COMPARISON OF THE MECHANISM OF GLYCEROL OXIDATION IN AEROBICALLY AND ANAEROBICALLY GROWN *STREPTOCOCCUS FAECALIS*[1]

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Oxygen is a requirement for glycerol dissimilation by the majority of the lactic acid bacteria (Gunsalus and Sherman, 1943). However, quite unique are certain strains of enterococci which are capable of growth either aerobically or anaerobically on glycerol. Glycerol utilization by these latter strains, although highly significant from a taxonomic standpoint, has not been thoroughly elucidated. Gunsalus and Umbreit (1945) studied the mechanism of glycerol oxidation by *Streptococcus faecalis* (*faecium*) strain F24, a strain which requires aerobic conditions for growth on glycerol, and found that glycerol is initially phosphorylated to α-glycerophosphate. This phosphate is then oxidized to yield hydrogen peroxide (H_2O_2) and lactic acid. Working with a strain of *S. faecalis* (strain 10C1) capable of anaerobic growth on glycerol, Gunsalus (1947) observed that the presence of an external hydrogen acceptor, e. g., fumaric acid, is required in the medium for the growth of this organism on glycerol in the absence of oxygen.

Studies in our laboratory of respiratory pathways in *S. faecalis* (strain 10C1) have indicated marked differences in the enzyme patterns of anaerobically and aerobically grown cells. Among these differences is an increase in the level of flavin enzymes, including a nonhemin peroxidase (Seeley and VanDemark, 1951; Seeley and Del Rio Estrada, 1951). The studies of Seeley and Del Rio Estrada (*unpublished data*) also gave evidence of differences in glycerol metabolism of cells grown anaerobically and aerobically on glucose. They observed that anaerobically grown cells of this organism reduce methylene blue with glycerol as the hydrogen donor at a markedly faster rate than cells grown in the presence of oxygen.

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The present study is an attempt to explain these differences by determining glycerol degradation in the two types of cells, as well as to elucidate the mechanism by which fumaric acid serves as an external hydrogen acceptor during the anaerobic growth of this organism on glycerol.

MATERIALS AND METHODS

Culture and growth conditions. S. faecalis strain 10C1, from the departmental collection was used in this study. AC medium (Wood and Gunsalus, 1942) consisting of 0.1 per cent glucose, 1.0 per cent tryptone, 1.0 per cent yeast extract, and 0.5 per cent K_2HPO_4, was inoculated with an 8-hr culture using a volume of inoculum equal to 0.5 per cent of the total volume. Aerobically grown cells were obtained by growing with constant shaking in Erlenmeyer flasks containing 0.1 volume of growth medium. Anaerobically grown cells were grown under stationary conditions in Erlenmeyer flasks containing 0.75 volume of growth medium. After 12 hr growth, cells were harvested by centrifugation and washed with one half the original growth volume of distilled water.

Preparation of cell-free extracts. Cell-free extracts were prepared by disrupting a 20 per cent suspension (wet weight) of cells in a 50 watt, 9 kc magnetostriction oscillator for 30 min and removing the debris and unbroken cells by centrifugation for 1 hr at 25,000 × G.

Determinations. Manometric determinations were made with a Warburg respirometer using conventional methods (Umbreit *et al.*, 1957). Spectrophotometric determinations were made at room temperature in a Beckman model DU spectrophotometer using a modification of the anaerobic cuvette and conditions described by Dolin (1955) due to the high dihydrodiphosphopyridine nucleotide (DPNH) oxidase activity of the cell-free extracts. The protein level of

crude extracts was determined by the trichloroacetic acid method of Stadtman *et al.* (1951). Hydrogen peroxide was measured by the method of Main and Shinn (1939). Glycerol was assayed by periodate oxidation as described by Neish (1952). Succinic acid was determined with pig heart succinoxidase as described by Umbreit *et al.* (1957).

Materials. All reagents and substrates used in this study were commercial preparations. Reduced flavin mononucleotide was prepared by the hydrosulfite method of Massey and Singer (1957) and determined spectrophotometrically at 470 mμ.

