The Differential Effect of Temperature on Gas Production by a Coliform Organism

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SUMMARY: The influence of the temperature of incubation on the course of formic acid and gas production by cultures of a coliform organism growing in buffered and unbuffered peptone media was studied. The evidence suggests that at 30° and at 87° the onset of gas production is determined by the concentration of undissociated formic acid in the culture, but that a higher concentration of acid is required to elicit response at 87° than at 30°. Undissociated formic acid is toxic to the organism and it appears that gas is not produced in unbuffered glucose medium at 37° because the environmental conditions do not allow elaboration of hydrogenlyase before inhibitory conditions of pH value and formate concentration are attained.

Coliform bacteria which ferment sugars at 37° with the production of acid and gas are believed to be mainly of intestinal origin and their presence in water is considered indicative of faecal contamination. In routine water analysis occasional coliform strains are encountered which ferment sugars at 37° without the production of gas but which produce acid and gas in the normal way at lower temperatures (Prescott, Winslow & McCrady, 1946). These strains are ordinarily disregarded in the assessment of purity since they are believed to be of non-faecal origin (Stuart, Mickle & Borman, 1940); their anomalous behaviour has received little attention. The gas consists of hydrogen and carbon dioxide and arises by the action of the adaptive hydrogenlyase system on the formic acid produced by carbohydrate breakdown. Wolf, Stickland & Gordon (1954) examined two coliform strains, B6 which produces acid and gas at 30° but only acid at 37°, and C59 which produces acid and gas at 87° but only acid at 44°, when grown in glucose broth. Organisms grown at the higher temperature were devoid of hydrogenlyase but the activity of preformed enzyme was similar at both temperatures; they concluded that enzyme synthesis was inhibited at the higher temperature. Growth of these organisms in glucose broth is noticeably poorer at the higher temperature than at the lower, which suggests that the change in the environmental conditions, which occurs as a consequence of the metabolism of the growing organisms, is more detrimental at the higher temperature. The toxicity of formic acid to coliform organisms increases with decrease in pH value (Gale & Epps, 1942; Dagley, Dawes & Foster, 1958) and since acid is still produced at the higher temperature, inability to synthesize hydrogenlyase may be an important contributory factor in growth inhibition.

Accordingly, the present investigations were concerned with the effect of temperature on the growth and glucose metabolism in peptone media of coliform organism B6, and in particular with its effect on the formic acid/gas system.
METHODS

Media. The medium used for the investigation of the inhibitory effect of formic acid on growth (Table 3) contained: glucose, 0.1% (w/v); citric acid + KH₂PO₄ buffer, 0.07 M; bacteriological peptone (Oxoid), 1% (w/v). In all other experiments the medium contained: glucose, 0.5% (w/v); KH₂PO₄ + Na₂HPO₄ buffer, 0.13 M or Na₂SO₄, 0.1 M; bacteriological peptone (Oxoid), 1% (w/v).

Peptone and buffer or sodium sulphate solutions of concentration twice that required in the complete medium were prepared and sterilized separately. Equal volumes of the solutions were mixed in the fermentation vessel (except in the inhibition study where 25 ml amounts of complete medium were distributed aseptically to 1 oz. screw-capped bottles) and to the mixture was added one twentieth its volume of sterile 10% or 2% (w/v) glucose solution.

Formate was included, when needed, by adding an appropriate volume of sterile sodium formate solution of concentration fifty times that required in the medium.

Fermentation procedures. Except for the single experiment where screw-capped bottles were employed, an atmosphere of oxygen-free nitrogen was maintained in the fermentation vessels. In all experiments the vessels were held in thermostatically controlled water baths.

In the initial studies of growth and glucose utilization 250 ml. or 500 ml. conical flasks were used as fermentation vessels. These contained 100 ml. or 200 ml. medium and were fitted with a rubber bung equipped with three short tubes. Two tubes were used for the continuous passage of nitrogen through the flask and the third was wide enough to allow the introduction of an 8 mm. diameter pipette for the removal of samples. When not in use the third tube was closed with a rubber stopper.

