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Membrane Associated Glucose and Gluconate Oxidase Enzymes in Pseudomonas Fluorescens: Characterization and Regulation

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MEMBRANE ASSOCIATED GLUCOSE AND GLUCONATE OXIDASE ENZYMES IN *PSEUDOMONAS FLUORESCENS*: CHARACTERIZATION AND REGULATION

by

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REVIEW OF LITERATURE

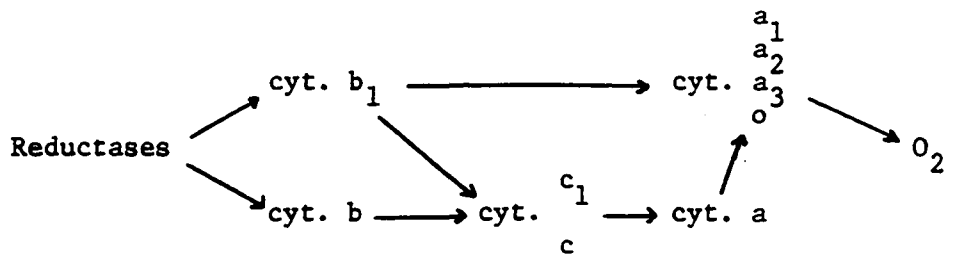
Membranes

Considerable effort has been expended towards determining the structure of membranes and various models of membrane structure have been postulated (L. Rothfield and A. Finkelstein, 1968). The Fluid Mosaic model (S. J. Singer and G. L. Nicolson, 1972) is presently viewed as the most generally acceptable. This model consists of a phospholipid bilayer with globular proteins interspersed throughout the plane of the lipid membrane. These globular proteins may or may not extend through the phospholipid bilayer. The Fluid Mosaic model as postulated by Singer and Nicolson describes the globular proteins as being mobile within a fluid phospholipid bilayer. This model is consistent with thermodynamic limitations and membrane protein functions (R. Coleman, 1972).

Membranes and membrane associated enzymes are implicated in essentially all major cellular activities including energy generation (F. M. Harold, 1972), nutrient transport (A. B. Pardee, 1968), DNA synthesis (A. Ryter, 1968), and protein synthesis (D. Schlessinger, 1963). The glucose and gluconate oxidase enzymes in Pseudomonas putida A3.12, formly P. fluorescens A3.12, are membrane bound (W. A. Wood and R. F. Schwerdt, 1953) although Burrows and Wood (1962) found gluconic acid dehydrogenase (oxidase) to be equally distributed in the cytoplasmic and membrane fractions. This discrepancy in gluconate

dehydrogenase localization was probably due to different methods used for enzyme preparation.

Since the early work of Weibull (1953) it has been known that the bacterial membrane represents the site of the organized cytochrome mediated electron transport chain. A schematic diagram of the possible pathways of electron transport in bacterial cytochrome systems is indicated below (L. Smith, 1961):



Abbreviations: cyt., cytochromes.

The oxidase and dehydrogenase enzymes of aerobic bacteria are coupled to the final electron acceptor, oxygen, via the cytochrome system (electron transport system, ETS). The cytochrome content of bacteria was extensively reviewed by Horio and Kamen (1970), and Kamen and Horio (1970). They thoroughly described the structural and functional characteristics of bacterial cytochromes. It has been demonstrated that Bacterium anitratum (J. G. Hauge and P. A. Hallberg, 1964), Acetobacter suboxydans (T. E. King and V. H. Cheldelin, 1957), and P. fluorescens (J. G. Hauge, 1961; and J. G. Hauge, 1966) contain b-type cytochromes. Lenhoff and Kaplan (1953) and Lenhoff (1963) demonstrated that P. fluorescens also contains a c-type cytochrome under certain growth conditions. These results illustrate two very important points in bacterial cytochrome systems. First, the

cytochrome composition can change both qualitatively and quantitatively as nutritional conditions vary and, secondly, aerobic metabolism can be associated with the absence, rather than the presence, of c-type cytochromes (T. Horio and M. D. Kamen, 1970).

Theories of Enzyme Regulation in Bacteria

The study of enzyme induction in micro-organisms has generated concepts which have come to be accepted as applicable to all of biology. Cohen et al., (1953) suggested the term "enzyme induction" to describe a relative increase in the rate of synthesis of a specific enzyme resulting from exposure to a chemical substance called the "inducer". The inducer of an enzyme is the metabolite which most directly causes enzyme synthesis. Repression may be defined, physiologically, as the opposite of induction, i.e., a relative decrease in the rate of synthesis of a specific enzyme resulting from exposure to a chemical substance.

Sequential induction is characterized by a chemical change in the inducer compound (L. N. Ornston, 1966). This type of induction is diagrammed below:

$$A \xrightarrow{\text{Enz. A}} B \xrightarrow{\text{Enz. B}} C \xrightarrow{\text{Enz. C}} D$$

Therefore, substrate A induces enzyme A which modifies substrate A chemically to compound B. Substrate B then induces enzyme B which catalyzes the conversion of compound B to compound C. Strict sequential inductive control offers cells a selective advantage since

they only induce an enzyme in a metabolic pathway when the enzyme substrate is present; thus, cells are able to conserve energy and amino acids for essential cellular components (L. N. Ornston, 1971).

Glucose Catabolism in *Pseudomonas fluorescens*

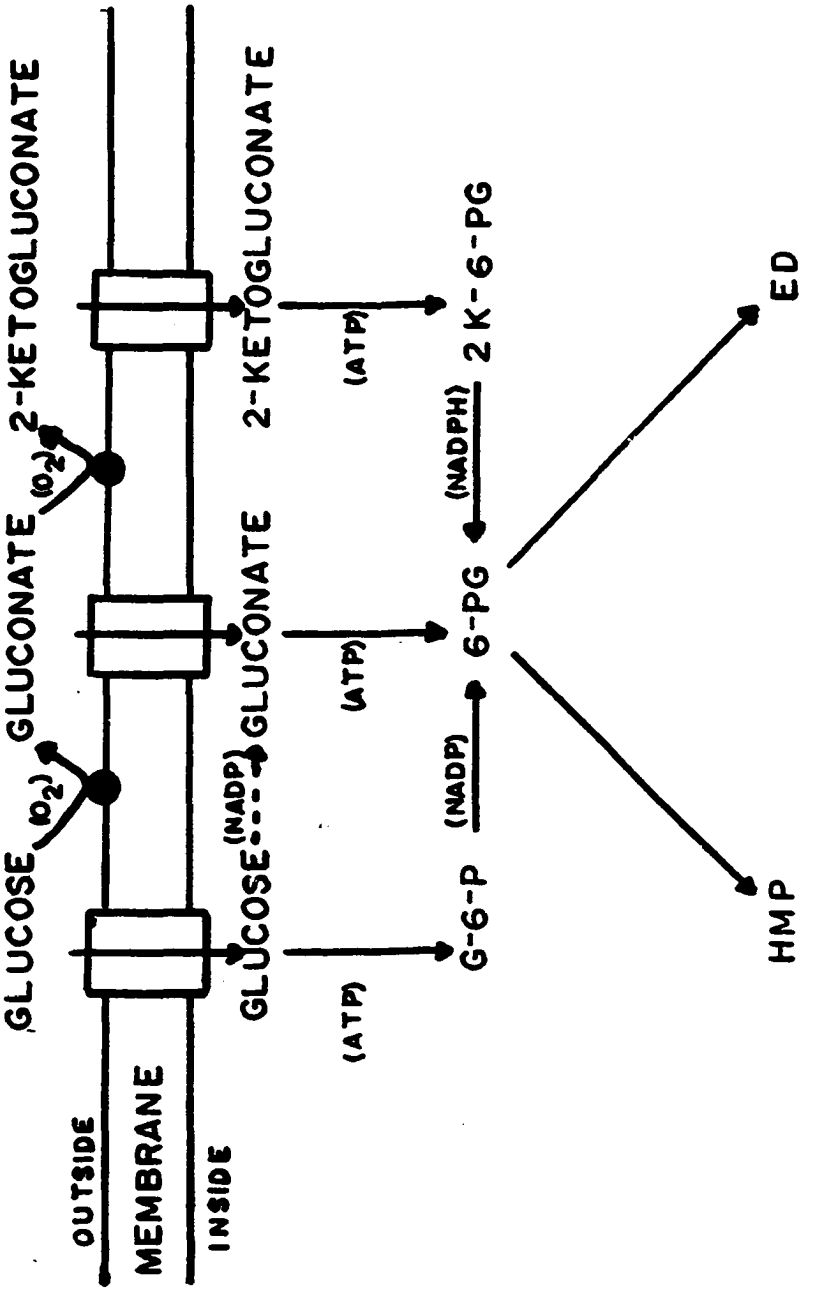
Glucose catabolism in *P. fluorescens* proceeds via a non-glycolytic oxidative mechanism to pyruvate and glyceraldehyde-3-phosphate (G-3-P) (J. DeLey, 1960; N. Entner and M. Doudoroff, 1951; and W. A. Wood and R. F. Schwerdt, 1953). This contrasts to the fermentative glycolytic mechanism of glucose degradation in *Escherichia coli* and a variety of other well studied bacteria.

P. fluorescens, *P. putida*, and *P. aeruginosa* have been grouped together by Stanier *et al.* (1966), on the basis of common physiological characters, as comprising the aerobic fluorescent pseudomonads. These three species are known to produce gluconate and 2-ketogluconate from glucose by the particulate glucose and gluconate oxidase enzymes (R. Bentley and L. Slechta, 1960; J. DeLey, 1960; N. Entner and M. Doudoroff, 1951; J. G. Hauge, 1966; F. N. Stokes and J. J. R. Campbell, 1951; M. Von Tigerstrom and J. J. R. Campbell, 1966; W. A. Wood, 1955; and W. A. Wood and R. F. Schwerdt, 1953). It has been shown that glucose and its oxidation products, gluconate and 2-ketogluconate, are actively transported, unaltered, via inducible transport proteins through the cells permeability barrier in these fluorescent pseudomonads (R. C. Eisenberg *et al.*, 1974; L. F. Guymon and R. G. Eagon, 1974; and B. K. Roberts *et al.*, 1973). The intracellular transport products are then phosphorylated via specific

cytoplasmic kinases to glucose-6-phosphate, 6-phosphogluconate, and 2-keto-6-phosphogluconate. The detection of a glucose kinase enzyme in P. fluorescens, catalyzing the metabolism of glucose to glucose-6-phosphate, was recently reported from this laboratory (R. C. Eisenberg et al., 1974). P. V. Phibbs (personal communication to R. C. Eisenberg) has also detected a glucose kinase enzyme in P. putida and thus all three species of the aerobic fluorescent pseudomonads have been shown to contain a cytoplasmic kinase activity for the phosphorylation of glucose. Glucose-6-phosphate is then oxidized to 6-phosphogluconate via an ATP regulated nicotinamide adenine dinucleotide phosphate (NADP) dependent glucose-6-phosphate dehydrogenase (T. Lessie and F. C. Neidhardt, 1967). 2-keto-6-phosphogluconate is reduced to 6-phosphogluconate via a reduced NADP ($NADPH_2$) dependent 2-keto-6-phosphogluconate reductase (E. W. Frampton and W. A. Wood, 1961). 6-phosphogluconate can then be further oxidized to a pentose phosphate via 6-phosphogluconate dehydrogenase and enter the hexose monophosphate shunt (pentose-phosphate pathway). 6-phosphogluconate can also be cleaved to yield pyruvate and G-3-P via the Entner-Doudoroff pathway enzymes. 6-phosphogluconate dehydrase produces 2-keto-3-deoxy-6-phosphogluconate (J. MacGee and M. Doudoroff, 1954; and R. Kovachevich and W. A. Wood, 1955), which is then cleaved by 2-keto-3-deoxy-6-phosphogluconate aldolase to yield pyruvate and G-3-P (N. Entner and M. Doudoroff, 1952; and R. Kovachevich and W. A. Wood, 1955). A diagrammatic summary of the above discussion on glucose catabolism in the fluorescent pseudomonads is shown in Figure 1.

Figure 1

Proposed unifying scheme for glucose utilization in the aerobic fluorescent pseudomonads as described by R. C. Eisenberg et al., J. Bacteriol. 120: 147 (1974). The closed circles represent particulate oxidase enzymes. Open rectangles represent transport proteins. The dashed line indicates a minor contribution to carbon flow (S. C. Quay et al., 1972). Abbreviations: G-6-P, glucose-6-phosphate; 6-PG, 6-phosphogluconate, 2K-6-PG, 2-keto-6-phosphogluconate; HMP, hexose monophosphate shunt (pentose-phosphate pathway); and ED, Entner-Doudoroff pathway.



The regulation of glucose catabolism in pseudomonads has recently been reviewed by Ornston (1971). The ED pathway is generally recognized as a major glucose inducible system for glucose dissimilation in pseudomonads (T. Lessie and F. C. Neidhardt, 1967; L. N. Ornston, 1966; and K. Kersters and J. DeLey, 1968). It has been reported, however, that the induction of ED pathway enzymes by growth on gluconate, but not glucose, occurs in a wide variety of bacteria, such as E. coli, Salmonella typhimurium, Enterobacter aerogenes (R. C. Eisenberg and W. J. Dobrogosz, 1966), and Vibrio natriegens (previously Pseudomonas natriegens [R. G. Eagon and C. H. Wang, 1962]). Quay et al. (1972) using P. fluorescens and oxidase deficient mutants derived from it, reported that gluconate, not glucose, regulates glucose catabolism in P. fluorescens. Their evidence was the inability of a glucose oxidase deficient mutant to induce the ED pathway when grown on glucose and the induction of these enzymes when the mutant was grown on gluconate. They concluded that the ability of P. fluorescens to induce the ED pathway enzymes when grown on glucose was due to the production of inducer (gluconate) from glucose via glucose oxidase. Hylemon and Phibbs (1972) postulated that the inducer for hexose catabolic enzymes in P. aeruginosa was a three carbon compound such as glycerol or glycerate. This type of coordinate, end-product induction is similar to the model for regulation of the β-keto-adipate pathway in P. aeruginosa (M. B. Kemp and G. D. Hegeman, 1968) and P. putida (L. N. Ornston and M. L. Wheelis, 1972).

