THE EFFECT OF pH ON THE FERMENTATION OF GLUCOSE AND GALACTOSE BY STREPTOCOCCUS PYOGENES¹

A. G. C. WHITE, R. H. STEELE, AND W. A. PIERCE, JR.

*Departments of Biochemistry and Microbiology, Tulane University School of Medicine,
New Orleans, Louisiana*

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Comparative studies on the fermentation of glucose and galactose by *Streptococcus pyogenes* strain S-23 have shown marked quantitative, but not qualitative, differences in the end products produced (Pierce and White, 1952; Steele *et al.*, 1954). The organism readily converts about 90 per cent of the glucose utilized during growth to lactic acid. When galactose serves as the energy source, a much smaller proportion of the fermented sugar is converted to lactic acid. In the first instance acetic acid, formic acid, and ethyl alcohol represent approximately 10 per cent of the end products while in the second case up to 80 per cent of the sugar used is accounted for in these three compounds.

We have also found (Pierce and White, 1954) a marked difference in the pH curves of glucose and galactose cultures of *S. pyogenes*. The pH of the glucose cultures fell rapidly below seven while that of the galactose cultures remained alkaline for a much longer period of time and then fell slowly to an acid value. Changes in medium pH are well known to have an effect on the metabolism of many microorganisms. This has been shown specifically for the enterococci by Gunsalus and Niven (1942). We have therefore attempted, in the series of experiments reported here, to ascertain whether the pH was responsible, at least in part, for the transformation of *S. pyogenes* from a homolactic to a heterolactic organism when galactose is substituted for glucose. The results of these experiments indicate that the pH of the medium, within the limits examined, is not responsible for this change.

MATERIALS AND METHODS

Organism. The organism used in these experiments was *S. pyogenes* strain S-23, a type 14

organism, maintained in the mucoid phase. The details of the maintenance of the organism have been reported earlier (Pierce and White, 1952).

Culture medium. The organisms were grown in heart infusion broth (Difco) containing glucose or galactose in a concentration of 1 per cent W/V, supplemented with 0.5 per cent W/V neopeptone (Difco). Incubation was at 37 C for the time indicated.

Initial attempts to maintain the pH of the cultures by incorporation of buffers in the medium failed due to inhibition of growth by the relatively high buffer concentration required. It was found, for example, that the use of a medium containing 0.08 M phosphate buffer (final concentration of phosphate) and adjusted initially to pH 7.8 resulted in a 50 per cent decrease in growth, as measured by bacterial nitrogen. The pH even under these circumstances decreased to 6.7 after 24 hours.

In the experiments reported here the pH was maintained either manually by the addition of 5 N NaOH at 30 minute intervals or automatically by the use of a Beckman titrimeter (Model K). This instrument proved very satisfactory under our conditions. The only modification of the apparatus was the construction of a shield around the electrode assembly unit. This shield was so constructed that it completely surrounded the incubation flask, thus minimizing the possibility of air borne contamination. The electrodes and stirring unit of the instrument were treated, prior to use, by immersion in a 1-2,000 solution of "aquet".² They were then rinsed twice with sterile distilled water. Three hundred ml of sterile culture medium were transferred to a sterile 400 or 600 ml beaker and placed in a 37 C water bath. The electrodes and stirrer were then placed

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² "Aquet" is a detergent sold by Emil Greiner Co., New York City. According to the manufacturer it is a 25 per cent solution of a polyglycol ether in water.

in the medium and the pH adjusted. This is a relatively easy matter since the instrument can be used as a conventional pH meter. The shield mentioned above now completely surrounded the beaker. The culture was then inoculated, the instrument set to titrate with 5 N NaOH in the burette, and incubation allowed to proceed. The culture, after incubation, was examined by direct smear and by plating on blood agar to detect contamination. Two of ten preparations maintained under these conditions were found to be contaminated after incubation and were discarded. With the apparatus described, pH control over a 6-8 hour period was maintained within ± 0.2 of a pH unit.

Chemical methods. After the prescribed incubation period the organisms were killed by the addition of 1 ml of 10 N H_2SO_4 per 100 ml of medium.³

Sugar was determined before and after fermentation either directly on the medium by the method of Morris (1948) or after $ZnSO_4$ -Ba(OH)₂ deproteinization by the method of Nelson (1944). Values given by the two methods were in good agreement.

Ethyl alcohol was determined quantitatively according to the procedure of Friedemann and Klass (1936).

The quantitative determination of acetic, formic, and lactic acids was carried out by the chromatographic analysis of an aliquot of a 40 hour ether extract of the medium on a silicic acid column according to the procedure previously described (Steele *et al.*, 1954).⁴

All values were corrected by a simultaneous analysis of samples of sterile, uninoculated broth adjusted to the same pH as the cultures and incubated for the same time interval at 37 C.

