THE ACID PRODUCTION OF BACILLUS WELCHII

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The studies recorded herein confirm and extend our previous observations with B. welchii,[1] but, in part, yield new interpretations.

We had observed that B. welchii, grown in a meat peptone mash medium, gives rise to an increased H + ion concentration culminating at about P_H 6.1 in from 12 to 24 hours, followed by a gradual decline toward neutrality (P_H 7) in about 200 hours. We ascribed the increase in H + ion concentration to the acid produced in the fermentation of the muscle sugar, and the decline in the curve to the neutralization of this acid by ammonia, presumably set free from the protein in the effort of the micro-organism to supply its carbon requirements. Kendall, Day and Walker[2] have since shown that B. welchii produces several times as much ammonia in mediums not containing fermentable sugars as in those containing them. In this respect the metabolism of this and other obligate anaerobes resembles that of the aerobes.

Corresponding to the increase in H + ion concentration there was also an increase in titratable acidity, followed, during the period of continued depression in H + ion concentration, by a slight decline, with a secondary, and usually greater, increase in titratable acidity, also followed by a fall. No such secondary peak occurred in mediums from which the meat particles had been filtered, and so we referred to the first peak as the "sugar phase" and to the second as the "protein phase."

Having secured these results in mediums to which no carbohydrates had been added, since we were then interested mainly in the elimination of muscle sugar in the preparation of "sugar-free" mediums, we now undertook to determine the effect of certain added fermentable sugars on the H + ion concentration and titrable acidity curves, and their interpretation with particular reference to the utility of the peaks of these curves as criteria of freedom from sugar.

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[1] Jour. Infect. Dis., 1921, 29, p. 344.
[2] Ibid., 1922, 30, p. 141.

EXPERIMENTS

Our general methods have been described.[1] The mediums now to be mentioned were made, however, with "Difco" peptone instead of that of Parke, Davis & Co., and 1 liter flasks containing 900 c c of medium were used instead of the smaller sizes to avoid any considerable alteration in volume through sampling.

The meat mash mediums were prepared by the addition of the meat residue, removed from the meat infusion by straining and filtration after boiling, to the clear 2% peptone meat infusion broth in the proportion of 100 gm. of meat per 800 c c of broth. Sugar mediums were resterilized on 3 successive days for 20 minutes daily in the Arnold sterilizer.

Sampling, titration, determination of H + ion concentration, and the usual check tests for purity, were as before, and the same culture of B. welchii (No. 2, for description and origin of which see Hall [3]) was used. Our data were analyzed and are presented as before by the graphic method; the charts are self-explanatory, H + ion concentration being shown in terms of P_H values and the corresponding titers in number of c c N/20NaoH required to neutralize 5 c c of a given sample to phenolphthalein, i. e., percentage normality.

Our first experiment was to duplicate the peculiar results with meat peptone mash medium under the slightly changed conditions of experimentation (peptone and larger flasks). There was no difficulty in this; the results practically coincided with those formerly reported [1] (charts 7 and 8). A similar test was included as a control in each of the experiments herein reported.

We then studied the effect of an excess (5%) of added glucose and lactose on the H + ion concentration and titratable acidity, as shown in chart 1.

It was no surprise to note in the plotted data the low P_H values of the sugar mediums as compared with those of the plain mash, but that there should be a peak in the curves for the former similar to that of the latter was not anticipated; we thought the sugar curves would be flat-topped. There was at any rate no secondary peak in the P_H curves, as in the titer curves, but it was particularly noteworthy that the P_H curves for glucose and lactose should practically coincide, while the titer curve for glucose was considerably higher than that for lactose, both being, of course, higher than that for the plain mash. We had expected that the secondary peak would be present only in the titer

[3] Ibid., 1922, 30, p. 445.