For experiments which involved the quantitative determination of gas evolved the constant pressure apparatus shown in Fig. 1 was used. The vessel contained 260 ml. medium. Samples were removed through the side arm. During the preliminary 20 min. gassing of the apparatus and during inoculation or removal of samples, nitrogen was passed continuously through the flask. When gas passage was to be discontinued the stopper was first pushed home and the tap immediately closed. This left a slight positive pressure in the flask.
The tap was then momentarily opened to relieve the pressure and the manometer level noted. Finally, the right-hand limb of the manometer was lowered to produce a slight reduction of pressure in the flask. Before a determination of hydrogen evolved was made, the vessel was swirled vigorously for about 30 sec. Where the culture was evolving gas there was a rapid initial depression of the manometer level which quickly steadied and was then little affected by continued shaking.

A similar apparatus containing distilled water was placed alongside the fermentation vessel to detect changes in volume caused by changes in atmospheric pressure. The determinations of gas evolved have not been corrected to N.T.P. It is important that the organisms be kept dispersed throughout the medium during the course of a fermentation if reproducible results are to be obtained. To achieve this the fermentation flask was swirled by hand every 20 min. during the phase of active glucose breakdown.

**Inoculum.** All inoculations were from cultures grown for approximately 18 hr. at the temperature of the main experiment. Where the constant pressure apparatus was used the inoculum was 1 ml. of culture grown in the phosphate-buffered medium (initial pH 6.9). Hydrogenlyase was present in the organisms of these inocula but its activity was much more pronounced in organisms grown at 30° than in organisms grown at 37°.

For other experiments the inoculum medium did not contain glucose but otherwise had the same composition as the medium used in the main experiment and was adjusted to pH 6.9 initially. The volume of inoculum was such that the initial count in the main fermentation was approximately 5 million viable organisms/ml. These organisms did not contain hydrogenlyase.

**Estimation of growth.** (a) For viable counts, selected dilutions of the culture were plated on nutrient agar (Oxoid) and counted after incubation for 48 hr. at 30°. (b) Opacity measurements of cultures were made with the EEL portable colorimeter, equipped with an orange filter.

**Yield and protein content of organisms.** The organisms from 200 ml. of culture were recovered by centrifuging, washed once with distilled water, again centrifuged and then suspended in distilled water. The EEL portable colorimeter, equipped with a yellow-green filter, was used for the determination of protein in a sample of suspension by Stickland’s (1951) method. The organisms in the remainder were recovered by centrifugation and dried at 100°.

**Glucose and formic acid.** The determinations were made on samples of culture fluid from which the organisms had been separated by centrifugation. For glucose the culture fluid was diluted 50 fold before analysis when it contained buffer, or 25 fold when it contained sodium sulphate. Glucose was determined by Somogyi’s (1945) method.

For formic acid 4 ml. of culture fluid were treated with 4 ml. of 15 % (w/v) CuSO₄·₅H₂O solution and 4 ml. 10 % (w/v) Ca(OH)₂ suspension and the precipitated glucose and protein sedimented in the centrifuge. Three ml. supernatant fluid mixed with 2 ml. 2 N-H₂SO₄ and 8 g. MgSO₄·₇H₂O were steam distilled in the Markham still and 50 ml. distillate collected. This was
neutralized to phenolphthalein with 0.01 N-NaOH and then boiled down to approximately 5 ml. The formic acid in this 5 ml. was determined by Hopton's (1953) method.

RESULTS

Influence of buffer capacity of medium

The amount of growth and of glucose consumption by the organism in peptone + sodium sulphate (unbuffered) medium at 30° and 37° are shown in Table 1(a). The adverse effect of the higher temperature is apparent, but at neither temperature had the glucose been completely consumed when multiplication had ceased. A series of qualitative experiments with media containing different concentrations of phosphate buffer and of glucose demonstrated that growth could be enhanced at both temperatures when measures were taken to check the decrease in pH value. The results shown in Table 1(b) indicate the improvement which was obtained when 0.1 M-sodium sulphate was replaced by 0.13 M-phosphate buffer. Moreover, in this medium gas was produced in the culture grown at 37° and the organisms showed hydrogenlyase activity when assayed in the Warburg apparatus.