RESULTS

Activity of cell-free extracts on glycerol. Manometric studies revealed that cell-free extracts of both aerobically and anaerobically grown *S. faecalis* fail to take up oxygen in the presence of glycerol. However, as shown in figure 1, on the addition of adenosine triphosphate (ATP) glycerol is readily oxidized by the extract of aerobically grown cells. The presence of ATP has no effect on glycerol oxidation in extracts of anaerobically grown cells. An analysis of the end products of the reaction illustrated in figure 1 revealed the accumulation of 4.0 and 7.0 μmoles of H_2O_2 during the consumption of 5.0 and 9.5

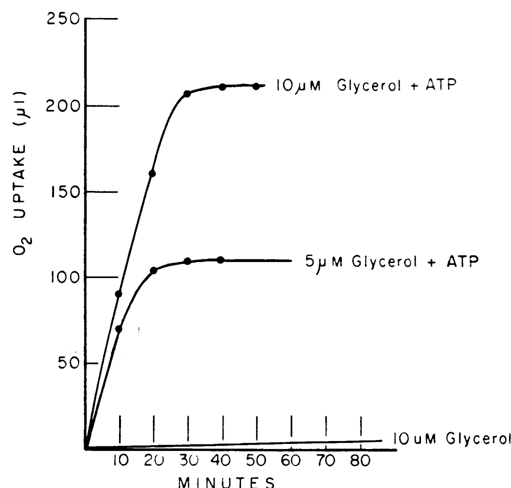

Figure 1. Oxidation of glycerol by extracts of aerobically grown cells. The reaction mixture contained 75 μmoles glycyl-glycine buffer, pH 7.4, and where indicated 20 μmoles adenosine triphosphate (ATP), and 25 mg enzyme protein, with water to 3.0 ml volume.

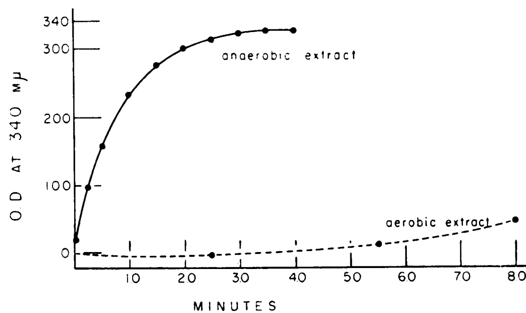

Figure 2. Dihydrodiphosphopyridine nucleotide reduction with glycerol. Each cuvette contained 150 μmoles phosphate buffer, pH 7.5; 0.28 μmoles diphosphopyridine nucleotide; 800 μmoles glycerol; and as indicated aerobic or anaerobic extract (2.5 mg protein), with water to 3.0 ml volume.

μmoles of O_2, respectively, with less than 1 μmole glycerol remaining per cup at the end of the reaction. Thus for each mole of glycerol utilized, one mole of oxygen is consumed and H_2O_2 approximating one mole is formed. This oxidation proceeds more rapidly in glycylglycine or Tris buffer than in phosphate buffer and is not stimulated by the addition of diphosphopyridine nucleotide (DPN). Therefore it would appear that aerobically grown *S. faecalis* possesses a glycerol kinase and the ability to oxidize the phosphorylated product.

Although the extracts of anaerobically grown cells appear to lack the above oxidative pathway, their ability to utilize glycerol is apparent as they reduce methylene blue with this substrate. Furthermore, as shown in figure 2, these extracts actively reduce DPN with glycerol as the substrate, whereas extracts from cells grown aerobically are weakly active at best. ATP has no effect on DPN reduction in either extract, nor will triphosphopyridine nucleotide replace DPN in this reaction. These data indicate the presence of a DPN-specific glycerol dehydrogenase in the anaerobically grown cells similar to that described in *Aerobacter aerogenes* (Burton and Kaplan, 1953; Magasanik *et al.*, 1953) and in *Lactobacillus brevis* (Eltz, 1957).