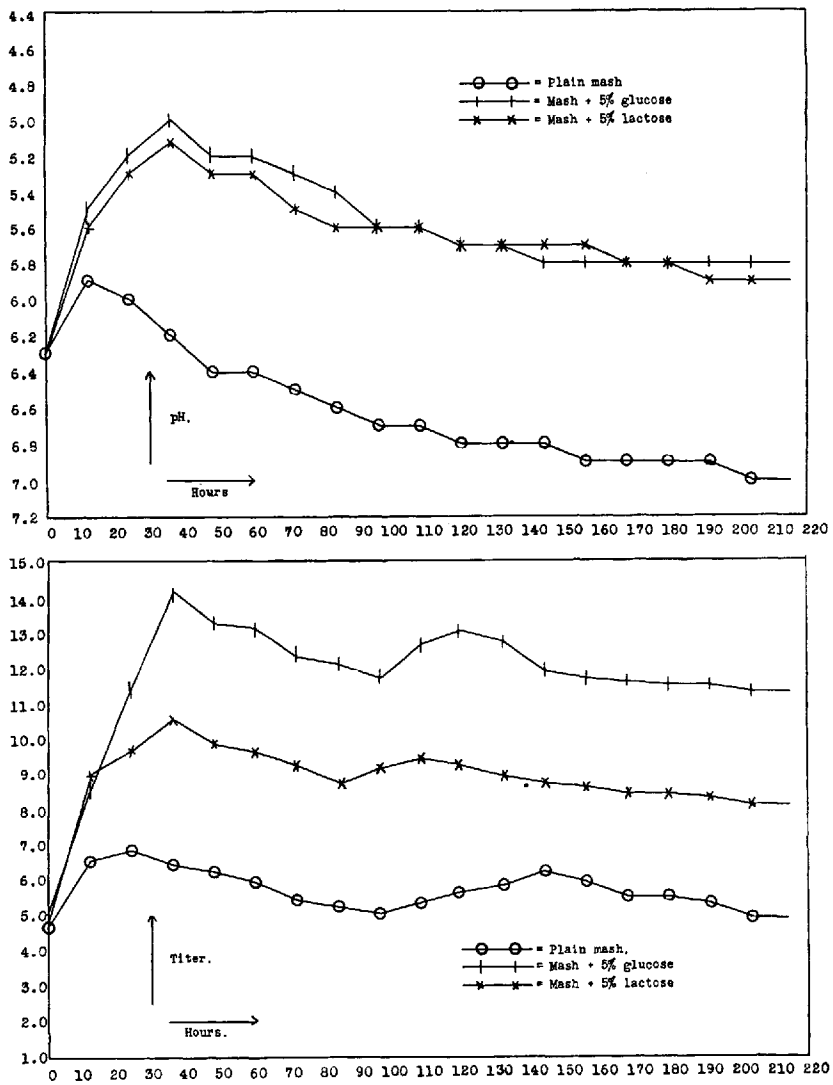

Chart 1.—Acid production of B. welchii in plain, glucose and lactose meat mashes.

curve for the plain mash; it occurred, however, also in the sugar medium curves, together with distinct depressions before and after, as in the plain mash medium. These findings raised doubt as to whether 5% of these sugars was really an excess, or, if so, whether the initial peaks of the curves really indicated the exhaustion of the sugar and whether our original interpretation of the secondary peak in the titer curve as due to deaminization was correct.

Turning first to the possibility that B. welchii was able to exhaust even 5% of sugar from the medium, a comparison was made of the acid curves for mediums containing in one case as high as 20% added glucose.

The P_H curves were found to be approximately alike for all mediums containing glucose, whether 5, 10 or 20%; a peak was reached and a depression occurred, somewhat tardily it is true, as in the medium without added sugar.

All of the titer curves in this experiment were more flat-topped than usual after the initial peak; those for 5 and 10% glucose were essentially alike and with the control without sugar showed the secondary peaks less plainly than usual. The 20% glucose titer curve showed no secondary peak, but the initial peak was more marked than in the mediums containing less sugar.

Yet the sugar was not exhausted in any of these except the control, as the following data show:

Added Glucose	Final Glucose	Glucose Utilized
%	%	%
0	0	?
5	3	2
10	8	2
20	15.5	3.5

Some attempts to use the usual chemical sugar tests failed, owing to the interference by the nitrogenous constituents of the mediums, but we were successful in removing these by Foster's [4] method of precipitation with tannic acid, lead acetate, and sodium oxalate, followed by the Folin-McEllroy [5] technic for quantitative estimation of glucose.

This experiment compelled us to discard the idea that the initial peaks of the curves, either of P_H or of titratable acidity, represent the "sugar-free" point and convinced us that even 5% glucose was an excess for this culture under the conditions of the test.

We immediately became interested in determining more exactly how much glucose B. welchii could utilize in mediums containing less than 5%. The curves in chart 2 show the result of such an experiment.

[4] Jour. Bacteriol., 1921, 6, p. 211.
[5] Jour. Biol. Chem., 1918, 33, p. 513.

