volatile Acids—

The total volatile acids formed were equivalent to 141 c.c. of an N/10 solution per 100 c.c.
 Thus there would be *per litre of medium—*
 8.56 grms. of acetic acid.

Experiment 11 (LABORATORY FERMENTATION, No. 62).

For this experiment a concentrated cooked-meat medium was prepared by adding to a quantity of finely minced horse's heart muscle its own weight of

water. The thick broth so obtained was sterilised in the usual way and inoculated with a culture of *B. histolyticus*.

During fermentation, gas production was noted, and samples of the fermenting liquid withdrawn at intervals and analysed. In addition to estimations of the amount of ammonia and amino-acid nitrogen, the quantity of volatile acid at each stage was determined by the exhaustive distillation of a portion of the broth.

From the data thus obtained, curves were plotted, showing the formation of the various products.

From Fig. 1 it is seen that the fermentation began shortly after inoculation. During the first six hours there was a very rapid formation of gas, which preceded any extensive production of the other measured product.

It may be that this large quantity of gas accompanied the formation of considerable amounts of fixed acids. In confirmation of this, measurements of the absolute reaction at this point showed the liquid to have become decidedly acid, although the amount of volatile acid formed was so far very small.

Table V. (EXPERIMENT 11).

B. HISTOLYTICUS ON COOKED MEAT.

Time in Hours.	Gas. C.c. per Litre.	Content of Amino-Nitrogen. Mgrms. per 100 c.c.	Amount of Amino-Nitrogen formed. Mgrms. per 100 c.c.	Content of Ammonia-Nitrogen. Mgrms. per 100 c.c.	Amount of Ammonia-Nitrogen formed. Mgrms. per 100 c.c.	Content of Volatile Acids. C.c. N/10 Solution per 100 c.c.	Amount of Volatile Acids formed. C.c. N/10 Solution per 100 c.c.
0	0	42	0	26	0	4.4	0
4.5	115	42	0	26	0	10.8	6.4
6	421	...	...	...	...	...	...
6.5	436	68	26	38	12	21.6	17.2
14	642	216	174	147	121	71.2	66.8
24	1001	...	...	...	...	168.0	163.6
30	1325	346	304	326	300	212.0	207.6
49	1977	388	346	554	528	300.0	295.6
72	2750	340	298	708	682	524.0	519.6
120	2905	...	...	...	...	...	...
144	2925	180	138	1020	994	582.0	577.6

At the twelfth hour, proteolysis was proceeding very rapidly. The rate of formation of amino-acids reached a maximum about this time, and then gradually fell off, until, fifty hours after inoculation, amino-acids began to disappear from the fermenting medium. Doubtless they were being transformed into ammonia, the production of which continued steadily. The average amount of amino-acid nitrogen which disappeared was about 14 mgrms. per six hours, whilst 26 mgrms. of ammonia were formed during this time. Thus, the ammonia at this stage was not entirely due to the breaking down of the disappearing amino-acids, but must have come in part from the higher complexes.

The maximum production of volatile acids, accompanied by a large formation of gas, extended over a period of twenty-four hours from the forty-eighth hour. At the seventy-second hour there was a considerable decrease in the rate of production of both volatile acids and gas, and at 144 hours this fell so as to be practically negligible.

The proteolysis during fermentation was very great, 77 per cent. of the total nitrogen having been degraded to the form of ammonia and amino-acid nitrogen.

No investigations were carried out as to the nature or quantity of the fixed acids formed. Volatile acids were present in large quantities. They consisted

Fig. 1.
B. HISTOLYTICUS ON COOKED MEAT.

mainly of acetic and butyric acids, the former being in excess. The presence of small amounts of other members of the volatile fatty acid series rendered the accurate estimation, by Dyer's method, of the proportions of the two main acids practically impossible.

In spite of the presence of so much volatile acid (sufficient to give an 0.5 normal solution), the fermented liquid at the close of fermentation had a reaction very close to neutrality. Contributing to this state of affairs were the presence of sufficient ammonia to give an 0.7 normal solution, the undetermined fixed acids, and the buffering influence of the amino-acids.

Unfortunately it was impossible to study closely the absolute reaction of the medium on account of the "poisoning" action of the hydrogen sulphide present on the palladium-hydrogen electrodes.

DATA (see also Figure 1).

800 grms. of horse's heart muscle to make 1600 c.c. of medium; sterilised at 110° C. for forty-five minutes; inoculated with H 38; incubated at 37° C.

Initial Composition of Medium—

Total nitrogen	1473 mgrms. per 100 c.c.
(This corresponds roughly to a protein content of 9.2 per cent.)	
Amino-nitrogen	42 mgrms. per 100 c.c.
Ammonia-nitrogen	26 „ „ 100 „
Proportion of total nitrogen originally present as amino-acid and ammonia-nitrogen	
	4.6 per cent.
Volatile acids	4.4 c.c. of an N/10 solution per 100 c.c.
Absolute reaction	$P_H = 6.77$.

