OXIDATION OF GLYCEROL BY STREPTOCOCCUS FAECALIS

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ABSTRACT


The nature of the factors in yeast autolysate essential for the oxidation of glycerol by Streptococcus faecalis F24 was examined. Two factors appear to be involved in the oxidation of glycerol. One factor was shown to be an inducer of the enzyme system required for glycerol oxidation; the other was shown to be α-lipoic acid. Minute quantities of glucose will "spark" growth of S. faecalis in a medium containing glycerol and acetate as carbon sources, probably by supplying sufficient energy for induction of the glycerol-oxidation system.

Certain lactic acid bacteria are known to utilize glycerol only under aerobic conditions (Gunsalus and Sherman, 1943). Gunsalus and Umbreit (1945) studied the mechanism of this oxidation in Streptococcus faecalis, strain F24, and showed that the over-all reaction was: glycerol + O2 → lactate + H2O. More recently, VanDemark (1950) examined this system in detail and concluded that several vitamins and an unidentified factor were essential for the oxidation of glycerol. Good sources of the unidentified factor were tryptic digest of casein, liver, and yeast autolysate.

We studied glycerol oxidation by S. faecalis as it relates to cofactor requirements. This paper describes the dual nature of the glycerol-oxidation factor and indicates that one of the factors is involved in the induction of an enzyme for glycerol oxidation, and that the other factor is lipoic acid.

MATERIALS AND METHODS

Growth of organism. S. faecalis strain F24 was used throughout this work. Stab cultures were maintained in a medium consisting of Tryptone, 1%; yeast extract, 1%; K2HPO4, 0.5%; glucose, 0.1%; and agar, 2%. An 8-hr broth culture (0.2 ml) in this same medium without agar, grown at 37 C, was used to inoculate 1-liter quantities of the chemically defined medium given in Table 1. After 16 hr of incubation at 37 C, the cells were harvested by centrifugation, washed twice with 0.033 M phosphate buffer (pH 6.5), and adjusted to an optical density equivalent to 1 mg of N per ml for Warburg studies.

Tube assays. In the tube-assay experiments, the medium employed was the synthetic medium in Table 1, modified as indicated in each experiment.

Determinations. Oxygen uptake was measured manometrically with conventional Warburg equipment. A 90-min period of oxidation, to allow for subsidence of endogenous activity, was employed in each experiment before glycerol was added from the side arm.

Lipoic acid assays were carried out by a modification of the method of O’Kane and Gunsalus (1948), employing S. faecalis F24.

Conventional ascending methods of paper chromatography were used with the butanol-acetic acid-water system (2:1:4:0.6). Optical densities were determined with a Lumetron photometer fitted with a 660-mu filter.

Materials. The supplement used in the Warburg experiments consisted of adenosine, 100 mg; riboflavine, 160 mg; nicotinic acid, 100 mg; thiamine HCl, 90 mg; glucose, 36 mg; glutamic acid, 2 g; magnesium sulfate heptahydrate, 1 g; and distilled water to 100 ml; pH 7.0. [As described by E. Oginsky, personal communication. This is probably based on the factors found to stimulate pyruvate oxidation by S. faecalis (Gunsalus, Dolin, and Struglia, 1952).]

Chloramphenicol was obtained from Parke, Davis & Co. (Detroit, Mich.).

RESULTS

Cells of S. faecalis grown in the chemically defined medium will not oxidize glycerol unless a
source of the glycerol-oxidation factor (GOF) is added (Table 2). When yeast autolysate was used as the source of GOF, a 90-min period was allowed for endogenous oxidation to subside before glycerol was added from the side arm. However, if the yeast autolysate was added at the same time as the glycerol, a lag period of approximately 30 min always preceded net O₂ uptake. This suggested that glycerol oxidation may be an adaptive process.

If S. faecalis is grown in the chemically defined medium supplemented with 1% glycerol, the cells will oxidize glycerol rapidly with no period of lag. This oxidation is further stimulated by the addition of yeast autolysate, suggesting a factor in addition to that required for the adaptation process (Fig. 1).

