Factors Affecting the Initiation of Respiration of *Streptococcus lactis*

R. SPENDLOVE, H. H. WEISER, AND W. J. HARPER

Department of Bacteriology and Department of Dairy Technology, The Ohio State University, Columbus, Ohio

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*Streptococcus lactis* is probably the most important organism used by the dairy industry. However, an examination of the literature indicates that this organism is incapable of many metabolic activities performed by other species of the genus *Streptococcus*.

The report of Farrell (1935) reports a characteristic of many of the streptococci. He used the reduction of indigotetrasulphonate in Thunberg experiments for the detection of dehydrogenases in 22 strains of *Streptococcus lactis* and found a rather general activation of carbohydrates, contrasted by an inability of these organisms to dehydrogenate 71 noncarbohydrate substances.

The noncarbohydrate compounds that seem to be oxidized most readily by the streptococci are glycerol (Barron and Jacobs, 1938; Gunsalus and Sherman, 1943; and Gunsalus and Umbreit, 1945); lactate and pyruvate (Barron and Jacobs, 1938); butyrate (Niven et al., 1945, and Wolin et al., 1952); and some of the alcohols (Gunsalus and Wood, 1942; Greisen and Gunsalus, 1943 and 1944; and Barner and O’Kane, 1952).

*Streptococcus faecalis* appears more versatile, however, and has been shown to utilize some of the tricarboxylic acid cycle intermediates as substrates (Campbell et al., 1943, and Dolin and Gunsalus, 1949 and 1951).

With the exception of the results of Kizer and Speck (1955) who showed a stimulation of *S. lactis* growth by citrate and acetate, the authors know of no other investigation reporting growth stimulation of *S. lactis* by citric acid. A stimulation of growth of *S. lactis* by citrate has not been shown in the absence of fermentable carbohydrate; this has been accomplished with other streptococci by Campbell and Gunsalus (1944).

This investigation was undertaken to determine if *S. lactis* was capable of any previously undetected metabolic activities that may be important in flavor production in dairy products. As lactic acid is practically the sole end product of lactose degradation by this organism, a study was made of the possible dissimulation of lactate.

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**Materials and Methods**

*Cultures*. The streptococci used were *S. lactis* strains 8 and 9 received from Dr. F. E. Nelson of the Dairy Technology Department at Iowa State College and *S. lactis* strain 32 obtained from the Chr. Hansen’s Laboratory Incorporated, Milwaukee, Wisconsin.

Most of the investigations were carried out using lyophilized cultures which had previously been stored at room temperature for one year.

The best growth was obtained when milk cultures of *S. lactis* were transferred every 12 hr using an inoculum of 0.5 per cent. These cultures, incubated at 30 C, coagulated milk in about 8 hr. To obtain the cells used in these studies, an inoculum was prepared by transferring 1 ml of recently coagulated milk into the bottom of a tube containing 20 ml of broth. After 3 hr incubation at 30 C, a band of growth about one-fourth of an in. deep formed above the milk layer. This growth was used as inoculum for broth medium containing not more than 0.3 per cent glucose and having an initial pH of 7.5 followed by incubation for 8 to 10 hr at 30 C.

*Media*. The medium for the cultivation of the *S. lactis* was a modification of the one designed by Barbo and Foster (1952). Its ingredients are: glucose, 3.0 g; peptonized milk, 2.5 g; neopeptone, 2.5 g; K2HPO4, 5.0 g; Tween 80, 0.5 g; yeast extract, 5.0 g; distilled water, 1,000 ml; pH to 7.5.

Substituting yeast extract for the liver and carrot extracts resulted in a medium that gave comparable growth and was prepared more easily.

*Warburg studies*. Oxygen uptake was determined by the Warburg manometric technique according to the procedure described by Umbreit et al. (1949). The microliters of oxygen consumed were calculated by the interval method to show more clearly the changing rates of uptake.

Following the desired incubation period the cells were collected by centrifugation, washed once with 0.067 M phosphate buffer (pH 7.0), and resuspended in buffer.

**Results**

Preliminary studies indicated no significant oxygen uptake by phosphate buffer suspensions of *S. lactis* 9 in the presence of various tricarboxylic acid cycle inter-
mediates. Numerous attempts were made to activate the cells without success. However, it was subsequently discovered that the cell suspensions would begin to respire actively if the time period of the experiment was extended over 3 hr. Some of the characteristics of these changing oxygen uptake rates can be seen in figure 1.