In earlier studies on the regulation of the glucose and gluconate oxidase enzymes, Entner and Stanier (1951) reported glucose oxidase to be constitutive. Their conclusion was based on the results of enzyme activities from P. fluorescens A3.12 grown on asparagine or glucose. Cells grown on asparagine oxidized glucose at once, to gluconate, and inducible enzymes participated in the oxidation of gluconate. Cells grown on glucose oxidized glucose and gluconate without a noticeable lag period (N. Entner and R. Y. Stanier, 1951). Later, chemostat studies by Ng and Dawes (1972) on the regulation of glucose metabolism in P. aeruginosa by citrate resulted in their conclusion that glucose oxidizing enzymes were regulated by induction with glucose or its metabolites and repressed by citrate or its metabolic products. In a study on the regulation of the tricarboxylic acid cycle (TCA), the glyoxylate cycle, and the enzymes of glucose oxidation in P. aeruginosa, Von Tigerstrom and Campbell (1966) reported the glucose oxidase enzyme to be induced by glucose and repressed by α-ketoglutarate and acetate. The TCA cycle enzymes were constitutive. This conclusion contrasts results obtained from facultative anaerobes (C. T. Gray et al., 1966) where catabolite repression (glucose effect [B. Magasanik, 1961]) of the TCA cycle enzymes occurs when cells are grown on glucose. Tiwari and Campbell (1969) reported that the ED pathway enzymes were induced by growth on glucose and absent in succinate grown cells of P. aeruginosa. The enzymes necessary for succinate utilization were present, however, in cells grown in either glucose or succinate medium.

An interesting observation by Hamilton and Dawes (1959) showed the existence of a "reverse" diauxic growth pattern when P. aeruginosa was grown in a mixture of glucose and organic acids; these investigators found that organic acids are utilized preferentially to glucose. Three possible explanations for this "reverse" diauxie were examined by Hamilton and Dawes (1960, 1961): (1) the enzymes of glucose catabolism are constitutive but entry of glucose is repressed by the growth on organic acids; (2) the enzymes of glucose catabolism are repressed by the presence of organic acids but the glucose transport proteins are constitutive; or (3) both glucose catabolic and transport proteins are inducible. Evidence was obtained for the third explanation as being the correct hypothesis.

When P. aeruginosa was grown on limiting equimolar amounts of glucose, gluconate, or 2-ketogluconate and molar growth yields (J. C. Senez, 1962) compared, it was concluded that no energy was gained by the cells in the oxidative steps: glucose ⟶ gluconate ⟶ 2-ketogluconate (J. J. R. Campbell et al., 1956). They also reported that no high-energy phosphate compounds were formed during the oxidation of glucose to 2-ketogluconate in sonic extracts. In a similar study by Mackechnie and Dawes (1969) similar results were reported. They also showed that these oxidations (glucose to 2-ketogluconate) were not a source of energy in the form of ATP or NADPH, which is in agreement with work reported earlier by Stokes and Campbell (1951) where they were unable to observe the formation of any phosphate esters in the oxidative steps from glucose to gluconate in P. aeruginosa.

Although neither P. aeruginosa nor P. fluorescens reduce either NAD or NADP during the oxidation of glucose to 2-ketogluconate, the conclusions that high-energy phosphate bonds (i.e. ATP) are not produced seems untenable. Hauge (1964a) and Wood (1955) demonstrated the presence and involvement of cytochromes b and c during the oxidation of glucose by particulate membrane preparations. The energy gained by the sequential oxidation and reduction of the cytochromes to the terminal electron acceptor, oxygen, can be stored in high-energy phosphate bonds of ATP via coupling to oxidative phosphorylation (F. M. Harold, 1972). This coupling of electron transport and oxidative phosphorylation is similar to the mechanism in mitochondria (A. L. Lehninger, 1966; A. L. Lehninger, 1970; and H. R. Mahler and E. H. Cordes, 1971).

The energy generated in the electron transport chain can also be used to energize active transport. Barnes and Kaback (1970) and Kaback and Barnes (1971) have described a specific mechanism for active transport in bacteria which involves the coupling of transport to membrane bound dehydrogenases (oxidases) via the ETS. It has been demonstrated that E. coli actively transports β-galactosides and amino acids via a D-lactate dehydrogenase coupled transport system (E. M. Barnes, Jr. and H. R. Kaback, 1970; A. S. Gordon et al., 1972; W. L. Klein and P. D. Boyer, 1972; and H. H. Winkler and T. H. Wilson, 1966). Barnes (1972, 1973, and 1974) has recently reported Azotobacter vinelandii, a strict aerobe, transports glucose via an inducible active transport system energized by the oxidation of L-malate by a membrane bound malate oxidase enzyme.

Reasons for Study

Following an extensive taxonomic re-evaluation of the aerobic pseudomonads by Stanier, Palleroni, and Doudoroff (1966), it was noted that all previous work using P. fluorescens A3.12, (S. E. Burrows and W. A. Wood, 1962; N. Entner and R. Y. Stanier, 1951; W. A. Wood, 1955; and W. A. Wood and R. F. Schwerdt, 1953) was in fact performed with P. putida (ATCC 12633). It was therefore necessary to re-evaluate the particulate glucose and gluconate oxidases from P. fluorescens (proposed neotype strain, ATCC 13525) and to rigorously characterize these enzymes with respect to optimum assay conditions, kinetics, their relationship to the cytochrome chain, and regulation of their biosynthesis. In addition to the confusion caused by the taxonomic re-evaluation, growth conditions and the medium (chemically defined or complex) used to cultivate the organisms have varied. This research was initiated, therefore, to describe reproducible assay conditions, association [illegible]ochromes, and inductive regulation of membrane bound glucose and gluconate oxidases in P. fluorescens.

MATERIALS AND METHODS

Source of Organism

Pseudomonas fluorescens ATCC 13525 (proposed neotype strain, R. Y. Stanier et al., 1966) and a particulate glucose oxidase mutant derived from this parent strain were employed in this study. The glucose oxidase mutant is designated gox-7 and has been described previously (S. C. Quay et al., 1972).

Growth of Organism

Stock cultures were maintained on Trypticase Soy Broth without Dextrose (BioQuest) plus 1.5% agar (Difco) slants. The bacteria were transferred bimonthly to new slants.

Cells used for preparation of membranes and all other experiments were grown in a modified Vogel-Bonner (1955) basal salts medium containing (grams/liter): K_2HPO_4, 5.0; KH_2PO_4, 3.94; $Na(NH_4)HPO_4 \cdot 4H_2O$, 3.50; and $MgSO_4 \cdot 7H_2O$, 0.2. The pH of the medium was 7.0. Carbon and energy sources were added as indicated in the Results section. Cultures were incubated at 26°C in a New Brunswick gyratory incubator shaker.

Bacteria were obtained in one of two ways depending on the type of study. Method A: For enzyme characterization and induction studies, bacteria from a 24 hour culture were used to inoculate 250 ml of the basal salts medium (inoculated $O.D._{600}$, 1 cm light path

equal to 0.03) in 2.8 liter Fernbach flasks containing the indicated carbon source; cells used as inoculum were always grown in the same medium that they were to be transferred into. The cultures were incubated overnight (about 14 hours) and then equal volumes of fresh medium was added and incubation continued for an additional three hours before harvesting. Method B: Bacteria for growth rate and enzyme level studies were grown as described above for Method A in 500 ml of the Vogel-Bonner basal salts medium in 2.8 liter Fernbach flasks containing the designated carbon source. Growth rates were determined using a Gilford 2000 recording spectrophotometer at a wavelength of 600 nm, a 1 cm light path, and measuring absorbance between 0.01 and 0.30 using diluted cell suspensions where necessary. Concentration of cells was estimated as μg dry weight per ml [μg dry wt bacteria/ml = $(O.D._{600})$ (Dil. factor) (299 μg)] from a standard curve relating absorbance to dry weight. Bacteria were harvested directly during exponential or stationary phase rather than after incubation in fresh medium for 3 hr as in Method A.

Preparation of Cell Extracts for Enzyme Assays and Cytochrome Spectra

Bacteria were harvested at the appropriate time or concentration (depending on the type of experiment) by centrifugation in a Sorvall RC2-B refrigerated centrifuge at 13,200 x gravity (g) for 5 min. The cells were washed twice in phosphate buffer (30 mM potassium phosphate, pH 6.7, for enzyme characterization studies; 10 mM potassium phosphate, pH 7.65, for enzyme induction studies) containing 2 mM

$MgSO_4$. Twice washed cell pellets were resuspended in a minimal volume of the appropriate buffer containing 5 mM β-mercaptoethanol and disrupted with a Branson model W185 sonifier at 6 amps (68 watts) with three 15 sec. treatments. Fifteen seconds were allowed for the suspension to cool after each 15 sec sonication period. Sonication vessels were immersed in an ice bath during cell breakage. Sonically treated cell suspensions were centrifuged at 12,000 x g for 15 min to remove whole cells and large cell debris, as determined by phase-contrast microscopy, from the cell extract. For induction studies an aliquot of the cell-free extract was retained for analysis of Entner-Doudoroff pathway enzymes. The cell extract was drawn off carefully using a Mohr-type pipet and a propipet bulb. This cell extract was centrifuged at 133,570 x g for 71 min in a Beckman L2 or L3-50 preparative ultracentrifuge to obtain the supernatant soluble fraction and the particulate membrane fraction. The particulate membrane fraction was resuspended and washed once in the appropriate wash buffer to remove residual β-mercaptoethanol. The washed membrane fractions were then resuspended in the appropriate enzyme assay buffers described in the Results section and retained for particulate oxidase analysis. All centrifugations and wash buffers were at 0-4°C.

Enzyme Assays

Particulate glucose dehydrogenase (D-glucose:dichlorophenol-indophenol [DCPIP] 1-oxidoreductase, EC 1.1.99.a) was determined spectrophotometrically, using a Gilford 2000 recording spectrophotometer, by following the coupled reduction of DCPIP at 600 nm. The

molar absorbancy of DCPIP at 600 nm was taken as 21 mM^{-1} cm^{-1} at pH 7.3. The reaction mixture contained: DCPIP, sodium salt, 143 µmoles; 90 µmoles potassium phosphate buffer, pH 7.3; 6 µmoles $MgSO_4$; 30 µmoles glucose; particulate fraction (in 30 mM potassium phosphate buffer, pH 6.7, plus 2 mM $MgSO_4$) and water to 3.0 ml. The reaction was measured at 26°C.

Particulate gluconate dehydrogenase (D-gluconate:DCPIP 2-oxidoreductase, EC 1.1.99.3) was determined spectrophotometrically as described for the glucose dehydrogenase except that 3 µmoles potassium gluconate was substituted for glucose.

6-Phosphogluconate dehydrase (6-phospho-D-gluconate hydrolyase, EC 4.2.1.12) was assayed as described by Kovachevich and Wood (1955).

Particulate glucose oxidase (D-glucose:O_2, cytochrome-linked oxidoreductase, EC 1.1.3.a), particulate gluconate oxidase (D-gluconate:O_2 oxidoreductase, EC 1.1.99.3), particulate malate oxidase (L-malate:O_2 oxidoreductase, EC 1.1.3.3), particulate D(-) lactate oxidase (D(-) lactate:O_2, cytochrome-linked oxidoreductase, EC 1.1.2.a), and particulate L(+) lactate oxidase (L(+) lactate:O_2, cytochrome-linked oxidoreductase, EC 1.1.2.b) activities were determined polarographically by measuring the consumption of dissolved oxygen at 26°C with a Gilson oxygraph fitted with a Clark-type oxygen electrode in a 1.50 ml reaction volume vessel. Oxygen saturation in distilled water at 26°C was estimated as 231 nmoles O_2 per ml. The contents of the reaction mixtures are indicated in the text (see Results). All values were reported as units of oxidase activity obtained by titration using at least three different enzyme

concentrations. Units of activity (nmoles of O_2 consumed per minute) were directly proportional to enzyme concentration.

Cytochrome Analysis

For the detection of the presence of cytochromes and flavoproteins in pooled glucose and gluconate oxidase membrane preparations (stock membrane preparations were mixed together), spectral assays were performed with a Coleman-Hatachi model 124 spectrophotometer equipped with a Coleman model 164 recorder. Absorption spectra were obtained at room temperature (ca. 23°C) between 660 nm and 400 nm. Total cytochrome reduction was measured by 10 min dithionite reduced minus oxidized difference spectra. For the reduced plus CO minus reduced spectra, CO was gently bubbled for 3 min through a 23-gauge hypodermic needle into a 3.0 ml cuvette containing 2.5 ml of dithionite or substrate reduced membrane particles. The cytochrome concentrations were estimated using the following wavelength pairs and millimolar extinction coefficients: cytochrome *b*, (562-574 nm) = 20 mM^{-1} cm^{-1}; cytochrome *c*+c_1, (552-540 nm) = 19 mM^{-1} cm^{-1} (R. F. Rest and D. C. Robertson, 1975; and K. Tochikubo, 1971), and cytochrome *o*, (417-432 nm, i.e. peak-trough) = 170 mM^{-1} cm^{-1} (R. M. Daniel, 1970). The presence of flavins within the ETS was indicated spectrally by the reduced minus oxidized absorption trough observed at 455 nm.

Respiratory and Electron Transport Inhibitors

The effect of respiratory and electron transport inhibitors on glucose oxidase was determined polarographically as described above. The inhibitors were added to the 1.50 ml reaction vessel following the buffer, magnesium, and membrane material. The reaction was initiated after a 3 min incubation period by addition of glucose. None of the inhibitors tested were oxidized by membrane enzymes.

Rotenone, 2,4-dinitrophenol (2,4-DNP), and 8-hydroxyquinoline were prepared in 95% ethanolic solutions. The inhibitor 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) was dissolved in 0.001 N KOH. Atabrine, sodium azide, acriflavin, and potassium cyanide were prepared as aqueous solutions.

Irradiation experiments were performed with a Long Wave UVL-22 Black-Ray black light lamp (Ultra-Violet Products, Inc., San Gabriel, Calif.). Membrane material (8.0 ml, 5 mm deep) was placed in an aluminum weighing dish and irradiated at a distance of 4.5 cm for the times indicated.