Measurements of the yield and protein composition of organisms obtained under the various cultural conditions (Table 2) when considered with those in Table 1 show that glucose fermentation in buffered medium was accompanied throughout by multiplication. A decrease in the yield of organisms at a temperature higher than the optimum is a common feature of bacterial growth.

Table 1. Glucose consumption, multiplication and pH change in phosphate-buffered and unbuffered peptone medium at 30° and 37°

<table>
<thead>
<tr>
<th>Time (hr.)</th>
<th>Glucose consumed (mg./100 ml.)</th>
<th>pH value</th>
<th>Viable count (in 10⁶/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°</td>
<td>37°</td>
<td>30°</td>
</tr>
<tr>
<td>(a) Unbuffered culture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>(479)*</td>
<td>(484)*</td>
<td>6.90</td>
</tr>
<tr>
<td>3.5</td>
<td>5</td>
<td>9</td>
<td>6.36</td>
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<td>6</td>
<td>49</td>
<td>28</td>
<td>5.40</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>96</td>
<td>48</td>
<td>5.04</td>
</tr>
<tr>
<td>10</td>
<td>152</td>
<td>-</td>
<td>4.68</td>
</tr>
<tr>
<td>25</td>
<td>282</td>
<td>38</td>
<td>4.30</td>
</tr>
<tr>
<td>(b) Buffered culture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>(462)*</td>
<td>(455)*</td>
<td>6.97</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>33</td>
<td>6.90</td>
</tr>
<tr>
<td>5.5</td>
<td>53</td>
<td>115</td>
<td>6.74</td>
</tr>
<tr>
<td>7.5</td>
<td>197</td>
<td>319</td>
<td>6.55</td>
</tr>
<tr>
<td>8</td>
<td>272</td>
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<td>-</td>
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<td>8.5</td>
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<td>455</td>
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<td>9</td>
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<td>6.31</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>-</td>
<td>6.45</td>
</tr>
</tbody>
</table>

* Figures in parentheses are initial concentrations of glucose.
Gas production by a coliform organism

In unbuffered medium at 30° fermentation can continue when conditions become unfavourable for cell synthesis, whereas at 37° inhibition of glucose breakdown accompanies inhibition of cell synthesis.

Table 2. Yield and protein content of coliform B6 organisms grown in phosphate-buffered and unbuffered peptone medium at 30° and 37°

<table>
<thead>
<tr>
<th>Medium and temperature</th>
<th>Time (hr.)</th>
<th>Glucose consumed (mg./100 ml.)</th>
<th>Dry wt. organisms (mg./100 ml.)</th>
<th>Protein/2 mg. dry wt. organism (colorimeter units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unbuffered, 30°</td>
<td>7-75</td>
<td>105</td>
<td>19.9</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>211</td>
<td>21.4</td>
<td>1.18</td>
</tr>
<tr>
<td>Unbuffered, 37°</td>
<td>7</td>
<td>42</td>
<td>6.3</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>56</td>
<td>5.7</td>
<td>1.26</td>
</tr>
<tr>
<td>Buffered, 30°</td>
<td>0.25</td>
<td>456</td>
<td>70.1</td>
<td>1.08</td>
</tr>
<tr>
<td>Buffered, 37°</td>
<td>9</td>
<td>450</td>
<td>56.1</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Quantitative determination of hydrogen evolution and formate production in growing cultures

When organism B6 was grown in unbuffered medium at 37°, formic acid accumulated in the medium (Fig. 2), but no hydrogen had been evolved when glucose breakdown ceased. It is possible that a little free formic acid was formed in the initial stages of the fermentation at 30° but during the phase of brisk hydrogen production the gas-producing system was active enough to prevent the accumulation of formic acid. Stephenson & Stickland (1932) reported that the optimum pH value for hydrogenlyase in Escherichia coli was 7.0; consequently the efficiency of formic acid breakdown at such low pH values seems, at first, surprising. In a later publication, however, Stephenson (1937) mentioned that the optimum pH value of hydrogenlyase changed with the concentration of formate and moved towards the acid side as the amount...
of formate was decreased. Studies with washed suspensions of organism B6 have shown that the optimum pH value for hydrogenlyase in this organism is similarly dependent on the formate concentration.