The longer period of time required for maximum respiratory activation by cells in the presence of glucose is of major importance in this investigation. Also worthy of note is the respiratory activity with succinate. This experiment was repeated numerous times during the early phases of the investigation, but the organism gradually lost its ability to activate its respiratory mechanism, and simultaneously lost its ability to utilize succinate. However, this culture was carried in broth not milk. Therefore, a milk culture of this organism that had been lyophilized for a year was used throughout most of the investigation as it was easier to activate.

To determine whether some substance necessary for a high endogenous respiration was being synthesized during the period of little activity, supernatant buffer from actively respiring cells was added to freshly harvested cell suspensions. The results in figure 2 indicated that some material had been excreted from the actively respiring cells which served as an activator for \( S. \) \( lactic \). This material was either oxidized when added to the freshly harvested cells or was capable of activating the endogenous respiration of these cells. The latter conclusion seems more probable as there was a direct relationship between the amount of supernatant added and the time required for respiration to commence.

Two other cultures of \( S. \) \( lactis \) (strains 8 and 32) were examined to determine whether they could be activated in a similar manner. In both cases the freshly harvested cell suspensions respired actively in the presence of the supernatant from \( S. \) \( lactis \) 9. The results with \( S. \) \( lactis \) 8 are presented in table 1.

**Factors Affecting Activation**

*Glucose concentration and pH.* It was assumed that a high endogenous respiration in the early part of some experiments was due to the oxidation of some carbohydrate as there had been no other reports of respira-

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**FIG. 1.** The prolongation of respiratory inactivity by glucose. Six-tenths ml of \( n/50 \) substrates were used. Thirty-six mg (dry weight) of cells were added per flask. 1 = succinate; 2 = endogenous control; and 3 = glucose added (\( Streptococcus \) \( lactic \) strain 9).

**FIG. 2.** The effect of activator concentration (supernatant buffer from \( Streptococcus \) \( lactis \) cells) on the period of respiratory inactivity. No substrates added. Fifty-two mg (dry weight) of cells per flask. 1 = 1.6 ml activator; 2 = 0.8 ml activator; 3 = 0.4 ml activator; and 4 = 0.2 ml activator added.

**TABLE 1.** The activation of \( Streptococcus \) \( lactis \) strain 8 by supernatant buffer from active \( Streptococcus \) \( lactis \) strain 9 cells

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Endogenous</th>
<th>Glucose†</th>
<th>Lactate†</th>
<th>Succinate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11</td>
<td>64</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>60</td>
<td>15</td>
<td>75</td>
<td>42</td>
<td>11</td>
</tr>
<tr>
<td>160</td>
<td>36</td>
<td>180</td>
<td>42</td>
<td>44</td>
</tr>
</tbody>
</table>

†Minus endogenous activity.
tation with other substrates for this organism. Because this type of activity inhibited the oxidation of other endogenous substrates, the amount of glucose used in the culture medium by Baribo and Foster (1952) was reduced to 0.3 per cent and the pH of the medium adjusted to 7.5. Following these two changes, the pH of the medium after growth was approximately 4.5 compared to 3.9 when using a glucose concentration of 0.5 per cent and an initial pH of 6.8. Cells grown in this medium were found to be more easily activated.

Cell age. Cells from 7-, 9-, and 11-hr-old cultures were tested for oxygen uptake. Data of figure 3 indicate that 9 hr appeared to be the optimal time for cultivation as the increasing rate of respiration began after approximately 2 hr. The 7-hr culture had a higher endogenous activity; however, this might have been due to endogenous glucose. There appear to be two controlling factors determining the time period before the increasing rate of oxygen uptake begins. First is the age of the cells, the 7- and 9-hr-old cells being more active than the 11-hr-old cells. Second is the amount of endogenous glucose; the 7-hr-old cells, although they were younger, required more time before they became active than did the 9-hr-old cells because they contained more endogenous glucose.

Sodium fluoride stimulation. S. lactis 9 had been shown to consume oxygen due to glucose oxidation but at the same time this glycolysis seemed to inhibit the oxidation of some other endogenous material. Therefore, a series of experiments was carried out to determine the effect of a glycolytic inhibitor on respiration.