Miscellaneous Determinations

Membrane protein was estimated by the method of Lowery et al. (1951). Particulate protein fractions were precipitated with cold trichloroacetic acid (10% w/v) followed by hot alkali extraction (1.0 N NaOH, for 15 min at 100°C) and, following centrifugation, the supernatant fluid was assayed for protein content. Bovine serum

albumin (fraction V powder, Sigma Chemical, St. Louis, Mo.) served as the standard and was treated in the same manner as described for the membrane protein.

Pyruvate was determined by the method of Friedmann and Haugen (1943).

Glucose concentrations in particle-free supernatants from growth experiments were determined colorimetrically by both the Nelson reducing sugar assay (1944) and the Glucostat assay kit (Worthington Biochemical Corp., Freehold, N. J.). Both assays gave a linear response from 0.1 to 1.0 μmoles per ml.

Gluconate concentrations were determined enzymatically using gluconate-grown, gox-7 membrane preparations following the standard gluconate oxidase assay procedure. From established gluconate:O_2 stoichiometries (see Results) for gluconate oxidation by these membrane preparations, a linear response for nmoles gluconate oxidized per nmoles oxygen consumed was obtained. The response was linear from 40-400 nmoles gluconate per ml.

2-Ketogluconate concentrations were determined using the procedure of Avigad (1975) for determining hexuronic acids and some keto acids. Samples were adjusted to pH 4.7 in Vogel-Bonner medium before determinations were performed.

Chemicals

Rotenone, acriflavin, atabrine, and HQNO were purchased from Sigma Chemical Co., St. Louis, Mo.; 2,4-DNP was a product from Fisher Scientific Co., Fair Lawn, N. J.; sodium azide and carbon

monoxide (CO) were obtained from Matheson Company, Inc., Joliet, Ill. 8-Hydroxyquinoline was obtained from the Aldrich Chemical Co., Inc., Milwaukee, Wisc.; and potassium cyanide was purchased from Mallinckrodt Chemical Works, St. Louis, Mo. All other compounds used were commercially available reagent grade.

RESULTS

Enzyme Characterization Studies

To more fully understand the physiological significance and biosynthetic regulation of the particulate glucose and gluconate oxidase enzymes in P. fluorescens, as well as their respective dehydrogenase activities, it was necessary to first establish reliable enzyme assays for this specific organism. The glucose and gluconate oxidase enzymes were characterized for pH optima and substrate (electron donor) saturation kinetics. The results presented in Figures 2 and 4 show the effects of substrate concentration on the initial rate of enzyme activity. The apparent K_m (substrate concentration required for observed half maximal activity) for glucose and gluconate oxidase activities was 5.9×10^{-4} M and 3.35×10^{-4} M respectively.

The effect of pH on the activity of glucose and gluconate oxidases is shown in Figures 3 and 5 respectively. Maximum activity for glucose oxidase occurred at pH 6.7 and the maximum activity for gluconate oxidase was pH 6.0. Both oxidases were assayed at saturating substrate concentrations.

Particulate L-malate oxidase was included in this study to serve as a membrane marker enzyme. The results presented in Figures 6 and 7 show the L-malate oxidase enzyme exhibits an apparent K_m of 5.6×10^{-4} M and a pH optimum of 7.9.

Figure 2

Substrate saturation kinetics of glucose oxidase activity in membrane preparations from glucose-grown (0.1 M) P. fluorescens parent strain. Bacteria were grown (Method A) and membranes prepared as described in Materials and Methods. V (velocity) is defined as nmoles O_2 consumed per min per mg membrane protein. The insert is a Lineweaver-Burke (1934) plot of the data. The oxidase assays were performed at pH 6.7 using 30 mM potassium phosphate buffer containing 2 mM $MgSO_4$. Reaction mixtures contained 2.7 mg membrane protein per ml and were assayed at 26°C. The correlation coefficient (r) is included to indicate the experimental fit of these data to a straight line equation as determined by linear regression.

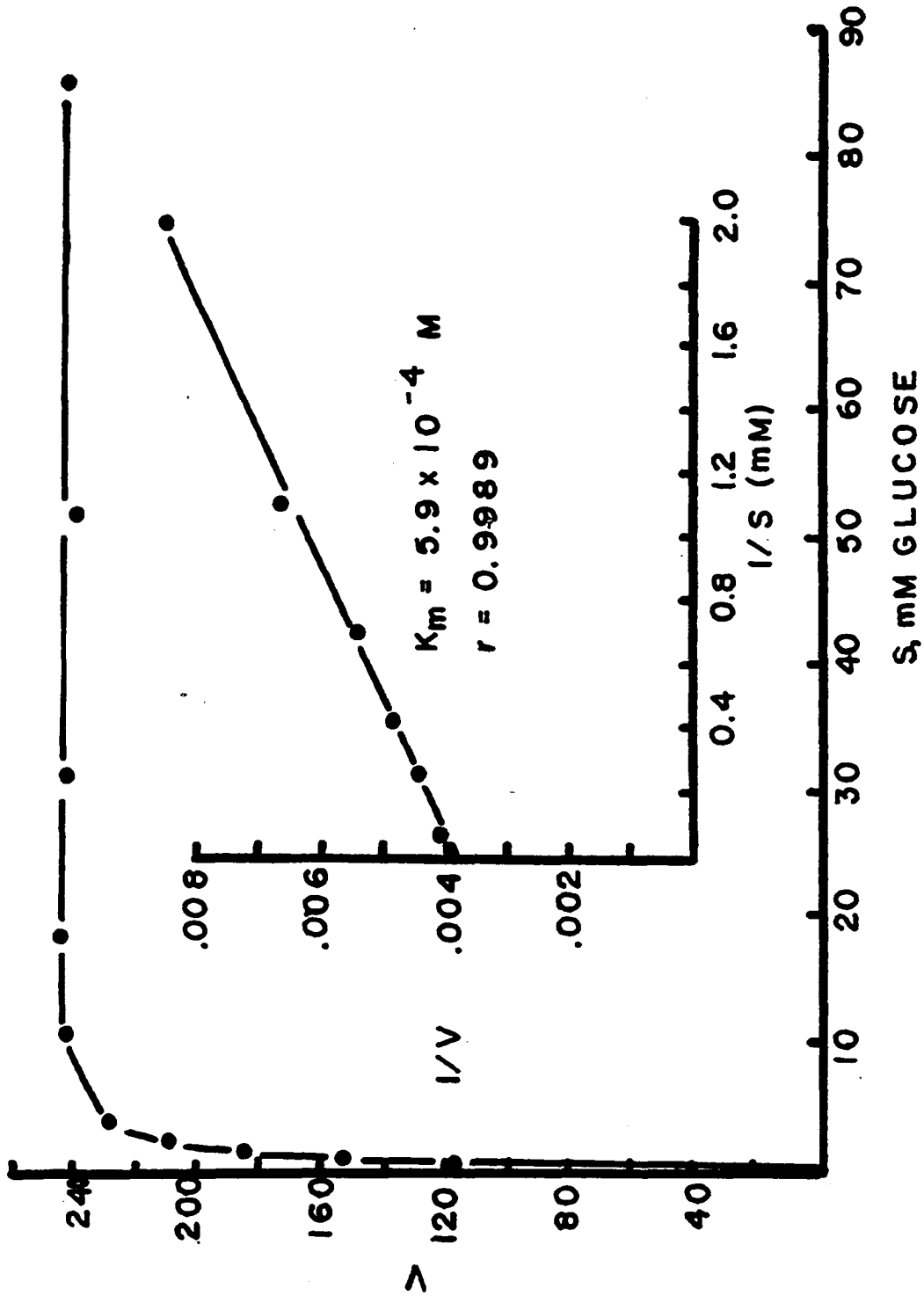


Figure 3

The effect of pH on glucose oxidase activity in membrane preparations from glucose-grown (0.1 M) P. fluorescens. Bacteria were grown using Method A, and membranes prepared as described in Materials and Methods. The reaction vessel contained (per ml): 30 µmoles of the buffer indicated, 2 µmoles $MgSO_4$, 50 µmoles glucose and 2.48 mg membrane protein. Oxidase activity is defined as nmoles O_2 consumed per min per mg membrane protein.

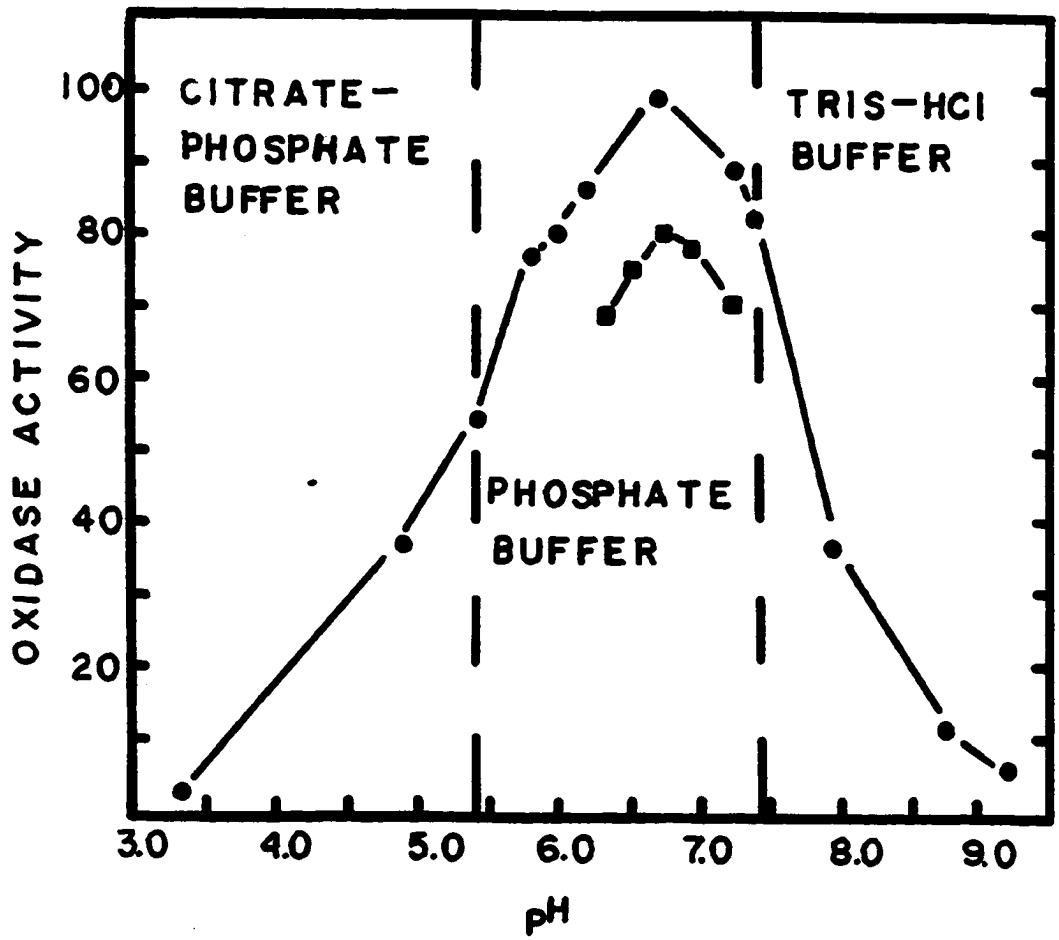


Figure 4

Substrate saturation kinetics of gluconate oxidase activity in membrane preparations from gluconate-grown (0.1 M) P. fluorescens parent strain. Bacteria were grown (Method A) and membranes prepared as described in Materials and Methods. V (velocity) is defined as nmoles O_2 consumed per min per mg membrane protein. The insert is a Lineweaver-Burke (1934) plot of the data. The oxidase assays were performed at pH 6.0 using 30 mM citrate-potassium phosphate buffer containing 2 mM $MgSO_4$. Reaction mixtures contained 5.89 mg membrane protein per ml and were assayed at 26°C. The correlation coefficient (r) is included to indicate the experimental fit of these data to a straight line equation as determined by linear regression.

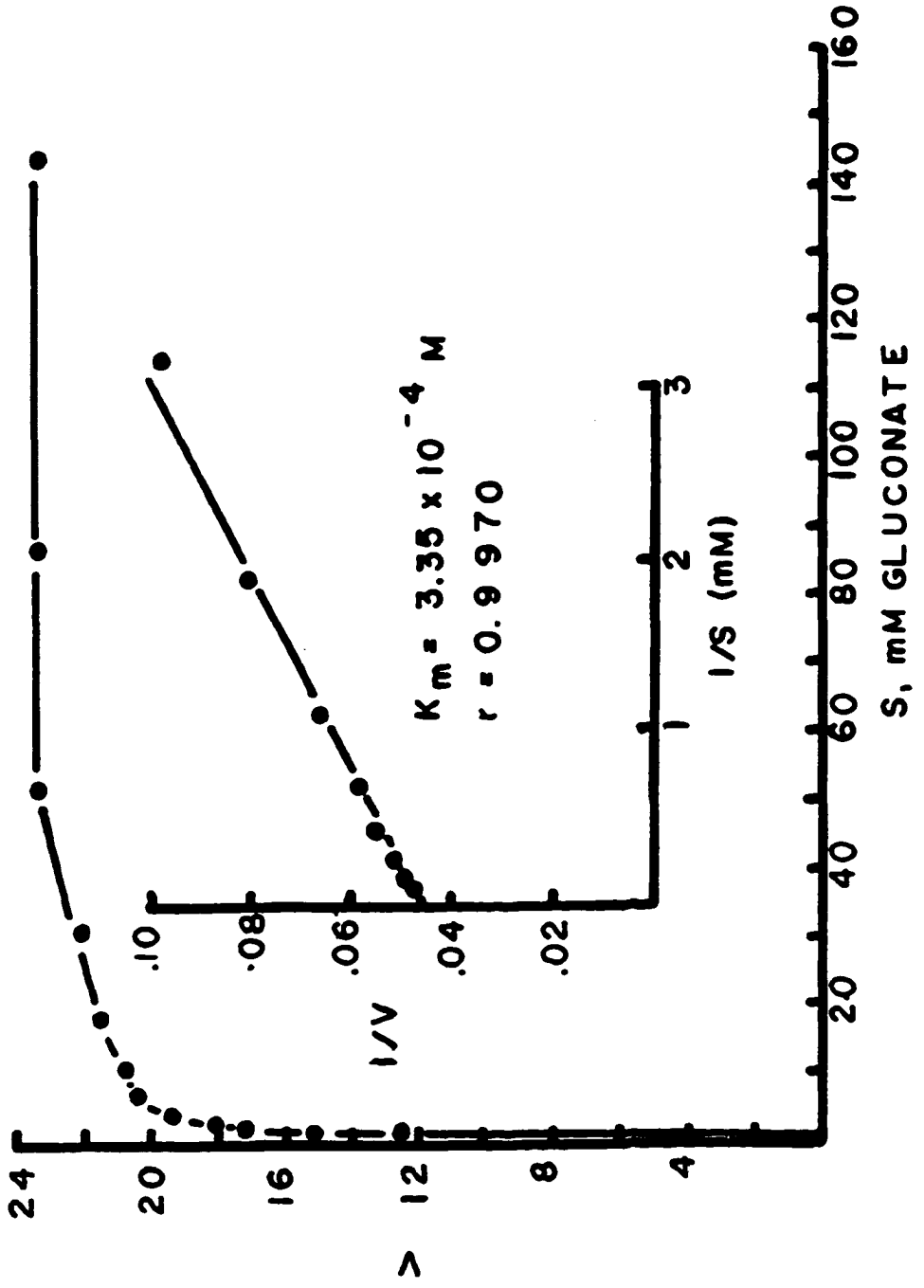


Figure 5

The effect of pH on gluconate oxidase activity in membrane preparations from gluconate-grown (0.1 M) P. fluorescens. Bacteria were grown using Method A and membranes prepared as described in the Materials and Methods. The reaction vessel contained (per ml): 30 μmoles of the buffer indicated, 2 μmoles $MgSO_4$, 50 μmoles gluconate and 6.47 mg membrane protein. Oxidase activity is defined as nmoles O_2 consumed per min per mg membrane protein.