The sequence of events when growth took place in a medium buffered with phosphate initially at pH 6-9 is illustrated in Fig. 3. Hydrogen evolution was only just evident in the 37° culture when the glucose was almost exhausted and when there was abundant formate in the medium. At 30° a trace of hydrogen had been produced when approximately 60 mg. glucose had been decomposed, at which stage a definite increase in the formate content of the medium was apparent. Subsequently the formate concentration increased still further but hydrogen continued to be evolved during the fermentation of the remainder of the glucose.

Comparison of the behaviour of buffered and unbuffered cultures at 30° suggests that the delay in the response of the gas-producing system (measured in terms of the amount of glucose consumed from the start of fermentation) lessens with decrease in pH value of the medium. This has been shown to be true for other coliform bacteria (Tikka, 1935; Mickelson & Werkman, 1938). That the pH value of the environment exerted a similar influence on the behaviour of organism B6 at both temperatures was made clear when fermentation in medium buffered initially at pH 6-8 was examined (Fig. 4). At both temperatures less glucose breakdown had taken place when hydrogen began to be evolved than in the corresponding cultures grown at the higher stabilized pH range. Here again, however, hydrogen production was not so readily evoked at 37° as at 30°.

It can be inferred from the foregoing experiments that the formate concentration and the pH value of the medium play interrelated parts in eliciting gas production by growing cultures of the organisms used; the evidence points to undissociated formic acid as having a central role in determining the response of the gas-producing system. No gas was produced for some time in the cultures buffered initially at pH 6-9 even though the concentration of formate

![Fig. 3. Dissimilation of glucose in phosphate-buffered peptone medium at 30° and 37°. Initial pH 6-9. Glucose, ●; hydrogen, ○; formic acid, ▲; pH, △.](image-url)
Gas production by a coliform organism

had increased and the inoculum organisms contained hydrogenlyase. This may indicate that de-adaptation occurred for a time because the concentration of undissociated acid was kept low and that not until further metabolism had decreased the pH value and increased the formate concentration did re-adaptation, and consequently gas production, begin. This behaviour is being further investigated. The delay in gas production (again measured in terms of

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**Fig. 4.** Dissimilation of glucose in phosphate-buffered peptone medium at 30° and 37°.
Initial pH 6.3. Glucose, ○; hydrogen, ○; formic acid, ▲; pH, △.

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**Fig. 5.** Dissimilation of glucose in phosphate-buffered peptone medium, containing added formate, at 37°. (a) Initial pH 6.9, initial concentration of formic acid 68 mg./100 ml.
(b) Initial pH 6.3, initial concentration of formic acid 40 mg./100 ml. Glucose, ●; hydrogen, ○; formic acid, ▲; pH, △.

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glucose consumed) was longer in the 37° culture than in the 30° culture. When growth took place in a medium buffered initially at pH 6.8 this delay was still quite evident at 37°, whereas at 30° it was very short (Fig. 6). This suggests that a higher concentration of undissociated formic acid is required for the initiation of gas production at 37° than at 30°. On this basis it would be expected that inclusion of formate in the medium initially would decrease the delay in gas production and that this would be easier to demonstrate at the
higher temperature. To investigate this the organism was grown in media buffered at pH 6.9 and 6.3 to which formate had been added. The initial concentration was approximately the same as that in the unsupplemented culture at the onset of gas production. Figure 5 shows that gas evolution began at a stage when less glucose had been consumed than in the corresponding culture not containing formate initially. The effect is better illustrated in Fig. 6 where hydrogen evolved is plotted against glucose consumed under the various cultural conditions. The plots have been confined to the results obtained during the period when the progress of fermentation was examined frequently.