Gas Production—

Volume of gas per litre of medium	2925 c.c.
Carbon dioxide	82 per cent.
Hydrogen	18 „

Amino-acid Formation—

Final content of amino-acid nitrogen	180 mgrms. per 100 c.c.
Therefore 138 mgrms. of amino-nitrogen were formed per 100 c.c.	

Ammonia Formation—

Final content of ammonia-nitrogen	1020 mgrms. per 100 c.c.
Therefore 994 mgrms. of ammonia-nitrogen were formed per 100 c.c.	
Proportion of total nitrogen present as amino-acid and ammonia-nitrogen at the end of fermentation	
	81.4 per cent.
Therefore 76.8 per cent. of the total nitrogen was transformed into amino-acid and ammonia-nitrogen during fermentation.	

Volatile Acids—

The total volatile acids formed were equivalent to 577.6 c.c. of an N/10 solution per 100 c.c.

The acids consisted mainly of butyric and acetic acids, with small quantities of other members of the volatile fatty acid series.

Absolute Reaction—

The absolute reaction, 4.5 hours after inoculation (during the first rapid evolution of gas), was $P_H = 6.80$.

No other determinations were made.

CONCLUSIONS.

The results detailed in the foregoing experiments tend to show that in *B. histolyticus* we possess an organism whose biochemical

characteristics account for the fulminating character of the pathological lesions described by Weinberg and Séguin.

B. histolyticus has a very remarkable proteolytic action which is not only concerned with the production of amino-acids. Its deaminating power is so great that, in a meat suspension containing 1473 mgrms. of total nitrogen per 100 c.c., 1000 mgrms. of ammonia-nitrogen are formed after 144 hours' fermentation.

During the production of ammonia the reaction of the medium remains fairly constant. Consequently there must be a large simultaneous formation of acids. We have not had the opportunity of investigating the fixed acid production, but the volatile acids formed reach the high level of an 0.57 normal acid solution. Reckoned as butyric acid this would be equivalent to a 5 per cent. solution of the acid. When one takes into account that large quantities of fixed acids are undoubtedly formed at the same time, it is evident that the capacity for acid production is very great indeed.

Weinberg and Séguin did not observe that any formation of gas took place when animals were infected with *B. histolyticus*. We find that the gas production is very greatly influenced by the nature of the medium, and not at all in the sense one might anticipate. Lactose is but slightly attacked and is partially hydrolysed. Peptone solution to which glucose has been added does not appear to give so good a growth as a simple peptone solution.

The behaviour towards protein is somewhat distinctive. Peptone water gives a minimal gas production. Alkaline egg is also not vigorously attacked. Whether this is associated with the high P_H of this medium we are unable to say.

The medium in which most vigorous catabolism and greatest gas production are found is that supplied by cooked meat. The course of the reaction is a complicated one, for it is apparently determined by the protein content of the suspension. The fermentation is less vigorous the lower the protein concentration. The gas production on cooked meat takes place in two phases, the earlier being the more vigorous. The mechanism of this initial gas production is difficult to explain. The sugar content of the medium is minimal. Glycogen estimations were made of the cooked horse's heart, and amounts of this substance less than 0.01 per cent. were found. Acids are produced, for the reaction of the medium becomes definitely more acid, and a quantity of volatile acid is shown to be present on analysis. But with this acid and gas production in the absence of obvious carbohydrate, there is no increase in ammonia and amino-acids. One is therefore forced to find an explanation other than a production from amino-acids or from carbohydrate. The only material which would appear to be available is the nitrogen-containing carbohydrate moiety of protein. It would seem, therefore, that the acid and gas production are preceded by a splitting off of a glucosamine group which is

Table VI.—*B. HISTOLYTICUS*.

Experiment.	Laboratory Fermentation.	Medium.	INITIAL COMPOSITION OF MEDIUM.						Laboratory No. of Culture used as Seed.	No. of Hours' Fermentation.
			Nitrogenous Matter.				Volatile Acids. C.c. N/10 Solution per 100 c.c.	Initial Reaction Ph.		
			Total Nitrogen. Mgrms. per 100 c.c.	Amino-Nitrogen. Mgrms. per 100 c.c.	Ammonia-Nitrogen. Mgrms. per 100 c.c.	Proportion of Total Nitrogen present as NH ₃ and NH ₄ .				
No.	No.					Per cent.				
1	51	Milk	..	5	5	..	0	..	H 6	384
2	52	"	448	13	5	4.0	..	6.16	H 14 (2)	720
3	53	"	448	13	5	4.0	0	..	H 14 (2)	1224
4	54	Peptone water	..	72	13	..	3	6.95	H 14 (1)	96
5	55	Alkaline egg	..	5	2	..	..	8.7	H 21	360
6	56	"	..	5	2	..	..	8.7	H 21	1176
7	57	"	..	5	2	..	..	8.7	H 21	840
8	58	Cooked meat	1056	32	16	4.5	3	6.46	H 25 (1)	191
9	59	"	1039	31	18	4.7	3	6.67	H 27 (1)	216
10	61	"	783	26	12	4.8	2	..	H 32 (2)	264
11	62	"	1473	42	26	4.6	4.4	6.77	H 38	144

* Amount of gas up to 71st hour only.