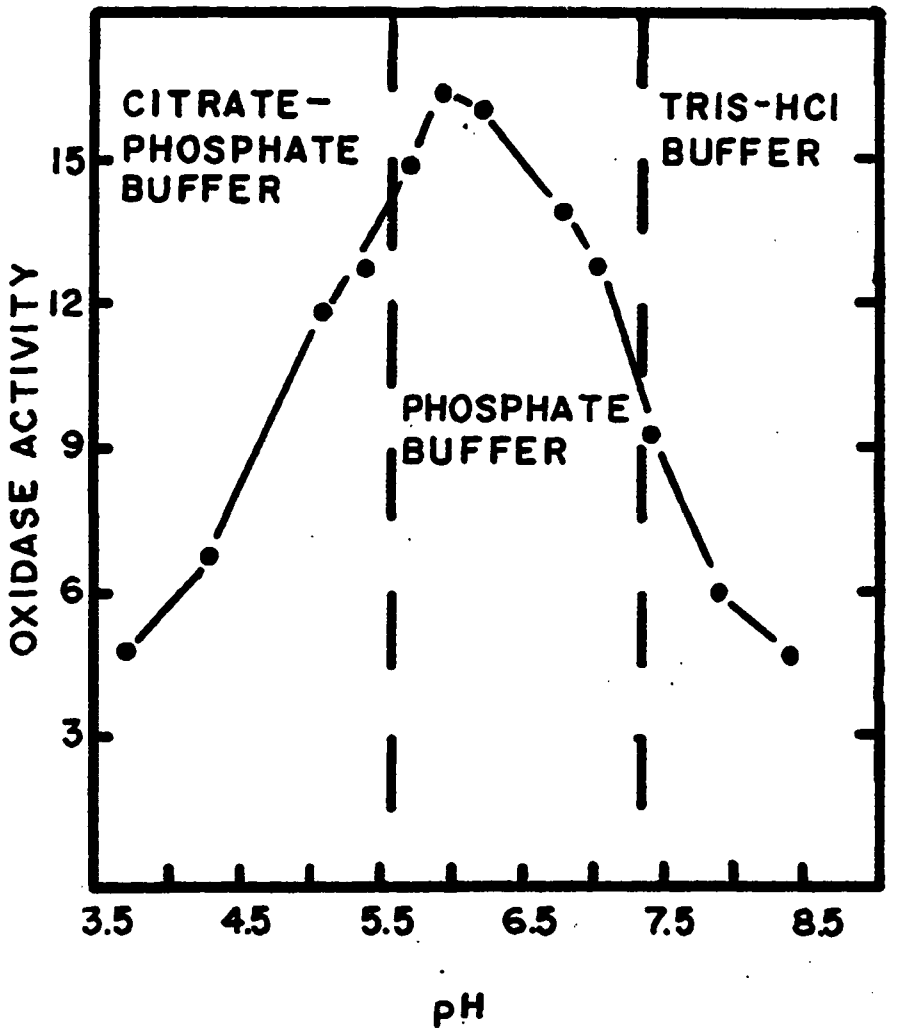


Figure 6

Substrate saturation kinetics of malate oxidase activity in membrane preparations from malate-grown (0.1 M) P. fluorescens parent strain. Bacteria were grown (Method A) and membranes prepared as described in Materials and Methods. V (velocity) is defined as nmoles O_2 consumed per min per mg membrane protein. The insert is a Lineweaver-Burke (1934) plot of the data. The oxidase assays were performed at pH 7.9 using 30 mM Tris (hydroxymethyl) aminoethane hydrochloride (Tris-HCl) buffer containing 2 mM $MgSO_4$. Reaction mixtures contained 3.39 mg membrane protein per ml and were assayed at 26°C. The correlation coefficient (r) is included to indicate the experimental fit of these data to a straight line equation as determined by linear regression.

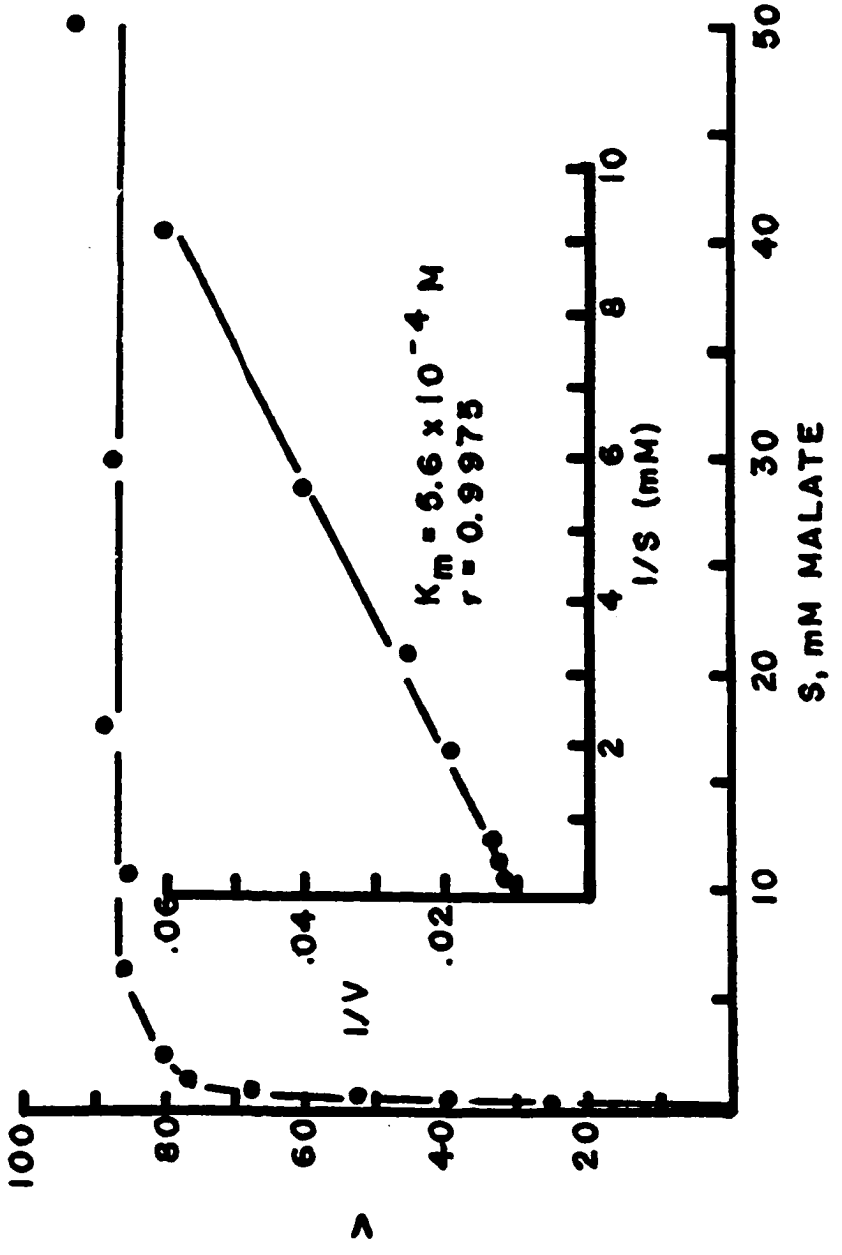
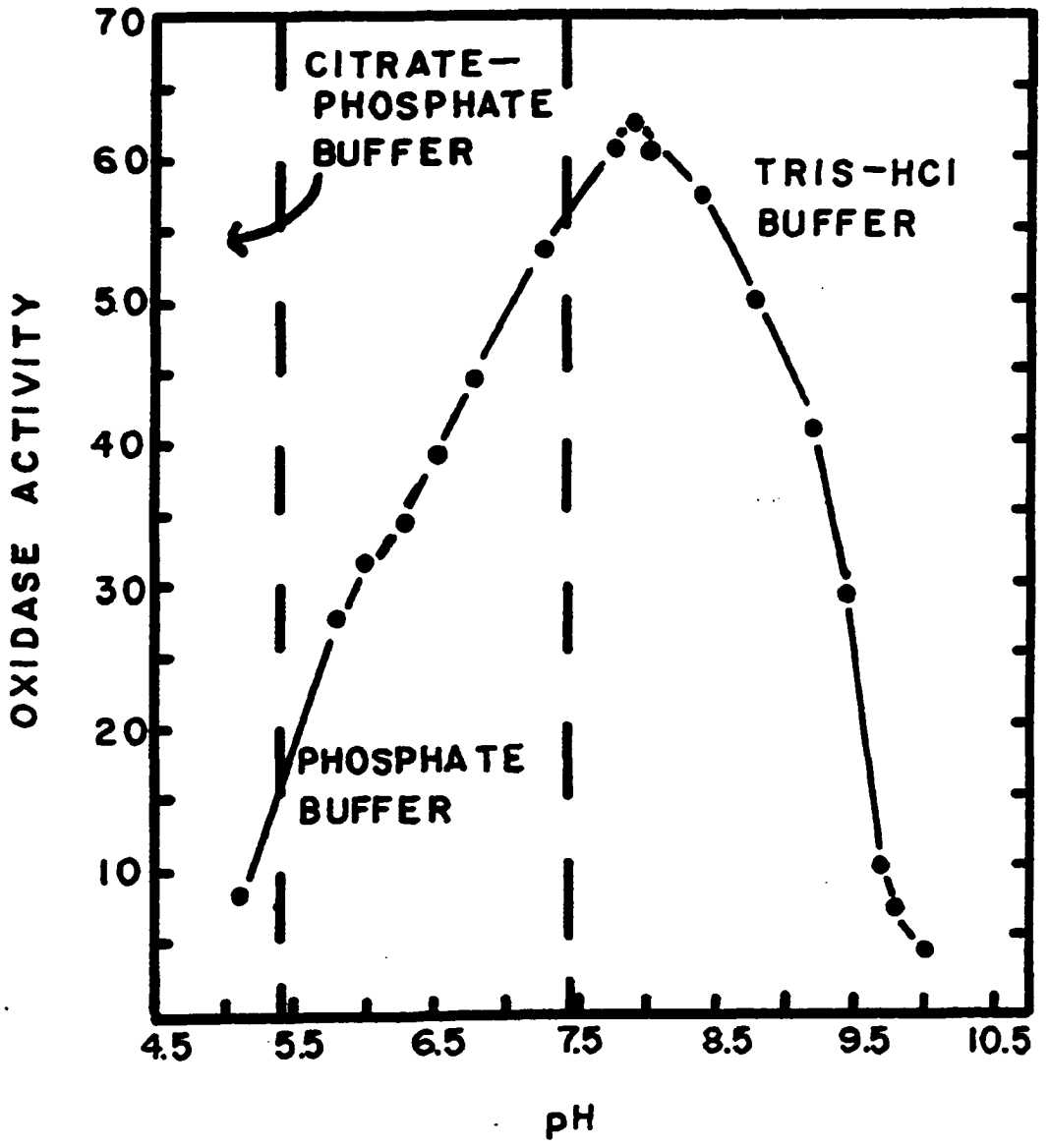


Figure 7

The effect of pH on malate oxidase activity in membrane preparations from malate-grown (0.1 M) P. fluorescens. Bacteria were grown using Method A and membranes prepared as described in Materials and Methods. The reaction vessel contained (per ml): 30 µmoles of the buffer indicated, 2 µmoles $MgSO_4$, 30 µmoles potassium malate, and 6.47 mg membrane protein. Oxidase activity is defined as nmoles O_2 consumed per min per mg membrane protein.



Particulate D(-) lactate oxidase and L(+) lactate oxidase enzymes were also characterized for apparent K_m's and pH optima. The effects of substrate concentrations on the initial rate of enzyme activity for D(-) lactate oxidase and L(+) lactate oxidase are shown in Figures 8 and 9 respectively. D(-) lactate oxidase exhibited an apparent K_m of 3.83×10^{-4} M and the L(+) lactate oxidase had an apparent K_m of 1.35×10^{-3} M. Both D(-) lactate and L(+) lactate oxidases had pH optima at 8.3 (Figure 10).

Table 1 summarizes the results of oxidase characterization studies on particulate glucose oxidase, gluconate oxidase, L-malate oxidase, D(-) lactate oxidase, and L(+) lactate oxidase enzymes.