Oxidation of α-glycerophosphate. As is shown in figure 3, α-glycerophosphate is metabolized only by the extract of aerobically grown cells. β-glycerophosphate is not utilized by this extract and does not inhibit the oxidation of α-glycerophosphate. Since the α-glycerophosphate used in

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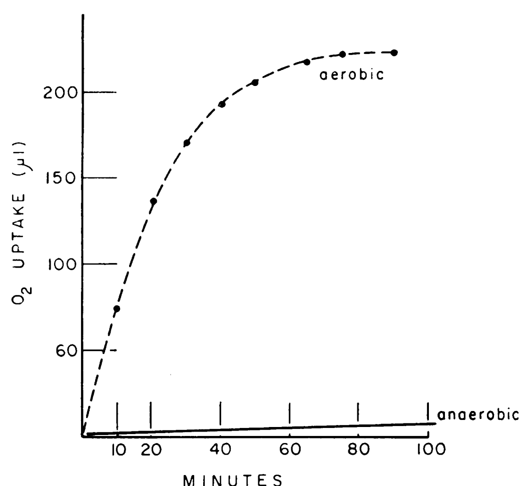

Figure 3. Oxidation of DL-α-glycerophosphate. Each flask contained 150 μmoles Tris buffer, pH 7.5; 20 μmoles α-glycerophosphate; and as indicated aerobic or anaerobic extract (2.5 mg protein) with water to 3.0 ml volume.

this study was a mixture of both the D- and L-forms, the uptake of only one-half mole of O_2 per mole of α-glycerophosphate indicates that only one of the isomers is being used. As previously shown (Magasanik *et al.*, 1953), the isomer utilized by *A. aerogenes* is the L-form.

The enzyme catalyzing the oxidation of α-glycerophosphate has been purified and its properties are being studied. The purified enzyme was found to be stimulated by flavin adenine dinucleotide, but not by flavin mononucleotide or riboflavin and therefore appears to be a flavin adenine dinucleotide-linked α-glycerophosphate oxidase. The enzyme has an optimum pH of 5.8. In table 1 the stoichiometric relationship between oxygen utilization and the appearance of end products is indicated. The alkali-labile phosphate produced by this purified preparation was identified as dihydroxyacetone phosphate after removal of the phosphate group with acid phosphatase and determination of the dihydroxyacetone by means of a modification of the Seliwanoff test for ketoses (Asnis and Brodie, 1953). The purification scheme and some properties of this enzyme will be more fully reported at a later date.

The ability of compounds other than oxygen to serve as hydrogen acceptors for α-glycerophosphate oxidation was studied. While ferricyanide and 2,6-dichlorophenolindophenol are reduced at an appreciable rate, methylene blue, and cytochrome *c* are reduced only slowly. This slow rate of methylene blue reduction differentiates this enzyme from a similar non-DPN linked α-glycerophosphate oxidizing enzyme system isolated by Green (1936) from rabbit muscle, which was described as cytochrome-linked.

The experiments illustrated in figures 1 and 3 demonstrate the presence of a glycerol kinase and an α-glycerophosphate oxidase in the extracts of aerobically grown cells, and the absence of an α-glycerophosphate oxidase in the anaerobically grown cells.

In order to determine if these latter cells possess glycerol kinase, a preparation of α-glycerophosphate oxidase purified from the extract of cells grown aerobically and possessing no glycerol kinase activity, was added to the crude extract of anaerobically grown cells. In the presence of glycerol, ATP, and the purified α-glycerophosphate oxidase, oxygen uptake should take place if an α-glycerophosphate generating system (glycerol kinase) were present. Since oxygen uptake did not occur under these conditions, it was concluded that glycerol kinase is not present in the anaerobically grown cells.