RESULTS

The fermentation data on galactose reported previously (Steele *et al.*, 1954) were obtained

³ This was erroneously reported as 1 ml of 1 N H_2SO_4 per 100 ml in a previous communication (Steele *et al.*, 1954).

⁴ A typical sequence of butyl alcohol-chloroform solutions of increasing polarity (increasing butyl alcohol concentration) as the mobile phase which provided satisfactory resolution and recoveries of the fermentation acids is as follows: Acetic acid, 500 ml, 5 per cent butyl alcohol in $CHCl_3$; formic acid, 250 ml, 10 per cent butyl alcohol in $CHCl_3$; lactic acid, 400 ml, 40 per cent butyl alcohol in $CHCl_3$. The acids are given in the order in which they appear in the effluent.

TABLE 1

Effect of time on the fermentation of galactose by Streptococcus pyogenes

	6 hr	24 hr
Lactic acid.....	73	110
Acetic acid.....	71	55
Formic acid.....	93	82
Ethyl alcohol.....	47	36
Per cent conversion to lactic acid....	36	55
Sugar used (mm).....	1.39	1.61
Redox index.....	1.01	1.14
Carbon recovery (%).....	92	98

Analytical values in millimoles per 100 millimoles of sugar used.

TABLE 2

Effect of pH on the fermentation of glucose and galactose by Streptococcus pyogenes

	Glucose				Galactose			
	pH of culture							
	7.8	6.6	6.0	8.2	7.8	6.5	6.0	
Lactic acid.....	179	184	190	49	59	42	114	
Acetic acid.....	23	10	6.6	121	114	210	86	
Formic acid.....	19	11	4.7	157	141	37	86	
Ethyl alcohol...	4.0	6.8	0	32	40	15	31	
Sugar used (mm).	2.49	1.33	1.00	1.51	1.18	0.67	0.29	
Per cent conversion to lactic acid.....	90	92	95	25	30	21	57	
Redox index....	2.4	0.80	—	2.4	1.8	1.2	1.4	
Carbon recovery (%).....	101	100	97	101	104	102	110	

Analytical values in millimoles per 100 millimoles of sugar used.

Incubation time 6-8 hours at 37 C.

after a 24 hour incubation period. On the basis of subsequent experiments we have found that, under our conditions, growth as measured by bacterial nitrogen ceases after 6 to 8 hours (Pierce and White, 1954). The pH at this time is between 5 and 6. Since we have been interested primarily in the fermentation by growing cells, all experiments reported here, except those given in the first table, were based upon a 6 to 8 hour incubation period. Table 1 gives a comparative analysis of the end products formed in the galactose medium after 6 hour and 24 hour incu-

bation periods. For ease of comparison the results are expressed on the basis of 100 millimoles of sugar used. The pH of both cultures was initially 7.8. At the end of the 6 hour incubation period, the pH had fallen to approximately 5.8 while that of the comparable 24 hour culture had fallen to 5.2. It will be noted from the table that the major change in the end products is a small production of lactic acid in the shorter time interval. Both the redox index and carbon balance are in reasonable agreement with theory. The acetic acid, formic acid, and ethyl alcohol appear to retain a relatively constant relationship to one another which may indicate a single precursor.

A typical set of analytical values obtained under conditions of controlled pH is given in table 2. When glucose is the carbon source, there appears to be no marked difference in the fermentation pattern of the organism over the pH range tested. On the basis of the results of Gunsalus and Niven (1942) this finding was rather unexpected.

At an acid pH there does appear to be a change in the production of lactic acid with galactose as the substrate. This is most pronounced at a pH of 6.0. Unfortunately our strain does not initiate growth readily at this pH. In consequence, a relatively small amount of sugar was used, and the accuracy of the analyses was decreased. At pH values below 6 on either sugar no growth could be obtained.

While the carbon balances in all cases appear to be satisfactory, the redox indices which also serve as an indicator of completeness of the analysis differ markedly from the theoretical value of 1.0 in the experiments conducted under alkaline conditions. The values, which we believe to be reasonably accurate since they have been repeatedly obtained, indicate an excess of oxidized material, that is, formic acid. This finding was completely unexpected since in our earlier experiments, in which the pH was not controlled, the redox indices had been found to approximate 1.0, regardless of the presence or absence of oxygen in the gas phase. We have observed in the course of other growth experiments an uptake of oxygen by *S. pyogenes* when grown aerobically, but since our earlier experiments were satisfactory from the standpoint of carbon balance and redox index calculations, we had assumed that the oxygen taken up by the cells was used in processes other than the oxidation of carbohydrates. This may not be the case with cells main-