Previous work by Oginsky and Umbreit (pers.

**Table 1.** Chemically defined medium for growth of Streptococcus faecalis

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amt</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄</td>
<td>5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1 g</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>100 mg</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>2 g</td>
</tr>
<tr>
<td>Salts B³</td>
<td>10 ml</td>
</tr>
<tr>
<td>AGU mixture a</td>
<td>4 ml</td>
</tr>
<tr>
<td>Vitamin mixture a</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>Folic acid</td>
<td>100 µg</td>
</tr>
<tr>
<td>Biotin</td>
<td>2 µg</td>
</tr>
<tr>
<td>Amino acid mixture a</td>
<td>300 ml</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>200 mg</td>
</tr>
<tr>
<td>Cystine</td>
<td>800 mg</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>400 mg</td>
</tr>
</tbody>
</table>

³ Total volume, 1,000 ml; pH adjusted to 7.2.
³ Salts B: MgSO₄·7H₂O, 40 g; NaCl, 2 g; FeSO₄·7H₂O, 2 g; MnSO₄·4H₂O, 2 g; and 12 N HCl, 1 ml; in a total volume of 1,000 ml.
³ AGU mixture: adenine sulfate, 870 mg; guanine hydrochloride, 620 mg; uracil, 500 mg; and 12 N HCl, 10 ml; to a total volume of 500 ml.
³ Vitamin mixture (mg): nicotinic acid, 150; riboflavin, 30; calcium pantothenate, 30; pyridoxal hydrochloride, 60; and thiamine hydrochloride, 60.
³ Amino acid mixture: 2 g each of DL-leucine, DL-isoleucine, DL-valine, DL-methionine, DL-phenylalanine, DL-glutamic acid, DL-aspartic acid, DL-norleucine, DL-threonine, L-alanine, DL-serine, and glycine. The mixture also contained DL-tryptophan, 4 g; L-lysine hydrochloride, 1.4 g; L-arginine hydrochloride, 2.4 g; and L-histidine hydrochloride, 2.7 g. Total volume, 1,400 ml.

**Table 2.** Dependence of glycerol oxidation upon yeast autolysate a

<table>
<thead>
<tr>
<th>Yeast autolysate</th>
<th>Q₀ (N)</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>mg</td>
<td>0</td>
<td>0.32</td>
</tr>
<tr>
<td>2</td>
<td>288</td>
<td>0.34</td>
</tr>
<tr>
<td>4</td>
<td>464</td>
<td>0.32</td>
</tr>
<tr>
<td>6</td>
<td>648</td>
<td>0.32</td>
</tr>
<tr>
<td>8</td>
<td>800</td>
<td>0.32</td>
</tr>
<tr>
<td>10</td>
<td>896</td>
<td>0.32</td>
</tr>
</tbody>
</table>

a Each cup contained 0.2 ml of 20% KOH in center well; 0.5 ml of 0.5 M phosphate buffer (pH 7.0); 40 µg glycerol; 0.5 ml of cells at 1 mg of N/ml; and yeast autolysate as indicated. Total volume, 3.0 ml; temperature, 37 C. For optical density readings at 660 m, the cup contents (except KOH) were diluted to 40 ml.

sonal communication) showed that the unknown factor found in yeast consisted of at least two components, which they designated GOF "A" and GOF "B."

In view of our preliminary findings, it appeared highly probable that one of these factors might act as an inducer of the glycerol-oxidation system, and that the other might be a cofactor which further stimulates glycerol oxidation.

Evidence for an adaptive enzyme for glycerol oxidation: growth studies. In growth studies with S. faecalis, we noted early that the organism grew well in the chemically defined medium but not in such a medium in which glycerol was substituted for glucose. Growth in the glycerol medium occurred only if the medium was supplemented with either glucose or yeast autolysate. As little as 5 µg per ml of yeast autolysate or 1 to 2 µg per ml of glucose were sufficient to elicit half-maximal growth (Fig. 2). This ability to utilize glycerol only in the presence of minute amounts of sugar or yeast autolysate is reminiscent of the "sparking" of ethanol oxidation by Acetobacter suboxydans by small amounts of reducing sugar, reported by Raghavendra Rao and Stokes (1953).