Mechanism of fumarate reduction by anaerobically grown cells. Gunsalus (1947) found *S. faecalis* capable of growing anaerobically on glycerol when fumarate was added to the medium. Since the anaerobic oxidation of glycerol involves the reduction of DPN, demonstration of the oxidation of DPNH with fumarate was attempted. Figure 4 shows that the oxidation of DPNH with fumarate occurs in extracts from anaerobically, but not aerobically, grown cells.

TABLE 1

Products of α-glycerophosphate oxidation

Time	O_2 Utilized	Alkali-Labile Phosphate Formed	H_2O_2 Formed
min	*μmoles*	*μmoles*	*μmoles*
15	1.74	1.95	2.04
30	3.92	3.90	4.30
45	5.13	5.04	5.50
60	6.70	6.54	6.90

Each Warburg flask contained 9 μmoles α-glycerophosphate; 2 mmoles acetate buffer, pH 5.8; 0.072 mg purified α-glycerophosphate oxidase; and water to 3.0 ml.

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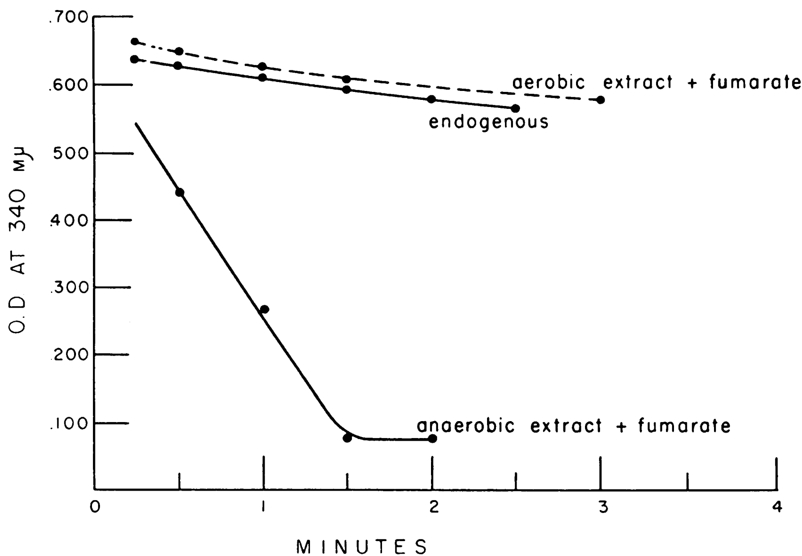

Figure 4. Oxidation of dihydrodiphosphopyridine nucleotide (DPNH) with fumarate. Each cuvette contained 150 μmoles phosphate buffer, pH 7.5; 0.28 μmole DPNH and as indicated aerobic or anaerobic extract (2.5 mg protein); and 20 μmoles sodium fumarate with water to a 3.0 ml volume. The endogenous activity of both the aerobic and anaerobic extracts was essentially the same.

The end product of this reaction, presumably succinate, was determined by assaying with pig-heart succinoxidase as described by Umbreit *et al.* (1957). In a typical experiment, on incubation of 7 μmoles DPNH and 40 μmoles sodium fumarate with the extract at 24 C for 35 min, 4.4 μmoles of sodium succinate accumulated.

An attempt was made to determine the mechanism of this fumarate reduction by DPNH. The question arose as to whether the reaction is catalyzed by one or several enzymes and whether some free carrier, such as free flavins, mediates hydrogen transport during the oxidation of DPNH and the subsequent fumarate reduction. In figures 5 and 6 the presence of at least 2 enzymes, which could account for the mechanism of fumarate reduction, is illustrated. Figure 5 shows the reduction of flavin mononucleotide by DPNH which takes place in the presence of the extracts of both the anaerobically and aerobically grown cells. No reduction of flavin mononucleotide was observed until DPNH was added to the reaction mixture containing unheated extract. Figure 6 shows the oxidation of reduced flavin mononucleotide by fumarate which takes place in the presence of the extract from anaerobically grown cells and very slightly with the extract from cells grown aerobically.