Sodium fluoride (NaF) did not inhibit but stimulated the endogenous respiration of the cell suspensions. The results are shown in figure 4. Evidently this organism has two respiratory mechanisms seemingly unable to function during glycolysis. Self-activated suspensions gave a greater total oxygen uptake than did the fluoride stimulated cells. Assuming that the initial O2 uptake of the controls with NaF is due to glycolysis of endogenous glucose and that glycolysis is inhibited by NaF, the total uptake of the controls should be equal to the sum of the uptake of the fluoride treated suspensions plus the uptake due to glycolysis. Examination of the curves indicates that this is what happened.

Sodium fluoride concentration from $8 \times 10^{-4}$ M to $2 \times 10^{-4}$ M were capable of stimulating respiration; higher and lower concentrations were not used. Another experiment showed immediate stimulation of respiration upon addition of NaF at various periods during the experiment. The longer the experiment had been running before the addition was made, the higher was the total oxygen uptake.

As the purpose of the investigation was to determine whether S. lactis was capable of lactate breakdown, the effect of NaF on lactate and acetate oxidation was determined. Table 2 illustrates that, although there was some activity on lactate alone, the oxidation of this substrate was greatly increased by the addition of NaF.

**Fig. 3.** The effect of cell age on Streptococcus lactis respiration. No substrates added. One hundred and fifteen mg (dry weight) of cells added per flask. Cells harvested from 7-, 9- and 11-hr-old cultures. 1 = 7-hr-old cells; 2 = 9-hr-old cells; and 3 = 11-hr-old cells.

**Fig. 4.** Stimulation of Streptococcus lactis respiration by an inhibitor of glycolysis. No substrates were added. Sixty mg (dry weight) of cells per ml. Results are shown only for the two extreme concentrations of NaF used. Flasks 1, 2 and 3 contained 1 ml of cells. 1 and 4 = controls with no NaF; 2 = 0.2 ml NaF; 3 and 5 = 0.8 ml NaF; and 6 = 0.4 ml NaF added.
Acetate metabolism in the presence or absence of NaF was only slightly greater than the control.

After 120 min, the oxygen uptake due to the addition of NaF alone was 15 microliters and the uptake due to the addition of lactate alone was 40 microliters. The sum of these two values plus the oxygen consumption of the control equals 84 microliters. The uptake with lactate and NaF combined was 153 microliters, therefore the rate of lactate oxidation was almost doubled in the presence of NaF. This assumes that the oxygen consumption rate due to fluoride stimulation of the endogenous respiration remained the same in the flask containing lactate and fluoride.

In an attempt to obtain some information concerning the nature of the activating material found in the supernatant buffer from actively respiring cells, a number of substances were added to freshly harvested cell suspensions. Compounds added were: cytochrome c, coenzyme I, catalase, yeast extract as a source of B vitamins, riboflavin phosphate, glutathione, adenine, guanine, uracil, xanthine, casein hydrolysate as a source of amino acids, methylene blue, inorganic salts, 2,4-dinitrophenol, ascorbic acid, and NaF. Ascorbic acid and NaF were the only compounds which when added to cell suspensions were capable of increasing respiration. The results of the NaF experiments have been discussed.

If ascorbic acid was involved in the respiration of S. lactis, the initial period of inactivity could be due to the synthesis of "sparking" amounts of ascorbic acid. In this experiment several concentrations of ascorbic acid were used. If ascorbic acid were reversibly oxidized and reduced, the endogenous concentration of the substrate that supplied the hydrogen for the reduction of the dihydroascorbic acid would be the limiting factor in the amount of oxygen consumed. Therefore, the initial rate of oxygen uptake would be independent of ascorbic acid concentration. If the ascorbic acid were oxidized but did not undergo a reversible oxidation-reduction, the quantity of ascorbic acid would be the limiting factor in determining the amount of oxygen uptake.

Figure 5 shows the amount of oxygen uptake to be dependent on the concentration of ascorbic acid. These curves were similar to those obtained with cell suspensions in the presence of glucose. Only in the lowest concentration of ascorbic acid did the cell suspensions become self-activated and, in this case, the period of time required was greater than that of the control without ascorbic acid added.

**DISCUSSION**

This investigation has shown that under certain conditions *S. lactis* is capable of active aerobic respiration. This was first observed when cell suspensions were shaken in a Warburg apparatus for an extended period of time. After several hours of lag, the cells began to oxidize some endogenous substrate.