The effect of temperature on the stability of the particulate glucose oxidase enzyme is indicated in Figure 11. These results show that glucose oxidase was rapidly inactivated at moderate temperature (45°C) whereas no significant activity was lost when the enzyme was stored in an ice bath at 0°C. The loss of ca. 27% glucose oxidase activity in 30 min at room temperature (26°C) indicates that care must be taken to retain these membrane preparations in an ice bath before measuring glucose oxidase activity. The effect of temperature on other oxidases described in this study was not established.

Enzyme Localization

Distribution of glucose and gluconate oxidase enzymes in the cell-free extract, particulate membrane, and soluble supernatant fractions is presented in Table 2. The results show that 91.5% of

Figure 8

Substrate saturation kinetics of D(-) lactate oxidase activity in membrane preparations from D,L,-lactate-grown (0.1 M) P. fluorescens. Bacteria were grown (Method A) and membranes prepared as described in Materials and Methods. V (velocity) is defined as nmoles O_2 consumed per min per mg membrane protein. The insert is a Lineweaver-Burke (1934) plot of the data. The oxidase assays were performed at pH 8.3 using 30 mM Tris-HCl buffer containing 2 mM $MgSO_4$. Reaction mixtures contained 8.17 mg membrane protein per ml and were assayed at 26°C. The correlation coefficient (r) is included to indicate the experimental fit of these data to a straight line equation as determined by linear regression.

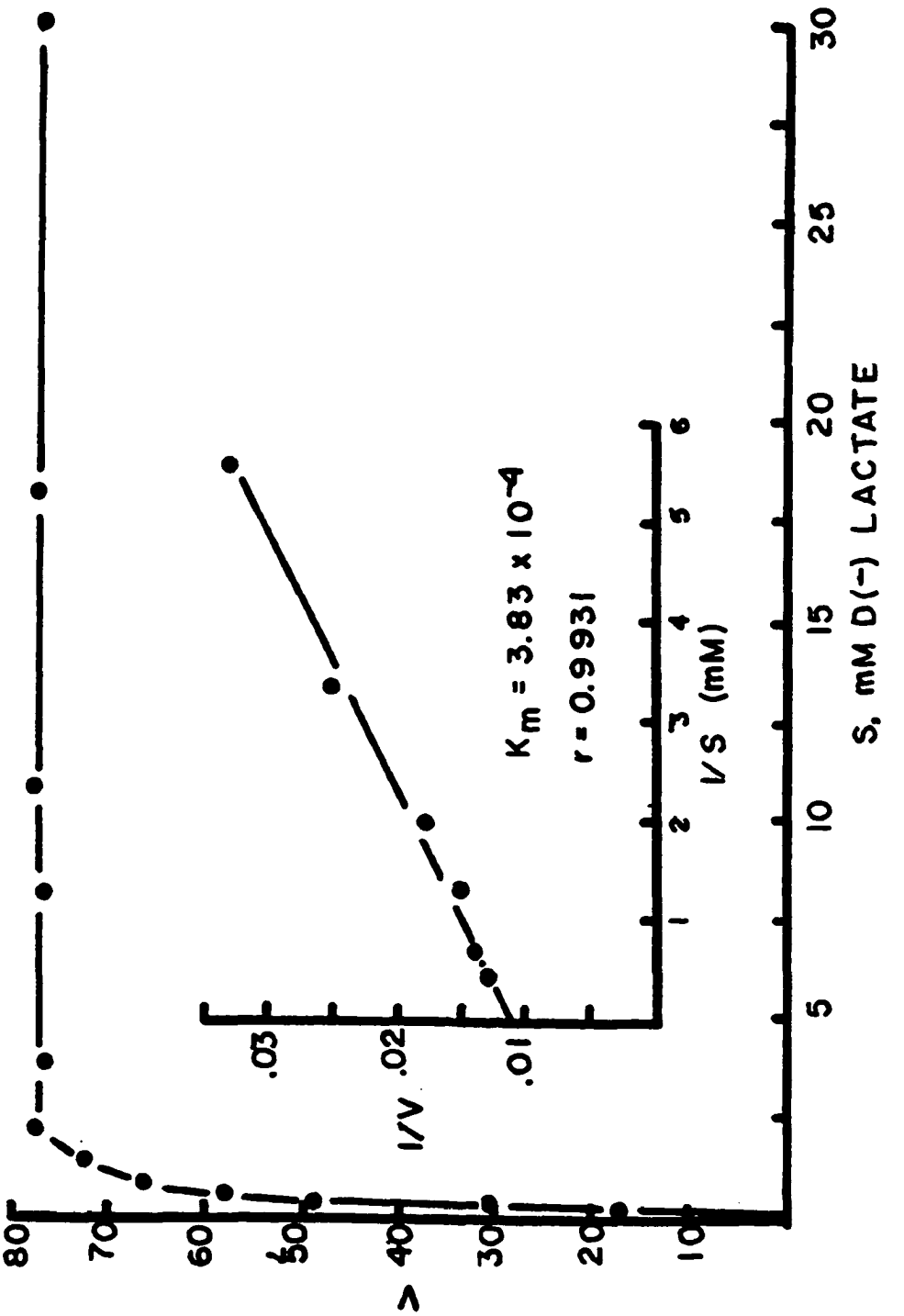


Figure 9

Substrate saturation kinetics of L(+) lactate oxidase activity in membrane preparations from D,L-lactate-grown (0.1 M) P. fluorescens. Bacteria were grown (Method A) and membranes prepared as described in Materials and Methods. V (velocity) is defined as nmoles O_2 consumed per min per mg membrane protein. The insert is a Lineweaver-Burke (1934) plot of the data. The oxidase assays were performed at pH 8.3 using 30 mM Tris-HCl buffer containing 2 mM $MgSO_4$. Reaction mixtures contained 6.79 mg membrane protein per ml and were assayed at 26°C. The correlation coefficient (r) is included to indicate the experimental fit of these data to a straight line equation as determined by linear regression.

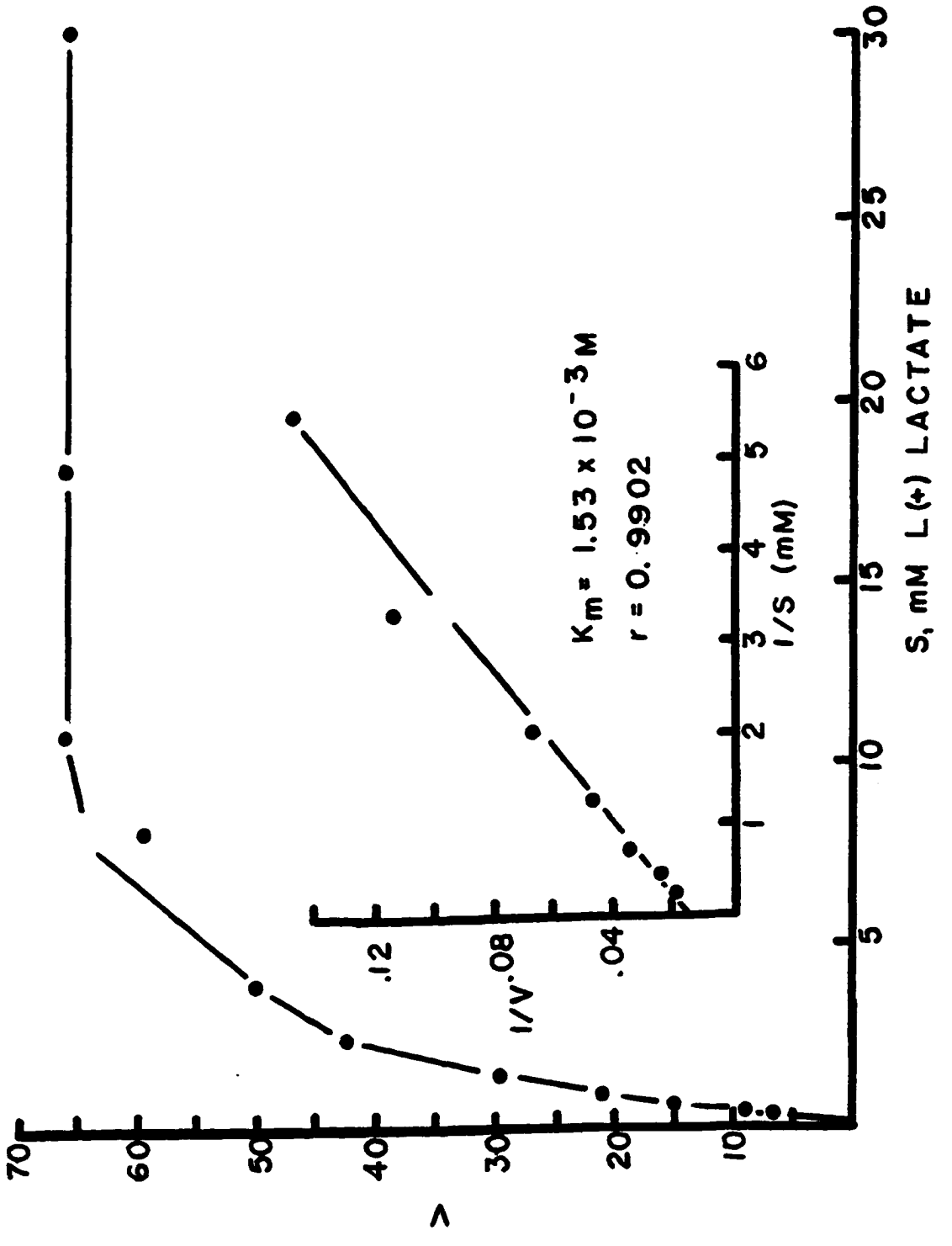


Figure 10

The effect of pH on D(-) and L(+) lactate oxidase activities in membrane preparations from D, L-lactate-grown (0.1 M) P. fluorescens. Bacteria were grown using Method A and membranes prepared as described in Materials and Methods. The reaction vessel contained (per ml): 30 μmoles of the buffer indicated, 2 μmoles $MgSO_4$, 20 μmoles lithium lactate and 8.17 mg membrane protein. Oxidase activity is defined as nmoles O_2 consumed per min per mg membrane protein. Symbols: ● — ● , D(-) lactate; ▲ — ▲ , L(+) lactate.

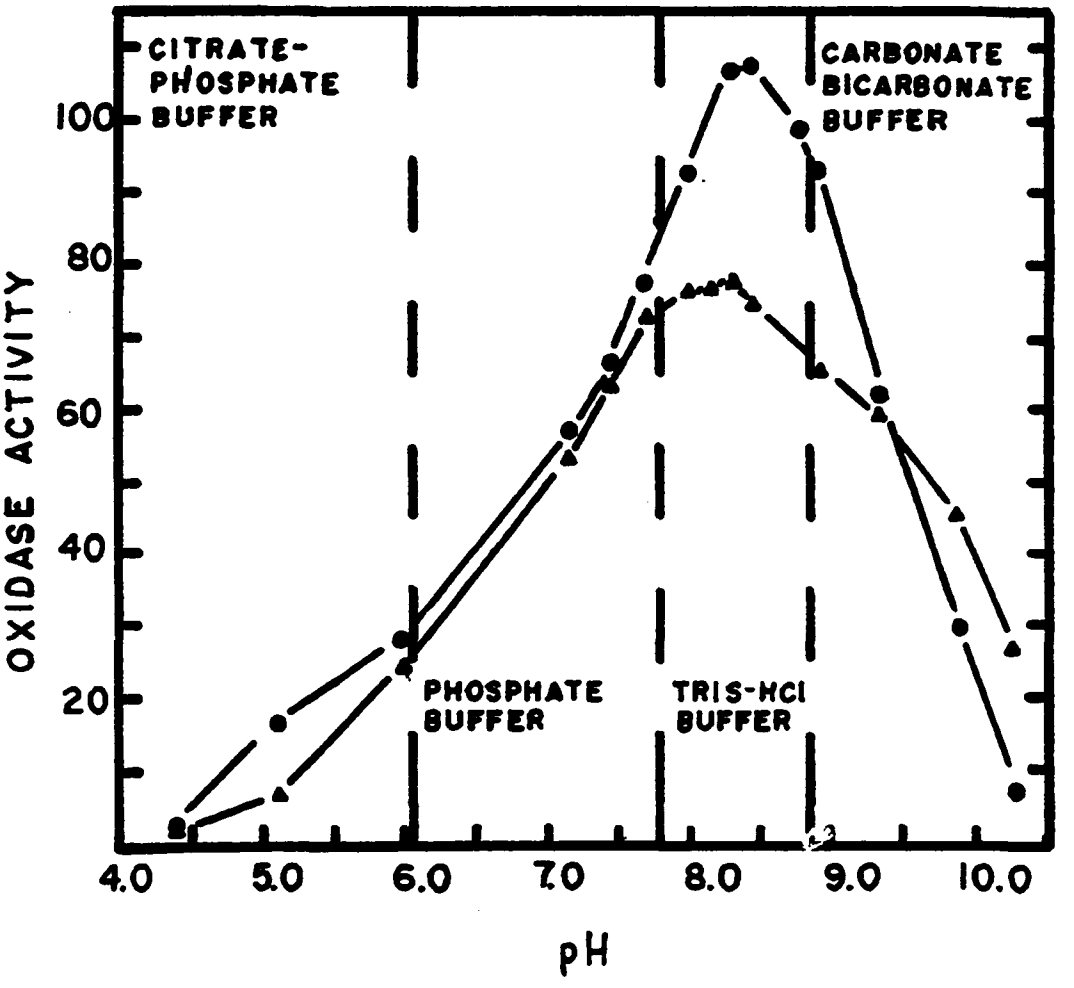


Table 1

Summary of pH Optima, V_{max}, and Apparent K_m Values for Various *P. fluorescens* Membrane Associated Oxidase Enzymes

Oxidase enzyme	Assay parameters		
	pH Optima	K_m	V_{max} [b]
Glucose	6.7	5.9×10^{-4} M	242.3
Gluconate	6.0	3.35×10^{-4} M	23.3
Malate	7.9	5.6×10^{-4} M	86.5
D(-) lactate	8.3	3.82×10^{-4} M	76.7
L(+) lactate	8.3	1.53×10^{-3} M	66.2

[a]Conditions for growth of bacteria and enzyme assay conditions were as described in the legends to Figures 2-10.