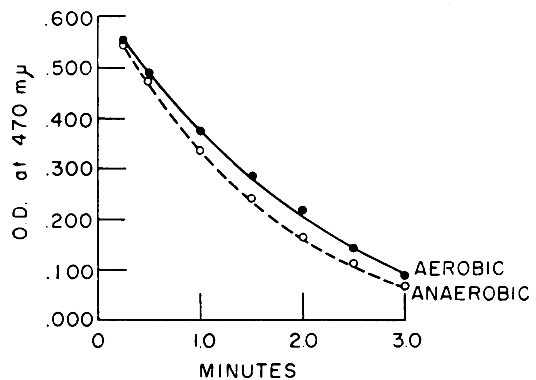

Figure 5. Reduction of flavin mononucleotide with dihydrodiphosphopyridine nucleotide (DPNH). Cuvettes contained 200 μmoles phosphate buffer, pH 7.4; 0.72 μmole DPNH; 0.2 μmole flavin mononucleotide; and as indicated either anaerobic (1.4 mg protein) or aerobic (1.8 mg protein) extract, with water to 3.0 ml volume.

Riboflavin or flavin adenine dinucleotide can replace flavin mononucleotide in both of the reactions illustrated in figures 5 and 6. The oxidation of reduced flavin mononucleotide by fumarate has been demonstrated in purified mammalian succinic dehydrogenase by Massey and Singer (1957). It appears then, that the

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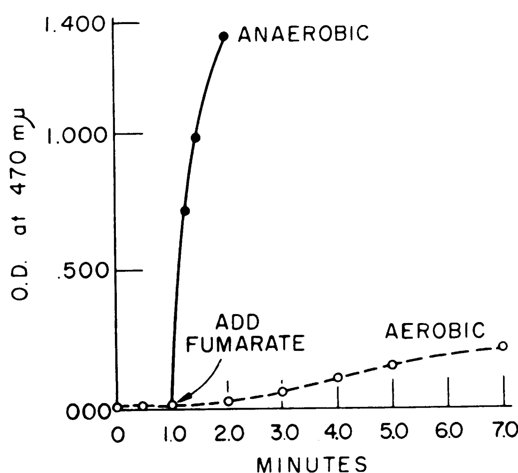

Figure 6. Oxidation of reduced flavin mononucleotide with fumarate. Cuvettes contained 120 μmoles phosphate buffer, pH 7.4; 0.6 μmole flavin mononucleotide; 20 μmoles sodium fumarate; and as indicated either aerobic (2.0 mg protein) or anaerobic (1.38 mg protein) extract with water to a 3.0 ml volume.

mechanism of fumarate reduction by DPNH may be dependent on the enzymatic reduction of free or bound flavins by DPNH and the enzymatic reoxidation of these flavins by fumarate. This is in agreement with the results of Gunsalus (1947) which showed that abundant growth on glycerol was dependent upon the presence of riboflavin and fumarate. No further evidence in support of the role of free flavins as mediators in this reaction has as yet been obtained.

Attempts to demonstrate succinic dehydrogenase activity by the methods of Singer and Kearney (1957) in the extract of cells grown anaerobically were unsuccessful even in the presence of DPN, phenazine methosulfate or ferricyanide.

DISCUSSION

The present study indicates another significant alteration in the metabolic pattern of *S. faecalis* induced by aeration, i. e., the adaptive formation of glycerol kinase and α-glycerophosphate oxidase during aerobic growth on glucose. This enzyme pathway would appear to be similar or the same as is constitutive in those strains of lactic acid bacteria which utilize glycerol only aerobically (Gunsalus and Umbreit, 1945).