Inhibition of glycerol oxidation with chloramphenicol. Chloramphenicol has been shown by several investigators to inhibit protein synthesis and, in particular, adaptive enzyme formation (Hahn and Wiseman, 1951; Koppel, Porter, and Crocker, 1953; Wiseman, Smadel, and Hopps, 1954). For this reason, we examined its
Effect on the glycerol-oxidation system. In the presence of adequate GOF (4 mg per flask of yeast autolysate), glycerol oxidation by S. faecalis grown in the chemically defined medium was inhibited 83% by as little as 17 µg per ml of chloramphenicol. In contrast, glucose oxidation by these cells, which is not stimulated by yeast autolysate, was not affected by as much as 200 µg per ml of chloramphenicol (Fig. 3).

When S. faecalis cells were grown in medium supplemented with 1% glycerol, they vigorously oxidized glycerol; such oxidation was affected only slightly by chloramphenicol (Fig. 3).

Koppel et al. (1953) demonstrated that β-galactosidase formation in Escherichia coli is inhibited by chloramphenicol without, at the same time, its interfering with the activity of the preformed enzyme. Experiments were conducted to see if glycerol oxidation is similarly affected. At various times during the 90-min adaptation period, 33 µg per ml of chloramphenicol were

**FIG. 1.** Stimulation of glycerol-grown Streptococcus faecalis by yeast autolysate or lipoic acid. Each cup contained 0.2 ml of 20% KOH in center well; 0.5 ml of 0.5 M phosphate buffer (pH 7.0); 40 µM glycerol; 0.5 ml of supplement; 0.5 ml of cells at 1 mg of N/ml; yeast autolysate and lipoic acid as indicated. Total volume, 5.0 ml; temperature, 37°C.

**FIG. 2.** "Sparkling" of growth by glucose in presence of glycerol. Optical density recorded after 16 hr of incubation at 37°C.

**FIG. 3.** Effect of chloramphenicol upon glycerol and glucose oxidation. Each cup contained 0.2 ml of 20% KOH in center well; 0.5 ml of 0.5 M phosphate buffer (pH 7.0); 40 µM glycerol; 0.5 ml of supplement; 0.5 ml of cells at 1 mg of N/ml; 4 mg of yeast autolysate; chloramphenolic and substrate as indicated. Total volume, 5.0 ml; temperature, 37°C. A = glycerol-grown, glucose substrate; B = glycerol-grown, glycerol substrate; C = glucose-grown, glucose substrate; D = glucose-grown, glycerol substrate.
FIG. 4. Effect of chloramphenicol upon preformed enzyme. Each cup contained 0.2 ml of 20% KOH in center well; 0.5 ml of 0.5 M phosphate buffer (pH 7.0); 0.5 ml of supplement; 4 mg of yeast autolysate; 40 µM glycerol; 0.5 ml of cells at 1 mg of N/ml; and 35 µg/ml of chloramphenicol, added at times indicated. Total volume, 5.0 ml; temperature, 37 C. Adaptation was for 90 min prior to addition of glycerol at 0 min. Curves indicate min of adaptation before addition of chloramphenicol. O = Endogenous (no yeast autolysate); ♦ = no adaptation; △ = 10-min adaptation; ▲ = 20-min adaptation; □ = 30-min adaptation; ■ = 45-, 60-, 90-min adaptation; X = 90-min adaptation, no chloramphenicol.

FIG. 5. Effect of yeast autolysate on glycerol-adapted cells. Each cup contained 0.2 ml of 20% KOH in center well; 0.5 ml of 0.5 M phosphate buffer (pH 7.0); 0.5 ml of “adapted” cells or normal cells at 1 mg of N/ml; 40 µM glycerol; 4 mg of yeast autolysate where indicated. Total volume, 300 ml; temperature, 37 C. △ = Normal cells + 4 mg of yeast autolysate; O = “adapted” cells; ♦ = “adapted” cells + 4 mg of yeast autolysate.

autolysate is acting as an inducer, then preincubation of cells with yeast should eliminate the lag encountered in the oxidation of glycerol by fresh cells. To test this point, S. faecalis cells were shaken at 37 C in the presence of buffer, supplement, and yeast autolysate.