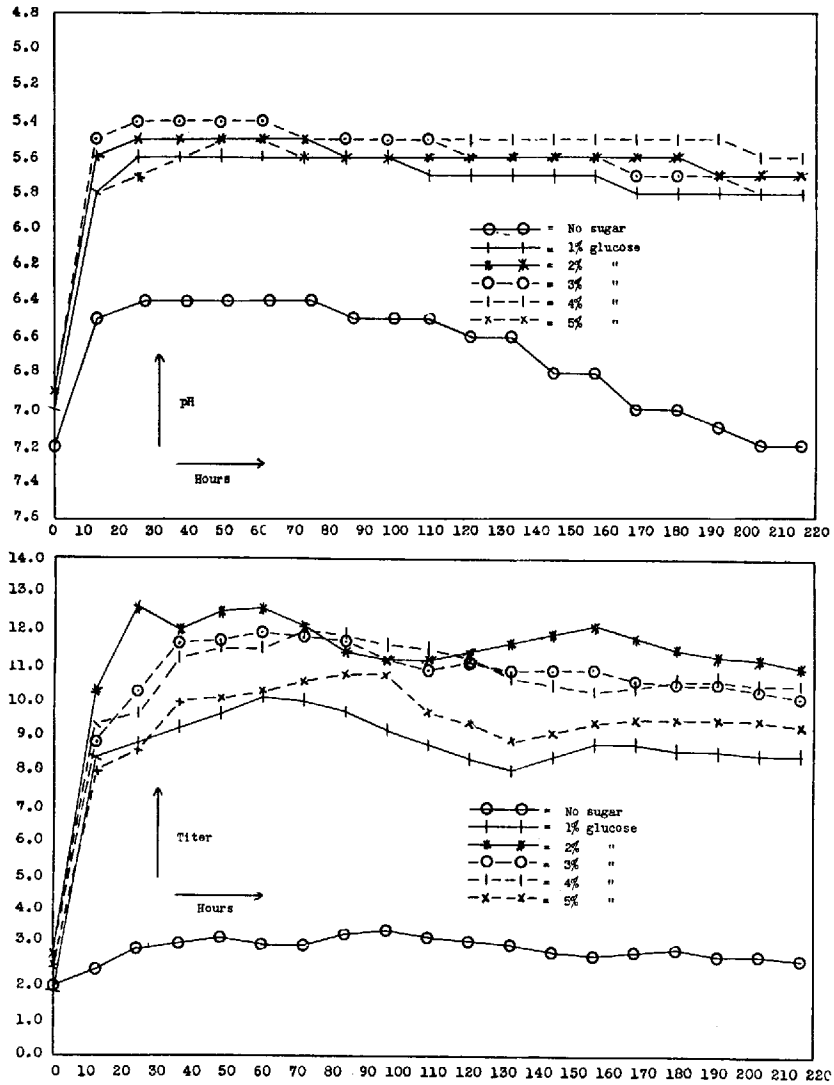

Chart 2.—Acid production of B. welchii in meat peptone mashes containing varying concentrations of glucose.

There was nothing significant in the P_H curves; those for the sugar mediums reached a higher acidity than that for the sugar-free mediums, but without marked differences among themselves.

The titer curves were more interesting, with the highest acidity recorded for 2% glucose; then 3, 4, 5, 1 and 0% in the order named.

It is clear that 2% glucose provided optimum conditions for acid production in our tests; more than 2% gave evidence of inhibition and 1% was completely exhausted, as shown by the final quantitative tests whose results follow:

Added Glucose	Final Glucose	Glucose Utilized
%	%	%
0	0	?
1.0	0.0	1.0
2.0	0.4	1.6
3.0	1.5	1.5
4.0	2.5	1.5
5.0	3.4	1.6

These data show that a remarkably definite amount (1.5-1.6%) of glucose was utilized in the various flasks of this experiment, but comparing the results with those of the previous test one might reason that the glucose utilized is within certain limits proportionate to the amount present even though that amount is an excess.

Secondary peaks are prominent in the titer curves for 1, 2 and 5% glucose, less so in those for 3 and 4%. The relatively close correlation of the curves for 1% glucose (less than excess) and for 5% glucose (more than 3 times the amount utilized) again emphasizes the difficulty of employing the titer curve as a criterion of freedom from sugar.