The reason for the induction of α-glycerophosphate oxidase and its function in cells grown aerobically on glucose is not clear. The possibility exists that under aerobic conditions the glycolytic pathway from triose phosphate to lactic acid is impaired resulting in the accumulation of α-glycerophosphate which induces the formation of α-glycerophosphate oxidase. Chefurka (1958) has presented evidence that in glycolyzing insect muscle lactic acid dehydrogenase activity is negligible and a DPN-linked α-glycerophosphate dehydrogenase functions in regenerating DPN from DPNH thus enabling the glycolytic pathway to function. However in the present study no evidence was found for a DPN-linked α-glycerophosphate dehydrogenase in aerobic cell extracts. The possibility also exists that the α-glycerophosphate oxidase represents a respiratory site in aerobically grown cells of this bacterium. Since this organism shows greater growth aerobically, if α-glycerophosphate oxidase is a respiratory site under aerobic conditions it might indicate that oxidative phosphorylation is occurring at this point. Dolin (1955) was unable to demonstrate oxidative phosphorylation in this organism. A similar α-glycerophosphate oxidase has been described in flight muscle (Estabrook and Sacktor, 1958), and its role in the respiratory control of flight muscle hypothesized.

Anaerobically grown cells of this organism are deficient in glycerol kinase and α-glycerophosphate oxidase, but they oxidize glycerol by means of a DPN-linked dehydrogenase, which is coupled with flavin-linked DPNH oxidizing enzyme systems, one of which is the system resulting in the reduction of fumarate. The reduction of fumarate may occur stepwise via the reduction of free flavin with DPNH and the subsequent oxidation of the resulting reduced flavin by a fumaric reductase to form succinic acid. In connection with these results, it is of interest to note that free flavins have been shown to mediate hydrogen transport between two flavoprotein oxidases from *Lactobacillus delbrueckii* (i. e., lactic and pyruvic oxidases) (Hager *et al.*, 1954). This system differs from fumarate reduction in heart muscle with DPNH (Slater, 1950) in that neither cytochromes or other hemin-containing enzymes enter in the pathway of fumarate reduction in *S. faecalis*. This flavin-linked fumaric reductase may be similar to a fumarate reducing enzyme demonstrated by Peck *et al.* (1957) in *Micrococcus lactilyticus*. However, the latter organism is known to possess demonstrable succinic dehydrogenase activity.

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The elucidation of the enzyme pathway involved in glycerol oxidation and fumarate reduction by this strain would seem to support the division of the present species, *S. faecalis* (Breed *et al.*, 1957) into two individual species, i. e., *S. faecalis* capable of anaerobic growth on glycerol and *Streptococcus faecium*, which is lacking in respiratory enzymes necessary for utilization of glycerol anaerobically. *S. faecalis* having a DPN-linked glycerol dehydrogenase coupled via a flavin-linked reductase to fumarate enables the species to utilize glycerol anaerobically. The species *S. faecium*, while it may plausibly be a mutant of *faecalis*, lacks this latter enzyme pattern and can use glycerol only aerobically.

SUMMARY

Streptococcus faecalis strain 10C1 grown aerobically on glucose contains a phosphorylated system for the oxidation of glycerol, including a glycerol kinase and a flavin adenine dinucleotide-linked α-glycerophosphate oxidase. This enzyme does not reduce diphosphopyridine nucleotide. Anaerobically grown cells, deficient in this phosphorylated pathway, possess a diphosphopyridine nucleotide-linked glycerol dehydrogenase which is coupled with flavin-linked dihydrodiphosphopyrindine oxidizing enzyme systems. The glycerol dehydrogenase appears to be lacking in aerobically grown cells. Extracts of anaerobically grown cells were shown to reduce fumaric acid with either dihydrodiphosphopyridine nucleotide or reduced free flavins. The reduction of free flavins by dihydrodiphosphopyridine nucleotide was observed in extracts of cells grown both aerobically and anaerobically. Some taxonomic and metabolic implications of these findings are discussed.

REFERENCES

Asnis, R. E. and Brodie, A. F. 1953 A glycerol dehydrogenase from *Escherichia coli*. J. Biol. Chem., **203**, 153–159.

Breed, R. S., Murray, E. G. D., and Smith, N. R. 1957 *Bergey's manual of determinative bacteriology*, 7th ed. The Williams & Wilkins Co., Baltimore.