After 90 min, the cells were removed by centrifugation, washed thoroughly, and tested in a Warburg flask for their ability to oxidize glycerol. Preincubated cells oxidized glycerol immediately, whereas untreated cells, even when supplied with yeast autolysate, were unable to oxidize glycerol (Fig. 5). In addition, it can be seen that glycerol oxidation by “adapted” cells is further stimulated by yeast autolysate. This observation is consistent with the hypothesis that yeast contains two factors.

It may be argued that the adaptation to glycerol by S. faecalis during the 90-min period of endogenous activity prior to the addition of glycerol may be due to a preselection of a glycerol-oxidizing mutant rather than to synthesis of a
new enzyme by resting cells. Such is not the case, since optical-density readings of Warburg-cup contents taken at the beginning of the experiment and after the 90-min adaptation period and the 15-min period of glycerol oxidation indicated no significant change (Table 2).

Lipoic acid cofactor. As indicated above, S. faecalis cells grown in glycerol-supplemented medium were capable of oxidizing glycerol rapidly. Such oxidation was stimulated by the addition of yeast autolysate. The stimulation by yeast autolysate was completely replaced by small quantities of α-(+)-lipoic acid (Fig. 1). A concentration of 30 μg of lipoic acid was sufficient to elicit a response equivalent to 4 mg of yeast autolysate.

Paper chromatograms containing yeast autolysate and α-lipoic acid were developed and tested, by shredding segments of the paper and placing the shreddings directly into Warburg flasks. Duplicate strips were made; one was tested for GOF activity and the other for pyruvate oxidation stimulation (lipoic acid). By this procedure, yeast autolysate was found to contain two active materials. One material (Rf 0.95) proved active in both the GOF and lipoic acid assays, and was identical to α-lipoic acid. A second material (Rf 0.3) active in the GOF assay was also detected. This component was not tested in the pyruvate oxidase assay, but may well be one of the several lipoic acid derivatives reported to be present in yeast extracts (Reed et al., 1951).

DISCUSSION

Early studies by Gunsalus and Sherman (1943) showed that the oxidation of glycerol by S. faecalis strain F24 is a highly aerobic process. Later, Gunsalus and Wood (1942) demonstrated that serial transfer in glycerol-containing medium increased the ability of the organism to oxidize glycerol. Although at first this observation was considered to be indicative of adaptation, Gunsalus and Umbreit (1945) in later studies reported no evidence for adaptation to glycerol. Data presented in this paper indicate that glycerol is oxidized by S. faecalis strain F24, at least in part, by an adaptive process. This conclusion is based on the following observations. Growing cells utilize glycerol as a carbon and energy source only when the medium is supplemented with small amounts of yeast autolysate or μg-quantities of glucose. A 30-min lag is noted in the presence of yeast in the glycerol-oxidation curve with cells grown on glucose. No oxidation occurs in the absence of yeast. Cells grown on glycerol oxidize glycerol immediately. Chloramphenicol, an inhibitor of protein synthesis, inhibits glycerol oxidation by glucose-grown cells, but does not inhibit glucose oxidation. Glycerol-grown cells are not affected by chloramphenicol.

Recent studies by Oginsky and Umbreit (personal communication) demonstrated the dual nature of yeast in the stimulation of glycerol oxidation. In our hands, one of these factors is involved in the formation of an adaptive enzyme capable of oxidizing glycerol. The second factor is lipoic acid. The latter is amply demonstrated by the ability of μg-quantities of α-lipoic acid to enhance glycerol oxidation to a level equal to that obtained with yeast; paper chromatograms indicate that the GOF activity of yeast autolysate is identical with lipoic acid.

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