Fig. 6. Plot of hydrogen evolved against glucose consumed during growth under various cultural conditions. (a) 30°: △-△, unbuffered medium; ○-○, buffered medium, initial pH 6.9; ●-●, buffered medium, initial pH 6.3. (b) 37°: ○-○, buffered medium, initial pH 6.9; ○-○-○, buffered medium containing added formate, initial pH 6.9. (c) 37°: ●-●, buffered medium, initial pH 6.3; ●-○-●, buffered medium containing added formate, initial pH 6.3.

Table 3. Effect of formic acid on growth of coliform organism B6 in citrate + phosphate-buffered peptone medium at 30° and 37°

<table>
<thead>
<tr>
<th>pH value</th>
<th>Initial concn. of formic acid (mg./100 ml.)</th>
<th>7 hr.</th>
<th>24 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30°</td>
<td>37°</td>
</tr>
<tr>
<td>5.2</td>
<td>0</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>5.7</td>
<td>0</td>
<td>0.41</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.19</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>6.2</td>
<td>0</td>
<td>0.58</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.42</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.33</td>
<td>0.38</td>
</tr>
<tr>
<td>7.1</td>
<td>0</td>
<td>0.85</td>
<td>0.59</td>
</tr>
</tbody>
</table>
Gas production by a coliform organism

The inhibitory effect of formic acid on growth

The effect of formate on growth at 30° and 37° in media buffered at different pH values is shown in Table 3. The lower total yield of organism at the higher temperature was here reflected in the opacity measurements. Growth in the presence of added formate was delayed at both temperatures but only in the medium buffered at the lowest pH value did formate have an effect on the total yield of growth at 30°. At 37° growth at pH 6.2 was unaffected by formate, but at pH 5.7 and 5.2 inhibition increased with increase in formate concentration and at the lowest pH value growth was severely curtailed.

DISCUSSION

The substrate of hydrogenlyase, formic acid, is a product of the metabolism of carbohydrate and the presence of the enzyme in the inoculum organisms is not a prerequisite for the initiation of growth. Thus the situation in the culture at the time of inoculation differs from that where initiation of growth is dependent on the organisms of the inoculum being able to elaborate an adaptive enzyme to attack a substrate which is the sole source of energy in the medium. The evidence of the above work and other observations (Dagley et al. 1958; Gale & Epps, 1942) indicate that the situation does become analogous when formic acid becomes potentially toxic, in which case previous formation of hydrogenlyase is obligatory for continued growth.

The experiments with cultures grown in buffered media revealed that the response, or the maintenance of activity, of the gas-producing system was conditioned by the status of the environment as regards pH value and formate concentration; the evidence points to undissociated formic acid as the determinant of activity. The effect of formic acid is qualitatively similar at both temperatures but less acid is required to elicit the response at the lower temperature. The results strongly suggest that the consequent activity is greater at the lower temperature than at the higher. Studies on tetraethionase and penicillinase, two adaptive enzymes which have a similar function to hydrogenlyase in that by their action their toxic substrates are removed, have shown that there is an optimum temperature for their formation (Pollock, 1945; Knox, 1950; Knox & Collard, 1952). With Bacillus cereus growth in the presence of penicillin is comparable with growth in its absence provided that the temperature of incubation is not inimical to penicillinase formation (Knox & Collard, 1952). The behaviour of coliform organism B6 with respect to temperature and concentration of undissociated formic acid is obviously similar. At the lower temperature formic acid only affects growth when present in relatively high concentration since the organism can readily form hydrogenlyase. At the higher temperature hydrogenlyase formation is suppressed and although this will be of little consequence where the concentration of undissociated acid is low it will impose a greater limitation where the concentration of acid is higher. Thus it was found that at 37° growth in medium buffered in the region of neutrality was comparable with growth in
the same medium at 30°, but inhibition became greater the lower the pH value
and the higher the concentration of formate. The evidence suggests that
coliform organism B6 does not produce gas in unbuffered glucose broth at 37°
because the environmental conditions have not allowed elaboration of hydro-
genlyase before the pH value has decreased enough to render the accumulated
formate toxic.

An appreciable part of this work was carried out in the Department of Agriculture,
University of Leeds, and was incorporated in a Ph.D. thesis submitted to the
University of Leeds in 1956.

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