tained under alkaline conditions. Barron and Jacobs (1938) and Barron and Lyman (1939) have studied several oxidations carried out by resting cells of hemolytic streptococci and reported an optimum pH of 7.0 for the oxidation of glucose. Under their conditions oxidation was apparently not complete, but no analyses for end products were reported. On the other hand, O'Kane (1950) using resting cells of *Streptococcus faecalis* 10 Cl found no effect of pH on the oxidation of glucose over the pH range 6 to 8 and no change in the proportion of end products. He did, however, note a marked increase in the utilization of oxygen and a change in the distribution of end products on the addition of lipoic acid to his preparations. In growth experiments in a synthetic medium thiamin appeared to serve as well as lipoic acid in stimulating the oxidative process. The effect of these two substances on oxygen consumption by our organism is unknown. Also of interest in this connection are the more recent observations of Dolin (1953) who has described the isolation from the same organism of an enzyme system capable of carrying out the oxidation of reduced diphosphopyridine nucleotide. An intermediate step in this reaction appears to be a peroxidation. The utilization of this mechanism by the organism could explain the coupling of oxygen with the oxidation of certain organic compounds by these cells.

Much more information is obviously needed before an understanding of the role of oxygen in the metabolism of these cytochrome deficient organisms is explained.

DISCUSSION

The change in the metabolism of *S. pyogenes* brought about by the substitution of galactose for glucose in the medium cannot be explained as a pH effect. Over the pH range 6 to 8 the organism grown in a glucose heart infusion broth produces the same end products in essentially the same proportion. These results are contrary to those reported by Gunsalus and Niven (1942) for two other homolactic organisms, *Streptococcus liquefaciens* and *S. faecalis*, but agree with the observation of DeMoss (1953) who used resting cells of *Leuconostoc mesenteroides*, a heterolactic organism.

The proportion of lactic acid produced from galactose is markedly less after six than after twenty-four hours (compare tables 1 and 2)

unless the pH of the medium is maintained below 6.5. The value obtained after a twenty-four hour incubation period can be explained, therefore, if one assumes that after the sixth or the seventh hour essentially resting cells are metabolizing the sugar at a pH of 6 or below.

We are unable to offer a reasonable explanation for the effect of pH on the distribution of the end products in the fermentation of galactose. Based upon the figures obtained for formic acid, acetic acid, and ethyl alcohol at pH 7.8 and 8.2 (table 2) one could postulate a three carbon precursor since the sum of the values for acetic acid and ethyl alcohol is approximately equal to the formic acid found. At pH 6.5, however, this relationship is not observed, and only the formic acid and ethyl alcohol are present in relatively the same molar ratio. The value for acetic acid in this case is so far out of line as to appear unreasonable, but a similar result was obtained in two additional, similar experiments.

According to Elsdon (1952) only two mechanisms are known for the production of formic acid by microorganisms. One is the hydroclastic or phosphoroclastic split of pyruvate which was first described by Neuberg (1914). The second is the reversal of the formic hydrogenlyase reaction.

Under aerobic conditions the second reaction would be expected to proceed to the right liberating to CO_2 and H_2 . We have found no indication of the formation of either of these gases in our cultures. This observation, therefore, would appear to rule out the second reaction. If the first reaction does occur in our organism, as has been shown for resting cells of other hemolytic streptococci by Barron and Jacobs (1938), then our results may be partially explained. Under these circumstances one might expect the production of some reduced product such as ethyl alcohol. This would presumably be accomplished by transfer of the hydrogens from DPNH_2 to a two carbon intermediate rather than to pyruvic acid as is the case in glycolysis.

The mechanism of formation of ethyl alcohol in these organisms is still unknown. It would appear possible on the basis of some of our present data that an aldehyde dehydrogenase-

alcohol dehydrogenase type of reaction, as suggested by Racker (1949), occurs since in all experiments both ethyl alcohol and acetic acid are found, though not in equivalent quantities. Changes in pH might be responsible for these variations.

With the information available at present it does not appear possible to propose a general scheme of metabolism which will account for the observed results. The suggestions which are presented are offered primarily as paths for future experiments.

SUMMARY

Automatic pH control of growing bacterial cultures within ± 0.2 of a pH unit has been possible by the use of a Beckman titrimeter (Model K).

Over a pH range of 6.0 to 7.8 no marked change in the over-all fermentation of glucose by *Streptococcus pyogenes* has been observed. At the pH values tested the organism converted to lactic acid 90 to 95 per cent of the glucose utilized.

With galactose as the carbon source a marked increase in the production of lactic acid was noted at a pH of 6.0 as compared with higher pH values. Under these conditions the production of lactic acid from galactose rose from approximately 25 per cent at pH 8.2 to 57 per cent at pH 6.0.

There is an indication, based upon the calculation of redox indices and the uptake of oxygen by growing cells, that under alkaline aerobic conditions *S. pyogenes* can utilize atmospheric oxygen in carbohydrate metabolism. This utilization appears to decrease with an increase in the acidity of the medium in which the cells are grown.

The change in the carbohydrate metabolism of *S. pyogenes* brought about by substituting galactose for glucose as the carbon source is still unexplained.

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