[b]Activity expressed as nmoles O_2 consumed per min per mg membrane protein.

Figure 11

Effect of various temperatures on the stability of particulate glucose oxidase. Bacteria were grown in the basal salts medium containing 0.1 M glucose (Method A) and membranes prepared as described in the Materials and Methods. Membrane preparations were brought to the indicated temperature from control membranes by vigorous shaking of test tube suspensions in a water bath. The control membrane preparation 0°C, was retained in an ice bath. When the membrane preparation reached the temperature of the water bath (between 30-60 seconds) a 0.1 ml aliquot was removed and assayed for glucose oxidase activity at 26°C; this is referred to as T_o and six additional 0.1 ml aliquots were taken at 5 min intervals thereafter. The control membrane suspension contained 2.87 mg membrane protein per ml and a specific activity of 309.6 nmoles O_2 consumed per min per mg membrane protein.

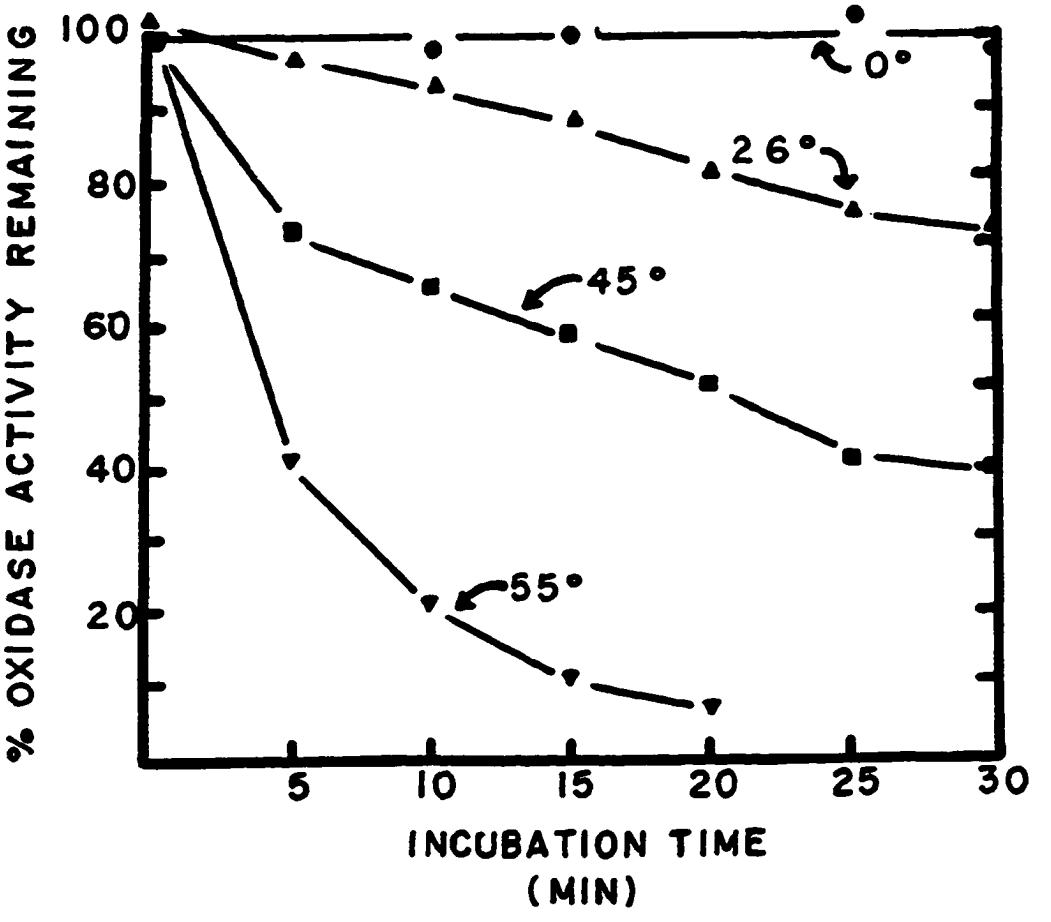


Table 2

Distribution of Glucose and Gluconate Oxidases in Particulate and Supernatant Fractions

Fraction	Total volume	Total protein	Total units glucose oxidase activity per fraction[a]	Total volume	Total protein	Total units glucose oxidase activity per fraction
Cell-free extract	20 ml	368 mg/ml	34,640	20 ml	614 mg/ml	22,640
Particulate (washed)	20 ml	127 mg/ml	31,680	20 ml	461 mg/ml	21,840
Supernatant	20 ml	204.4 mg/ml	62	20 ml	159 mg/ml	1,230

[a]Cell-free extracts, washed particulate membrane, and soluble fractions were prepared as described in Materials and Methods. Bacteria were grown using Method B and harvested at stationary phase. The parent strain of P. fluorescens grown in 0.1 M glucose was used for measuring glucose oxidase and a glucose oxidase deficient mutant, gox-7, grown in 0.1 M gluconate was used to measure gluconate oxidase activity.

the glucose oxidase and 96.5% of the gluconate oxidase activity in the cell-free extract were recovered in the particulate fractions. These data confirm the results of Quay et al. (1972) that these glucose and gluconate oxidase activities in P. fluorescens are localized in the particulate membrane fraction of these cells.

Estimation of Glucose and Gluconate Oxidase Stoichiometries

The stoichiometry for glucose and gluconate oxidases in P. fluorescens was estimated. The results obtained in this study using P. fluorescens agree with manometrically determined stoichiometries obtained earlier by Entner and Stanier (1951) using P. putida (ATCC 12633). The stoichiometry for oxidation of glucose to gluconic acid and 2-ketogluconic acid has been reported (W. A. Wood and R. F. Schwerdt, 1955; and J. DeLey and A. C. Stouthamer, 1959) as:

(1) glucose + 1/2 O_2 = gluconic acid, and

(2) gluconic acid + 1/2 O_2 = 2-ketogluconic acid + H_2O.

In order to estimate a stoichiometry for glucose oxidase using membranes prepared from the glucose-grown P. fluorescens parent strain it was necessary to substitute 2-deoxyglucose for glucose so the membrane preparation could not further oxidize the glucose oxidation product, gluconate, to 2-ketogluconate. Since 2-deoxyglucose lacks a free hydroxyl group at C_2, the oxidation product would be 2-deoxygluconate, a non-oxidizable substrate for gluconate oxidase. The gluconate oxidase stoichiometry was easier to estimate directly since 2-ketogluconate is reported to be a terminal product of glucose

oxidation in membrane preparations of these organisms (W. A. Wood, 1955).

Results from these experiments are presented in Figure 12. These data show that the expected stoichiometries of 0.5 moles O_2 consumed for each mole of glucose or gluconate oxidized are in good agreement with the experimentally obtained values of 0.53 moles O_2 consumed per mole 2-deoxyglucose and 0.51 moles O_2 consumed per mole gluconate. It should be noted that these results do not explain the path of electron flow to oxygen in these oxidase activities.

A tacit assumption in the above experiment, where glucose oxidase was estimated using 2-deoxyglucose, was that glucose oxidase does specifically oxidize 2-deoxyglucose in a manner comparable to glucose. The rate of O_2 consumption by these membrane preparations in the presence of different potential aldohexose electron donors was tested and the results are given in Table 3. The data show that 2-deoxyglucose is oxidized at about 87% the rate of glucose oxidation by these membrane preparations. It has not been rigorously established that 2-deoxyglucose is oxidized by the same enzyme that oxidizes glucose; this question, however, cannot be resolved until glucose oxidase (glucose dehydrogenase) is purified from these membrane preparations and such purification was beyond the scope of the present study.

Evidence Against Membrane Vesicle Formation in These Membrane Preparations

Estimation of apparent saturation kinetics for these membrane oxidase activities could be complicated by membrane diffusion

Figure 12

Estimation of glucose and gluconate oxidase stoichiometries. Glucose oxidase stoichiometry was determined with membranes prepared from stationary phase, 0.1 M glucose-grown (Method B) parent strain cells. Gluconate oxidase stoichiometry was determined with membrane material obtained from stationary phase, 0.1 M gluconate-grown (Method B) gox-7 cells. Assay contents are indicated in the legend to Table 1. The respective assays were initiated by addition of substrate and total O_2 consumed (corrected for electrode O_2 consumption at ca. 1.2 nmoles O_2 consumed per min) was recorded; O_2 consumption was complete after 10 min in all of these experiments.

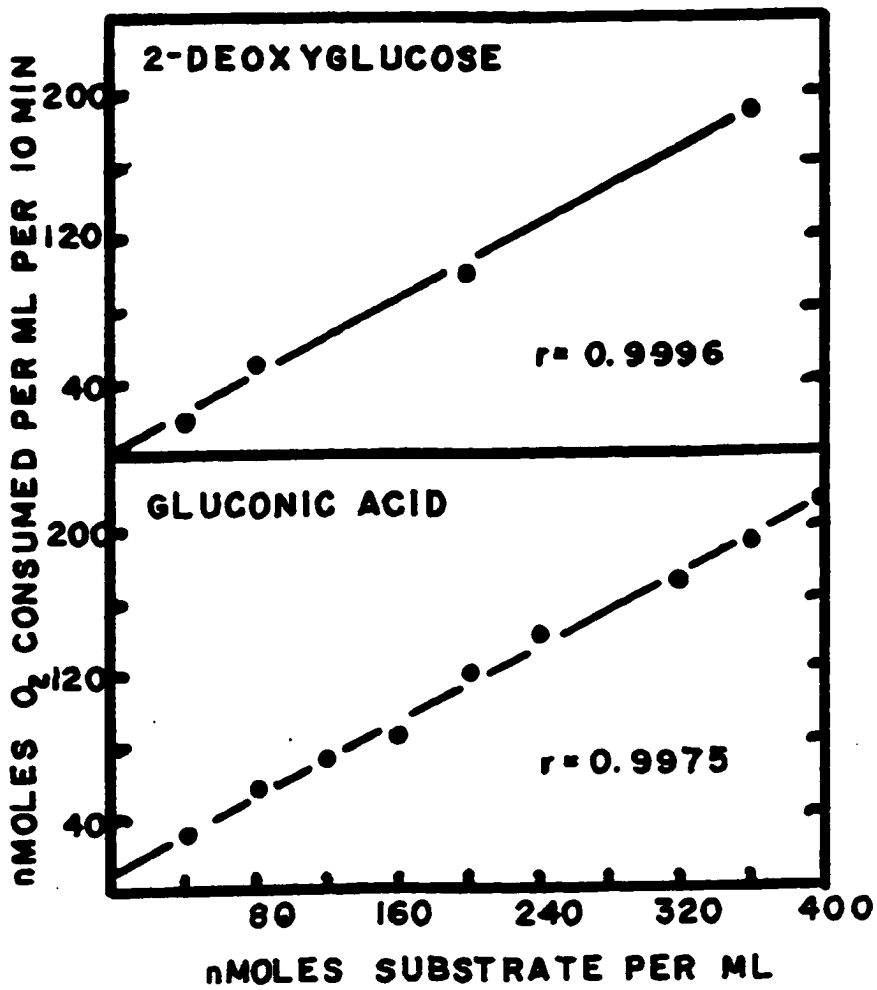


Table 3

Membrane Associated Hexose Oxidase Activity in P. fluorescens, Parent Strain [a]

Oxidase substrate	Oxidase activity[b]	Hexose oxidase / Glucose oxidase
Glucose	306.6	1.00
Galactose	279.4	0.91
Mannose	228.3	0.74
2-Deoxyglucose	265.8	0.87

[a]Bacteria were grown (Method B) in 0.1 M glucose and harvested in stationary phase. Membranes were prepared as described in Materials and Methods.

[b]The reaction vessel contained 30 mM potassium phosphate buffer (pH 6.7), 2 mM $MgSO_4$, 3.39 mg membrane protein per ml and 50 mM hexose substrate. Activity expressed as nmoles O_2 consumed per min per mg membrane protein.

barriers if these membrane fragments aggregate to form closed intact membrane vesicles. This possibility is a well recognized condition that must be taken into consideration when measuring bacterial membrane activities and has been thoroughly discussed by F. M. Harold (1972) and S. Razin (1970).

We found, during early preliminary experiments in this study, that addition of magesium ions to buffer systems used in preparation and storage of these P. fluorescens membrane fragments was essential to retain membrane oxidase activities. Presumably, this magnesium requirement is due to stabilizing these membrane oxidases as described previously for membranes of Bacillus megaterium KM (Eisenberg et al., 1970).

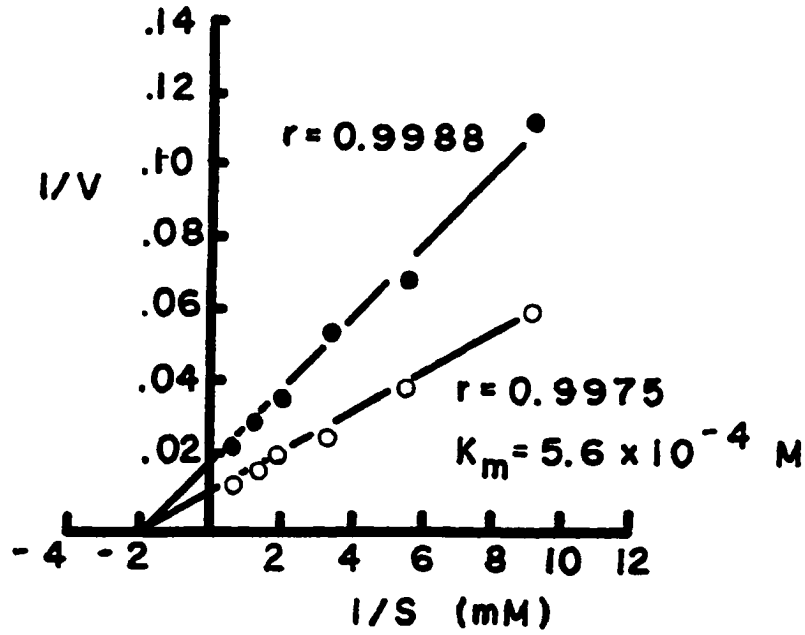
If the membrane fragments used in this study do aggregate to yield closed vesicles due to magnesium ions being present, we would expect to see a difference in apparent K_m values for glucose and L-malate oxidase activities between membranes obtained in the presence and absence of magnesium ions. Results from experiments designed to indirectly test for the presence of closed vesicles are given in Figure 13. These results show that the apparent K_m values for both glucose and L-malate oxidase are the same for membranes obtained in the presence of magnesium ions and membranes resuspended and further sonicated in the absence of added magnesium ions.