SUMMARY OF EXPERIMENTS.

FINAL COMPOSITION OF MEDIUM.									GAS.		
Nitrogenous Matter.						Volatile Acids.		Final Reaction Pa.	Volume of Gas. C.c. per Litre.	CO ₂ per cent.	H ₂ per cent.
Amino-Nitrogen.		Ammonia-Nitrogen.		Proportion of Total Nitrogen present as NH ₃ and NH ₄ .	Proportion of Total Nitrogen transformed during Fermentation.	Content. C.c. N/10 Solution per 100 c.c.	Amount formed. C.c. N/10 Solution per 100 c.c.				
Content. Mgrms. per 100 c.c.	Amount formed. Mgrms. per 100 c.c.	Content. Mgrms. per 100 c.c.	Amount formed. Mgrms. per 100 c.c.								
133	178	87	82	Per cent. ..	Per cent. ..	42·8	42·8	6·25	328	78·5	21·5
150	187	63	58	47·0	43·0	..	..	..	140	77·0	23·0
127	114	67	62	43·0	39·0	40·8	40·8	..	232	..	..
96	24	37	24	..	..	18·9	15·9	7·05	25	69·0	31·0
11	6	6	4	..	..	..	..	..	Traces.	..	..
56	51	28	26	..	..	..	..	..	400	25·0	75·0
22	17	6	4	..	..	..	..	..	Traces.	..	..
181	149	594	578	78·5	69·0	280·0	257·0	..	1132*	..	..
339	308	309	291	62·4	57·5	202·0	199·0	..	560†	96·2	3·8
323	297	216	204	68·8	64·0	143·0	142·0	..	654	96·0	4·0
180	138	1020	994	81·4	76·8	582·0	577·6	..	2925	82·0	18·0

† Amount of gas up to 50th hour only.

attacked to form acid and gas. The amount of nitrogen transformed to ammonia in a reaction of this kind would be small compared with the amount of carbon dioxide produced, provided that acid formation was not prominent in this reaction. The maximum comparative figure for gas production from glucosamine is where 100 mgrms. of nitrogen would yield 950 c.c. of carbon dioxide, but the actual ratio would be much lower owing to the formation of acids.

In the last cooked-meat experiment, the two curves which follow one another most closely are the gas curve and the volatile acid curve. The acid production takes place in two phases, as does the formation of gas, so that, as we have suggested in a previous paper, the process of carbon-dioxide formation is partly due to a degradation of organic acids of a type similar to the β -ketonic oxidation of Knoop (1904⁸). In the present instance the process must be a very specific one, for we find that the resulting acids are almost entirely butyric and acetic acids.

It is unfortunate that we are unable to compare the action of *B. histolyticus* on cooked meat with the action of *B. sporogenes* on this medium. Experiments with the latter organism are lacking, but we hope at a future date to supplement our previous study by an examination of the metabolism of *B. sporogenes* when grown on cooked meat.

SUMMARY.

B. histolyticus is essentially a protein-splitting organism, and attacks certain protein media with great intensity. Amino-acids, and especially ammonia, are formed in the reaction. At the same time large quantities of volatile acids are formed. These are chiefly butyric and acetic acids.

The composition of the gas produced varies with the medium; on cooked meat the carbon dioxide content may be as high as 96 per cent.

APPENDIX.

Cooked-Meat Medium.

Take 500 grms. of horse's heart, free from fat and gristle, cut into pieces, place in a casserole and just cover with tap water. Cook thoroughly over a small flame. Pour off the liquid into a tared beaker, and then mince the solid residue by passing two or three times through a fine mincer, till the meat is in a fine state of division. Add the minced meat to the cooking water in the beaker; add tap water till the total weight of the broth is 1000 grms. (This yields a very thick broth containing about 10 per cent. protein. If required, therefore, more water may be added at this stage, till the total weight is 1500 grms. or 2000 grms.)

When the water has been added, break down any small lumps in the mince between the fingers. Add normal sodium hydrate till the medium is faintly alkaline to litmus, or better, till it just fails to turn coralline paper yellow. Tube, and sterilise at 110°C . for forty-five minutes. After sterilisation, the reaction of this medium is from $\text{P}_\text{H} = 6.5$ to $\text{P}_\text{H} = 7.0$.

Alkaline-Egg Fluid.¹

The yolk of one egg and the whites of two are beaten up in a beaker; add 6 c.c. of normal sodium hydrate solution; add 500 c.c. of tap water by degrees. Heat very slowly to 95°C ., keeping the mixture at this temperature for an hour or longer; filter through cotton wool and muslin. Autoclave at 115°C . for twenty minutes.

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