We next endeavored to find an explanation for the recessions in curves representing mediums with excess glucose, in the escape of volatile acids, which Wolf and Telfer[6] have shown comprise about 50% of the total acidity in a 2% glucose peptone medium.[6] Tests were made, both with plain meat mash peptone medium and with a similar medium containing 5% glucose, using 2 flasks of each. In each set, one flask was stoppered, as usual, with a cotton plug wrapped in gauze; the other was provided with a sterile rubber stopper which was wired onto the neck of the flask. Through a perforation in this rubber stopper extended a glass tube reaching from near the bottom of the flask and bent over on the outside like a siphon to connect with a short piece of rubber tubing closed with a pinch cock. The purpose of this device was to prevent the escape of volatile acids whose loss we thought might account for the recession in both P_H and titer curves in our

[6] Bioch. Jour., 1917, 11, p. 197.

previous experiments. The pressure developed within the flasks so stoppered was ample within a few hours to force the culture fluid samples through the siphon when the pinch cock was opened.

The results, however, failed to support our supposition, for both the P_H and titer curves secured for plain meat mash and the 5% glucose meat mash were essentially the same in the rubber stoppered and cotton stoppered flasks.

Assuming from Kendall's work that ammonia cannot be held responsible for a recession in acidity so long as an excess of fermentable sugar remains, and having failed to account for such a recession by the escape of volatile acids, we are now inclined to attribute the loss of acidity that occurs to destruction of certain acids with formation of neutral, or at least less strongly acid compounds, as, for example, carbon dioxide and water from lactic acid. Some of these may recombine to form less dissociable compounds whose presence would be shown in the secondary peak of the titer curves, even while the H + ion concentration was falling. This explanation seems now more acceptable than that formerly advanced, in which we assumed the secondary titer curve to be due to an increase in amino acids.

None of our mediums containing excess sugar, in which a recession in the curves occurred, became more alkaline than P_H 6; therefore in utilizing a terminally increased H + ion concentration as a qualitative criterion of fermentation, where a neutral medium is used, as recently advocated,[7] there is no danger of misinterpretation attributable to such recessions.

We then returned to the curious differences in the height of the titer curves for lactose and glucose shown in chart 1. It will be recalled that the P_H curves were essentially alike.

Several ideas occurred to explain the differences in the titer curves. A seemingly remote one was that the primary hydrolysates of lactose, i. e., glucose and galactose, were unequally fermented, and we reasoned that by doubling the amount of lactose (10%) in one flask over that of glucose (5%) in the other, we should obtain identical results. We also compared a medium containing 5% galactose with these.

The P_H curves for glucose, galactose and lactose were practically alike, also the titer curves for glucose and galactose, but the titer curve for lactose (10%) again ranged below those for the monosaccharides.

[7] Jour. Infect. Dis., 1921, 29, p. 321.

The curves for the disaccharide, saccharose, and the monosaccharide levulose also resemble those for glucose and galactose, although in this experiment the P_H values as well as the titer values for lactose ranged slightly lower than those for the other sugars.

Notwithstanding, the H + ion concentration seems to constitute an important limiting factor in the utilization of these sugars by B. welchii, and the more marked differences in titratable acidities may some time be explained on the basis of different dissociation constants in the products of lactose fermentation. Possibly more highly ionized products are formed from lactose than from glucose, galactose, levulose or saccharose, and the transformation may be direct or at least without an intermediate hydrolysis of lactose into monosaccharides. We could not, at this time, undertake to determine whether there are differences in the acids formed from these sugars by B. welchii, and the literature gives no satisfactory clue.

SUMMARY

The fact that a distinct peak, followed by a depression in H + ion concentration and titratable acidity, occurs in peptone meat mash mediums containing an excess of glucose, levulose, galactose, lactose or saccharose undergoing fermentation by B. welchii, precludes the possibility of regarding the change in direction of such curves as proof of freedom from sugars.

The escape of volatile acids seems not to be responsible for the recessions in acidity, and the hypothesis is suggested that some of the acid first formed is subsequently destroyed. The resulting products being less dissociable may account for the recession in H + ion concentration, and at the same time for the increased titer observed in the secondary peak of the titer curves.

The recessions in acidity, when an excess of fermentable sugar is present, are insufficient to confuse qualitative fermentation tests.

The limiting P_H values for glucose, levulose, galactose, saccharose and lactose are approximately equal; also the titratable acidities for all but lactose; B. welchii never produces quite as much acid from lactose as from the other sugars, owing possibly to different dissociation constants in the end products.