Direct examination of the standard membrane preparations employed in this study with an electron microscope (Siemens Elmiskop 1A) using phosphotungstic acid staining failed to reveal any vesicle structures (data not shown) although small (< 0.1 μmeter) membrane

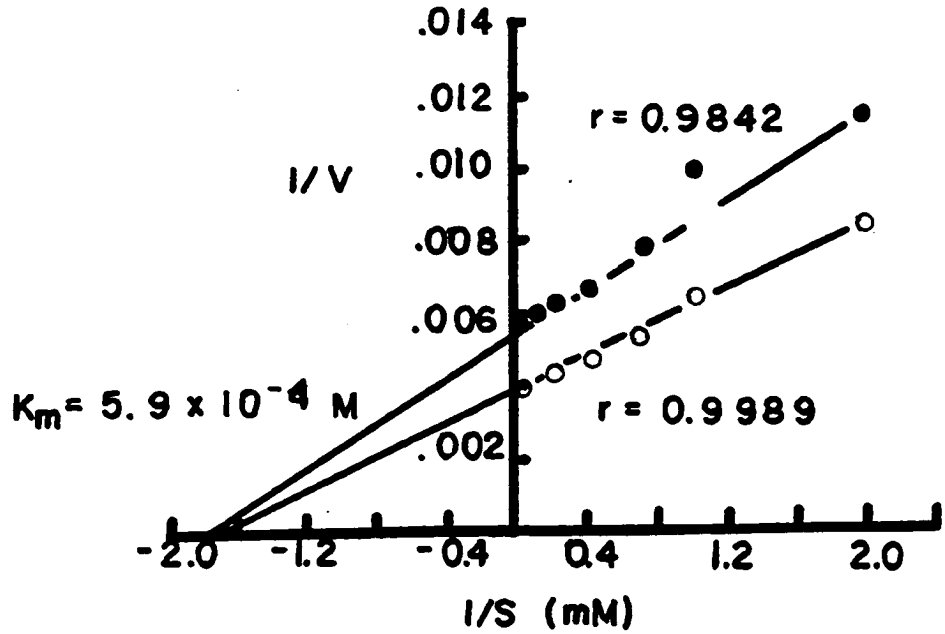
Figure 13

Kinetic evidence against membrane vesicles as diffusion barriers in these membrane preparations. Glucose-grown (0.1 M, Method A) P. fluorescens, parent strain, membranes were used. The membranes were prepared as described in Materials and Methods. Following the final ultracentrifugation wash, the membrane pellets were suspended in the appropriate oxidase assay buffer with (open circles) or without (closed circles) 2 mM Mg^{+2}. The membranes without 2 mM Mg^{+2} were sonicated again as described in the Materials and Methods to insure complete disruption of membrane particle aggregates or vesicles which may have been formed. The glucose and malate oxidase assays were performed as described in the legends to Figures 2 and 6, respectively. The correlation coefficient (r) is included to indicate the experimental fit of these data to a straight line equation as determined by linear regression.

MALATE OXIDASE



GLUCOSE OXIDASE



fragments were observed. We interpret these indirect and direct results as good evidence against significant membrane vesicularization in the membrane preparations used in this study.

Induction Studies

Entner and Stanier (1951) reported that the glucose oxidase enzyme in P. putida (referred to as P. fluorescens by these investigators) was constitutive with the gluconate oxidase enzyme being inducible. Quay et al. (1972) demonstrated both glucose and gluconate oxidase enzymes in P. fluorescens were inducible. The pH optimum and substrate saturation assay conditions described above should, therefore, provide a more reasonable basis for establishing the regulation of these membrane associated glucose and gluconate oxidase activities. 6-Phosphogluconate dehydrase, the rate limiting enzyme of the Entner-Doudoroff pathway, was also included since it has been shown to be gluconate inducible in P. fluorescens (S. C. Quay et al., 1972). This would be of value experimentally since gluconate, the product of glucose oxidase, should show induction of the enzyme if gluconate was produced from other oxidizable energy sources. Table 4 shows the results of the induction studies on the membrane oxidases in P. fluorescens. The data indicates that glucose oxidase enzyme was inducible since the highest specific activity for this membrane enzyme was from glucose grown cells. The units of activity in membranes prepared from cells grown on the other carbon and energy sources indicate a complex mechanism for the regulation of the particulate glucose oxidase enzyme. The glucose dehydrogenase

Table 4

Membrane Associated Oxidase Enzyme Activities
from P. fluorescens Grown on Various
Single Carbon and Energy Sources

[a]Bacteria were grown (Method A) and membranes prepared as described in the Materials and Methods. The carbon and energy sources were at a final concentration of 0.1 Molar except the Casamino Acids, which was 0.25%. The glucose dehydrogenase, gluconate dehydrogenase, and cytoplasmic 6-phosphogluconate dehydrase (Cyt. 6-PG dehydrase) enzymes were assayed as described in the Materials and Methods. The glucose oxidase (Figure 2), gluconate oxidase (Figure 4), malate oxidase (Figure 6), D(-) lactate oxidase (Figure 8), and L(+) lactate oxidase (Figure 9) enzymes were assayed as described in the legends to the figures indicated.

[b]Oxidase activity expressed as nmoles O_2 consumed per min per mg membrane protein; dehydrogenase activity expressed as nmoles DCPIP reduced per min per mg membrane protein at pH 7.3; 6-PG dehydrase activity expressed as nmoles pyruvate formed per min per mg protein at pH 7.65.

Carbon and energy source[a]	Specific activity[b]							
	Glucose oxidase	Glucose dehydro-genase	Gluconate oxidase	Gluconate dehydro-genase	Malate oxidase	D(-) lactate oxidase	L(+) lactate oxidase	Cyt. 6-PG dehydrase
Glucose	170.6	6.27	9.24	2.82	78	9.11	4.01	128.9
Gluconate	70.4	6.09	25.1	6.78	98.4	18.6	5.7	250.5
Glycerol	98.8	7.36	10.5	1.71	62.5	7.8	7.0	203.0
Malate	15.5	< 0.2	4.2	< 0.2	100.3	19.3	5.7	< 8.3
Succinate	17.1	< 0.2	3.7	< 0.2	90.7	11.8	8.2	< 8.1
D,L-lactate	39.4	2.3	2.7	< 0.2	98.0	92.5	96.0	18.0
Asparagine	27.8	2.0	3.6	< 0.2	80.9	11.88	8.4	11.3
Casamino acids	87.9	1.76	1.38	< 0.2	88.0	5.0	6.8	< 8.1

activity from glucose, gluconate, and glycerol grown cell membranes indicated the coupled DCPIP oxidoreductase assay was not a good indicator for the glucose oxidase enzyme. It was, however, capable of exhibiting lack of glucose oxidase inducibility when cells were grown on malate or succinate.

The data also indicates the inducibility of gluconate oxidase since the highest units of activity were obtained from gluconate grown cell membranes. The high gluconate oxidase activity in glucose grown cells was reasonable since the product of glucose oxidase is gluconate. The gluconate DCPIP coupled oxidoreductase data indicates this assay can serve as an indicator for gluconate oxidase inducibility. The high units of gluconate dehydrogenase activity in glucose grown cells are consistent using the same argument advanced for the gluconate oxidase activity. The high 6-phosphogluconate dehydrase specific activity from gluconate grown cells confirms gluconate induction of this enzyme. The high specific activity of 6-phosphogluconate dehydrase obtained from glucose grown cells is in accord with the proposed scheme as described earlier.

The data for L-malate oxidase activity suggests that this oxidase is constitutive. Both the D(-) and L(+) lactate oxidase data suggests that these enzymes are inducible although the D(-) lactate oxidase is present at a higher constitutive (basal) level than the L(+) lactate oxidase.

Enzyme Levels as a Function of Culture Age

The method of growing the bacteria (Method A, see Materials and Methods) for the above induction studies was used for convenience.

The reproducibility of these induction data became questionable and it was decided to further characterize the inducibility of the glucose and gluconate oxidase enzymes as a function of cell growth. Growth curves for malate, succinate, glucose, gluconate, glycerol, and acid-hydrolysed vitamin-free casamino acids (Difco) were established for the P. fluorescens parent strain. Table 5 shows the doubling times of P. fluorescens grown in the basal medium containing the indicated carbon and energy source. The shape of the curve for glucose and glycerol grown cells showed a biphasic nature whereas the other carbon sources resulted in monophasic growth curves. Characteristic growth patterns are shown in Figures 14 and 15 for glucose and malate grown P. fluorescens, respectively.

The bacteria grew best in the complex amino-acid medium as indicated by the 73 minute doubling time. An interesting observation from these growth studies was the similarity in doubling times for gluconate grown cells and the second phase of glucose grown cells, 87 minutes and 92 minutes, respectively. From the data in Table 4 and the scheme for glucose utilization in Figure 1, the biphasic nature of the growth curve for glucose grown cells could be explained, since the product of glucose oxidase is gluconate. In an attempt to understand the basis for the biphasicity of glucose grown cells, the pH, cell concentration and concentrations of glucose, gluconate, and 2-ketogluconate in the growth medium were measured as a function of time. These results are shown in Figure 16 and demonstrate that gluconate does appear in the growth medium coincident with a faster growth rate (Figure 16, at ca. 6 hours).

Table 5

Estimated Doubling Times of P. fluorescens Grown on Various Single Carbon and Energy Sources

Growth conditions[a]	Shape of growth curve	Estimated doubling time (min)[b]
Glucose (0.1 M)	Biphasic	160.0 (92.0)
Gluconate (0.1 M)	Monophasic	87.0
Glycerol (0.1 M)	Biphasic	154.0 (120.0)
Malate (0.1 M)	Monophasic	79.0
Succinate (0.1 M)	Monophasic	99.7
Casamino Acids (0.25%)	Monophasic	72.5

[a]Bacteria were grown using Method B as described in the Materials and Methods. Growth was measured turbidimetrically by measuring the optical density at 600 nm with water as a blank. Optical density was estimated by diluting, where necessary, bacterial cultures to an absorbance of 0.01 to 0.30 with phosphate buffer. Values in parenthesis indicate the concentrations of growth substrates used.

[b]Doubling times were calculated from exponentially growing cultures using the equation: $G_t = \frac{\log b/a}{0.301\ t}$ where G_t = number of generations per hour, b = O.D. at t_2, a = O.D. at t_1, and $t = t_2 - t_1$. Where biphasic growth is indicated the value in parenthesis represents the second growth phase estimated doubling time.

Figure 14

Growth curve for P. fluorescens parent strain grown on 0.1 M glucose. Bacteria were grown and doubling times calculated as described in the legend to Table 5. An absorbance of 1.0 at 600 nm was equivalent to 299 µg dry weight per ml bacterial cell suspension. The arrow indicates the cell concentration where bacteria were harvested for mid-log phase enzyme activities.

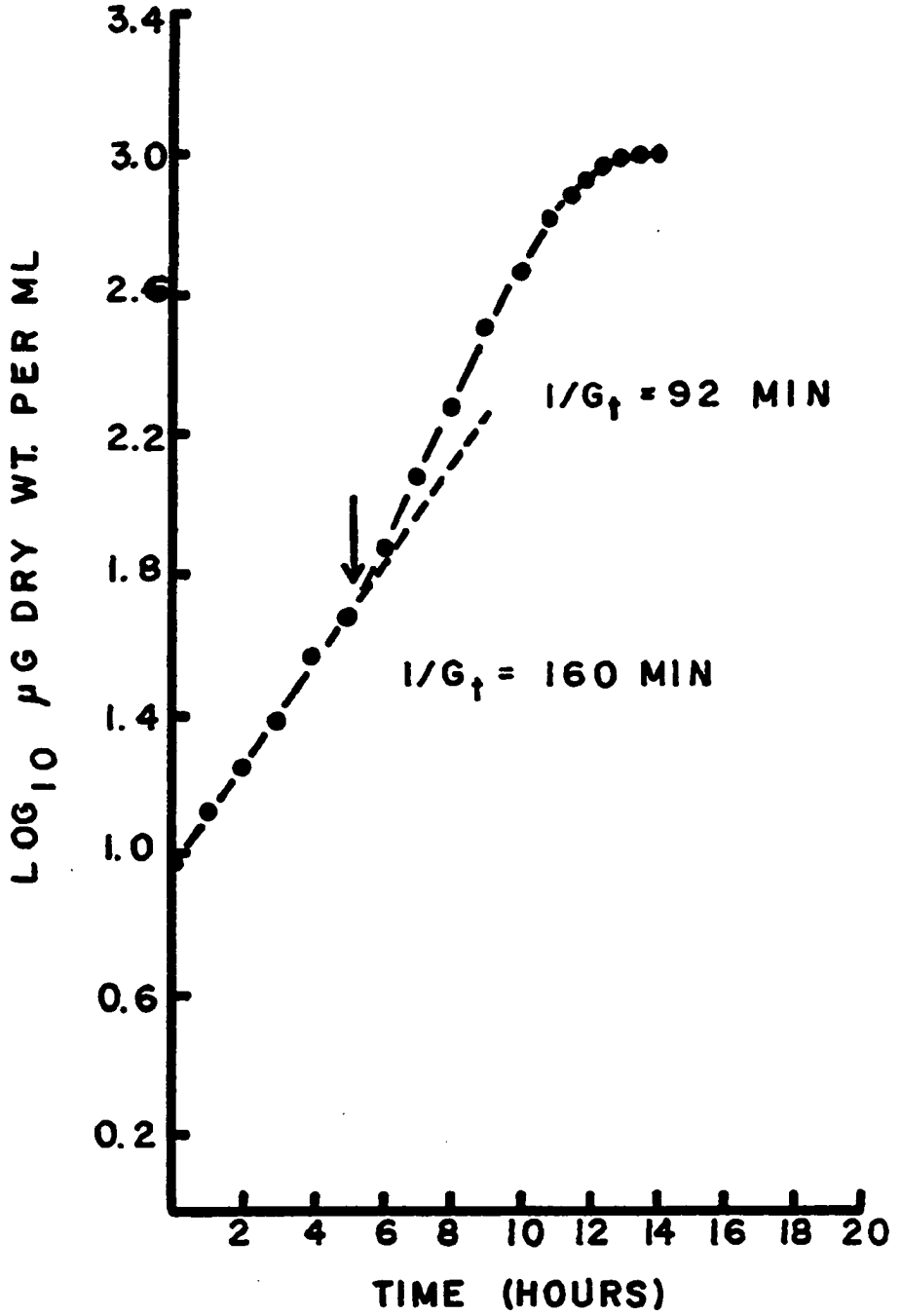


Figure 15

Growth curve for P. fluorescens parent strain grown on 0.1 M L-malate. Bacteria were grown and doubling times calculated as described in the legend to Table 5. An absorbance of 1.0 at 600 nm was equivalent to 299 µg dry weight per ml.