Burton, R. M. and Kaplan, N. O. 1953 A DPN specific glycerol dehydrogenase from *Aerobacter aerogenes*. J. Am. Chem. Soc., **75**, 1005–1006.

Chefurka, W. 1958 On the importance of α-glycerophosphate dehydrogenase in the glycolizing insect muscle. Biochim. et Biophys. Acta, **28**, 660–661.

Eltz, R. W. 1957 A study of the enzymes of fructose dissimilation of *Lactobacillus brevis*. Ph.D. Thesis, Cornell University.

Dolin, M. I. 1955 The DPNH-oxidizing enzymes of *Streptococcus faecalis*. II. The enzymes utilizing oxygen, cytochrome c, peroxide and 2,6-dichlorophenolindophenol or ferricyanide as oxidants. Arch. Biochem. Biophys., **55**, 415–435.

Estabrook, R. W. and Sacktor, B. 1958 α-Glycerophosphate oxidase of flight muscle mitochondria. J. Biol. Chem., **233**, 1014–1019.

Green, D. E. 1936 α-Glycerophosphate dehydrogenase. Biochem. J., **30**, 629–644.

Gunsalus, I. C. 1947 Products of anaerobic glycerol fermentation by *Streptococcus faecalis*. J. Bacteriol., **54**, 239–244.

Gunsalus, I. C. and Sherman, J. M. 1943 The fermentation of glycerol by streptococci. J. Bacteriol., **45**, 155–162.

Gunsalus, I. C. and Umbreit, W. W. 1945 The oxidation of glycerol by *Streptococcus faecalis*. J. Bacteriol., **49**, 347–357.

Hagar, L. P., Geller, D. M., and Lipmann, F. 1954 Flavoprotein catalyzed pyruvate oxidation in *Lactobacillus delbrueckii*. Federation Proc., **13**, 734–738.

Magasanik, B., Brooke, M. S., and Karibian, D. 1953 Metabolic pathways of glycerol dissimilation. A comparative study of two strains of *Aerobacter aerogenes*. J. Bacteriol., **66**, 611–619.

Main, E. R. and Shinn, L. E. 1939 The determination of hydrogen peroxide in bacterial cultures. J. Biol. Chem., **128**, 417–423.

Massey, V. and Singer, T. P. 1957 Studies on succinic dehydrogenase. III. The fumaric reductase activity of succinic dehydrogenase. J. Biol. Chem., **228**, 263–274.

Neish, A. C. 1952 *Analytical methods for bacterial fermentations*. Report No. 46-8-3 (Second Revision), p. 36. National Research Council of Canada, Saskatoon.

Peck, H. D., Smith, O. H., and Gest, H. 1957 Comparative biochemistry of the biological reduction of fumaric acid. Biochim. et Biophys. Acta, **25**, 142–147.

Seeley, H. W. and Del Rio Estrada, C. 1951 The role of riboflavin in the formation and disposal of hydrogen peroxide by *Streptococcus faecalis*. J. Bacteriol., **62**, 649–656.

Seeley, H. W. and VanDemark, P. J. 1951 An adaptive peroxidation by *Streptococcus faecalis*. J. Bacteriol., **61**, 27–35.

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Singer, T. P. and Kearney, E. B. 1957 Determination of succinic dehydrogenase activity. In *Methods of biochemical analysis*, vol. IV, pp. 307–333. Edited by D. Glick. Interscience Publishers, Inc., New York.

Slater, E. C. 1950 The components of the dihydrocozymase oxidase system. Biochem. J., **46**, 484–499.

Stadtman, E. R., Novelli, G. D., and Lipmann, F. 1951 Coenzyme A function in and acetyl transfer by the phosphotransacetylase system. J. Biol. Chem., **191**, 365–376.

Umbreit, W. W., Burris, R. N., and Stauffer, J. F. 1957 *Manometric techniques*. Burgess Publishing Co., Minneapolis.

Wood, A. J. and Gunsalus, I. C. 1942 The production of active resting cells of streptococci. J. Bacteriol., **44**, 333–341.

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