A significant finding was that glucose had an inhibitory effect on initiation of active respiration. Cell suspensions in the presence of glucose showed some initial oxygen uptake due to glucose oxidation, but the period required before the endogenous respiration began to increase was longer than those of the cell suspensions to which no glucose was added.

Young cells which have a low endogenous glucose concentration were optimum for respiration.

During the inactive period, the cell suspensions seemed to synthesize some material or system required for the oxidation of an endogenous substrate. This assumption was supported when supernatant buffer from actively respiring cells was found to be capable of activating freshly prepared cell suspensions. This unknown factor appeared to reach a certain concentration before activation occurred as the length of inactivity was determined by the amount of buffer added. The

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**Table 2. Stimulation of lactate oxidation by sodium fluoride**

<table>
<thead>
<tr>
<th>Time</th>
<th>Microliters of Oxygen Uptake*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control NaF Lactate Lactate NaF Acetate Acetate NaF</td>
</tr>
<tr>
<td>min</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>28</td>
</tr>
<tr>
<td>225</td>
<td>46</td>
</tr>
</tbody>
</table>

* Autoactivation of the control began at approximately 140 min. None of the other flasks showed an increased uptake at this time. Two-tenths ml of mg/25-solutions of substrate were added. The NaF concentration was 4 × 10⁻⁴ M. Fifty mg (dry weight) of cells were added per flask.

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**Fig. 5. Ascorbic acid oxidation by a cell suspension of Streptococcus lactis.** Side arms tipped after 1 hr. One hundred and five mg (dry weight) of cells added per flask. 1 = 3.5 mg ascorbic acid; 2 = 1.8 mg ascorbic acid; 3 = 0.9 mg ascorbic acid added; 4 = control with no ascorbic acid.
ability of *S. lactis* to respire seems to be limited by its capacity to produce this activating material as demonstrated by the activation of two other strains of *S. lactis* by supernatant buffer from strain 9.

To confirm the importance of glucose inhibition on endogenous respiration, an inhibitor of glycolysis was added to the cell suspensions. In the presence of NaF, glycolysis was inhibited and the endogenous respiration occurred immediately. This would indicate that the inactive period in the cell suspensions to which fluoride had not been added was a period in which the cells were removing their endogenous glucose by oxidation. Once the glucose was removed, the cells were then able to oxidize some other endogenous substrate.

This does not explain, however, the activating capacity of supernatant buffer from actively respiring cells. It may be possible that two mechanisms are functioning: first, the endogenous glucose is being removed; and secondly, some material is being synthesized that is capable of stimulating respiration. This material could be an inhibitor of glycolysis. Further investigation is needed to elucidate this activation.

Lactic acid accounts for approximately 95 per cent of lactose degradation in *S. lactis* cultures, therefore, lactic acid may be the oxidizable endogenous substrate. This supposition was not proved, but fluoride was found to stimulate lactate oxidation. The possible importance of such an oxidation in dairy products remains to be determined.

When ascorbic acid was added to the cell suspensions, they behaved in much the same manner as they did when glucose was added. Evidently these cells have two respiratory systems, one involved in glucose and ascorbic acid oxidation, and the other in the oxidation of noncarbohydrate substrates such as lactate and succinate.

The results of these investigations pose the problem of how common this type of respiration may be to *S. lactis*. During this study, a number of strains of *S. lactis* were examined. One strain demonstrated this phenomenon but not as readily as *S. lactis* 9. In addition, if the cultures of *S. lactis* 9 were left for a period of time without 12-hr transfer, several weeks of rapid transfer were required before they again became active.

It was observed that other strains of *S. lactis* were able to respire if supernatant buffer from actively respiring *S. lactis* 9 cells were added. It therefore appeared that most strains of *S. lactis* are capable of actively oxidizing certain noncarbohydrate substrates. All strains of *S. lactis* tested are limited, however, by their inability to remove endogenous glucose or to synthesize some activating material.

### Summary

Resting cell suspensions of *Streptococcus lactis* strain 9 were found capable of oxidizing certain noncarbohydrate substrates. Glucose and ascorbic acid had an inhibitory effect on these reactions. The oxidative reactions were stimulated by sodium fluoride. This organism seems to have two mechanisms for hydrogen transport, one utilized in glucose and ascorbic acid oxidation and the other in the oxidation of lactate and an endogenous substrate which may be lactate or succinate.

### References