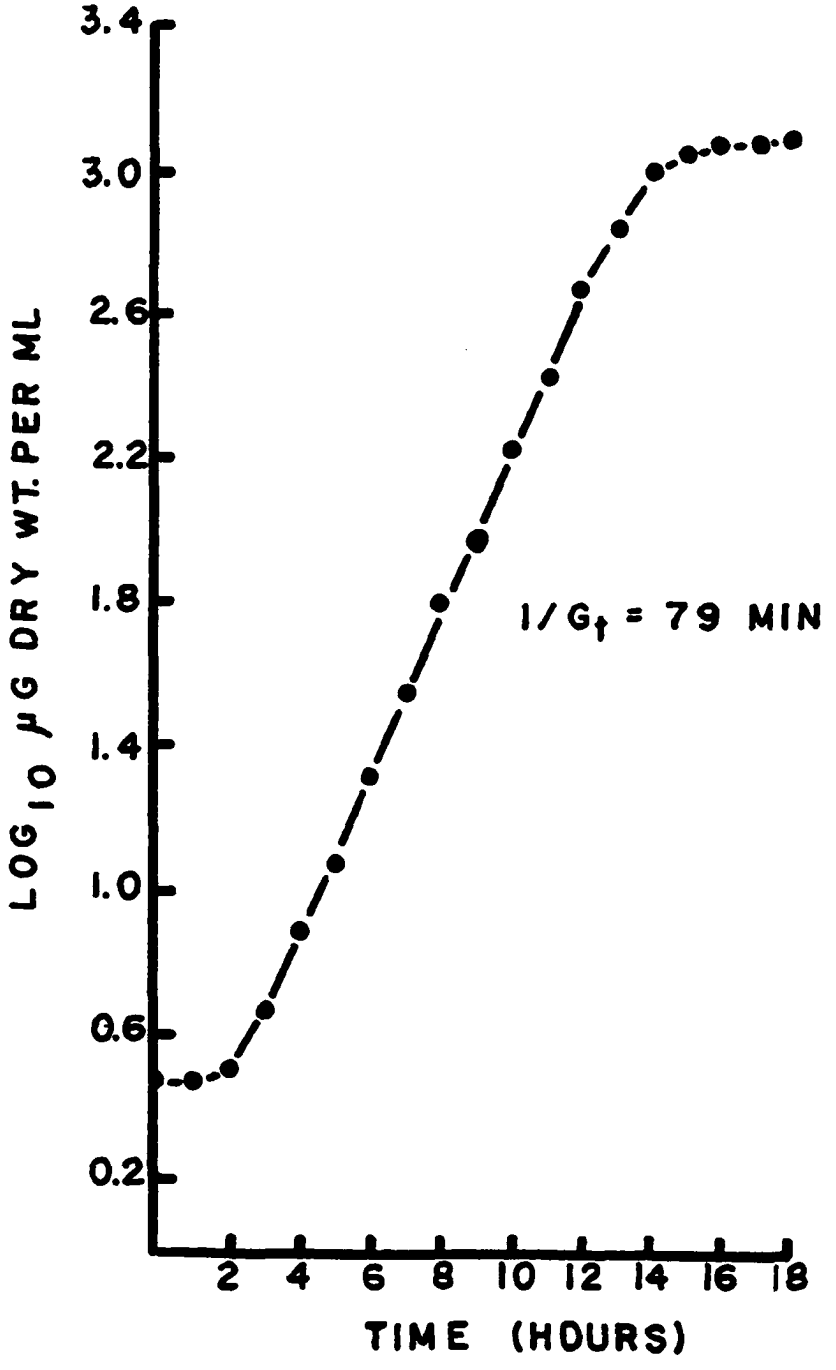
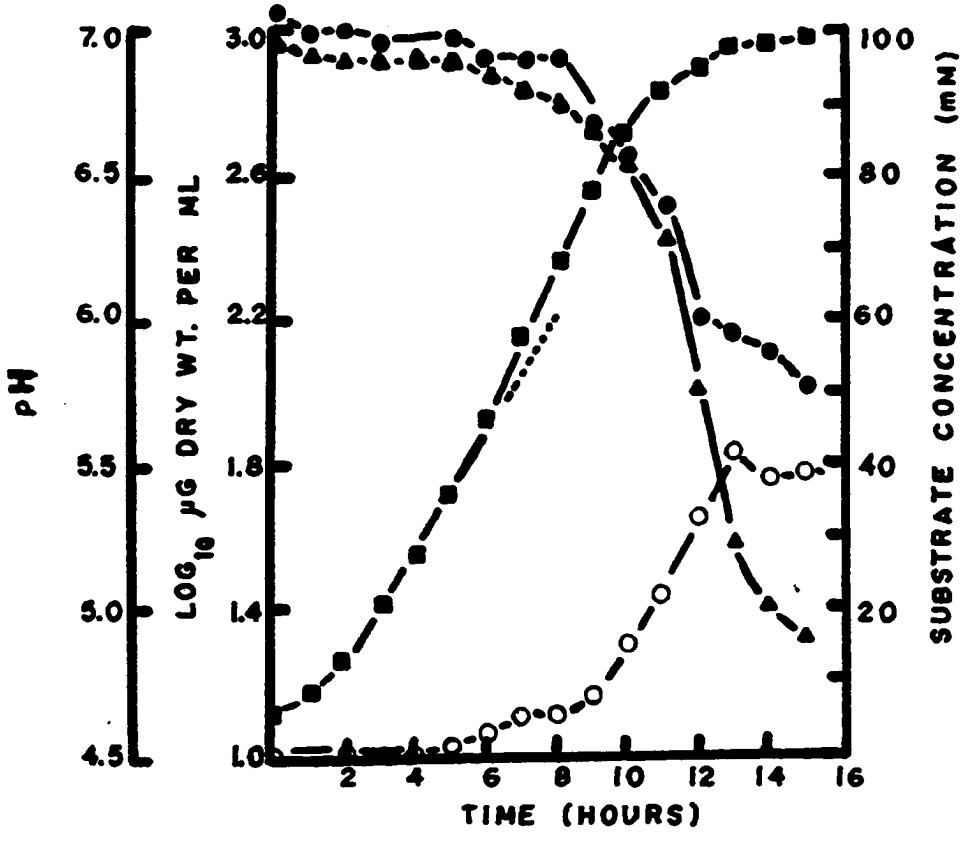


Figure 16

Determination of cell concentration, pH, and glucose, gluconate, and 2-ketogluconate concentrations as a function of growth. Pseudomonas fluorescens was grown (0.1 M glucose) using Method B and determinations were performed as described in the Materials and Methods. Symbols: ● —— ●, Glucose; O — O, gluconate; ▲ —— ▲, pH; and ■ —— ■, cell concentration. 2-Ketogluconate was not detected.



To determine the effect of culture age on glucose, gluconate, and malate oxidase enzyme activities and cytochrome composition in the P. fluorescens parent strain, bacteria were grown in the basal salts medium containing various single carbon and energy sources and membranes were prepared from cells harvested at mid-log and stationary phase. Results obtained from this survey are shown in Tables 6 and 7. The ratio of specific activities of stationary phase cell membranes to mid-log phase cell membranes for glucose oxidase was always greater than unity. The gluconate and malate oxidase activities always decreased with culture age except when these enzymes were assayed from cells grown on the substrates for these enzymes, and then the ratio of mid-log to stationary cell enzyme activities was approximately unity.

The cytochrome content also changed with culture age and carbon source. The cytochrome b content remained essentially constant during growth. The cytochrome c content in membranes from cells grown on substrates which resulted in the characteristic fluorescent green pigment, fluorescein, always had a lower stationary cell membrane to mid-log cell membrane cytochrome c content ratio. Malate grown cell membranes had a 6-fold increase in cytochrome c production in stationary phase cell membranes and did not produce any green pigment. A CO-binding cytochrome pigment (cytochrome o) was also observed in these preparations and its content also increased with culture age.

It is apparent from the data that the regulation of glucose and gluconate oxidase activities and the cytochrome content are dependent on both culture age and growth substrate.

Table 6

Effect of Culture Age and Sole Carbon and Energy Sources on Glucose, Gluconate, and Malate Oxidase Activities in P. fluorescens Parent Strain[a]

[a] Bacteria were grown (Method B) and membranes prepared from mid-log and stationary phase cells as described in the Materials and Methods.

[b] Growth on glucose or glycerol as sole sources of carbon resulted in biphasic growth patterns. The arrow in Figure 14 indicates the mid-log cell concentration for glucose grown P. fluorescens. The mid-log cell concentration for glycerol grown P. fluorescens was 69.34 µg dry wt/ml.

[c] The glucose (Figure 2), gluconate (Figure 4), and malate (Figure 6) oxidase enzymes were assayed as described in the legends of the figures indicated. The glucose, gluconate, and malate oxidases were assayed at substrate concentrations of 50 mM, 50 mM, and 30 mM, respectively.

[d] Activity expressed as nmoles O_2 consumed per min per mg membrane protein.

Growth substrate [Conc.]	Oxidase enzyme[c]	Activity[d] Mid-log	Stationary	Stationary / Mid-log
Glucose[b] [0.1 M]	Glucose	99.3	370.3	3.73
	Gluconate	51.8	16.6	0.32
	Malate	148.3	78.4	0.53
Gluconate [0.1 M]	Glucose	84.4	234.4	2.78
	Gluconate	36.5	33.5	0.92
	Malate	132.6	98.8	0.75
Glycerol[b] [0.1 M]	Glucose	24.3	180.0	7.41
	Gluconate	21.5	8.6	0.40
	Malate	75.2	52.0	0.69
Succinate [0.1 M]	Glucose	10.5	36.4	3.47
	Gluconate	4.0	1.3	0.33
	Malate	112.4	74.6	0.66
Malate [0.1 M]	Glucose	0.87	18.9	21.72
	Gluconate	9.71	5.7	0.59
	Malate	102.1	99.9	0.98
Casamino acids [0.25%]	Glucose	36.4	186.6	5.13
	Gluconate	8.7	4.8	0.55
	Malate	123.8	74.0	0.60

Table 7

Effect of Culture Age and Sole Carbon and Energy Sources on the Amount and Composition of P. fluorescens, Parent Strain, Cytochromes[a]

[a]Bacteria were grown (Method B) and membranes prepared from mid-log and stationary phase cells as described in the Materials and Methods.

[b]Cytochromes were detected in particles from dithionite reduced minus oxidized difference spectra recorded at room temperature (ca. 23°C) in 10 mm (light path) cuvettes as described in the Materials and Methods. Particles were obtained from pooled glucose and gluconate oxidase enzyme preparations.

[c]Amount of cytochromes present was calculated from millimolar extinction coefficients and wavelength pairs as described in the Materials and Methods and are expressed as nmoles cytochrome component per mg membrane protein. Particles were obtained from pooled glucose and gluconate oxidase enzyme preparations.

Growth substrate [Conc.]	Cytochrome component	Amount		
		Mid-log	Stationary	Stationary / Mid-log
Glucose [0.1 M]	b	0.184	0.205	1.11
	c	0.159	0.104	0.65
	o	0.071	0.120	1.69
Gluconate [0.1 M]	b	0.242	0.326	1.35
	c	0.208	0.286	1.38
	o	0.054	0.074	1.37
Glycerol [0.1 M]	b	0.181	0.255	1.41
	c	0.273	0.255	0.93
	o	0.060	0.187	3.12
Succinate [0.1 M]	b	0.220	0.212	0.96
	c	0.160	0.070	0.44
	o	0.059	Not detected	----
Malate [0.1 M]	b	0.163	0.352	2.16
	c	0.057	0.390	6.84
	o	0.042	0.054	1.29
Casamino acids [0.25%]	b	0.114	0.190	1.67
	c	0.090	0.163	1.81
	o	0.038	0.103	2.71

The results in Table 4 indicated the presence of oxidase enzymes capable of oxidizing aldohexoses. It was decided to test enzymatically whether these aldohexoses were being oxidized by the glucose oxidase enzyme or specific galactose and mannose oxidases. Also, since the specific activity for glucose oxidase from glucose-grown P. fluorescens cell membranes increased with culture age (see Table 6), it was postulated that if the ratio of glucose oxidase to galactose or mannose oxidase activity from mid-log and stationary phase cell membranes was constant, then the glucose oxidase enzyme could be assumed to be an aldohexose oxidase. The results of these experiments (Table 8) indicated the glucose oxidase enzyme in P. fluorescens may in fact be a non-specific aldohexose oxidase. A more definite conclusion could be reached, however, from purifying the glucose dehydrogenase (oxidase) enzyme. Enzyme purification studies were not attempted in this study.

Cytochromes in Particulate Fractions from P. fluorescens

Characteristic dithionite reduced minus oxidized difference spectra of cytochromes from membrane particles obtained from P. fluorescens grown on L-malate, glucose, and Casamino Acids are presented in Figures 17, 18, and 19, respectively. The reduced b-type cytochromes exhibited a peak in the α-region at 560 nm - 562 nm. Reduced cytochrome c showed a peak in the α-region at 551 nm - 553 nm. There was no indication of cytochromes a or d (a_2) in these reduced minus oxidized preparations since no peaks were observed in the 570 nm - 650 nm region or a shoulder in the Soret region.

Table 8

Evidence Suggesting Glucose Oxidase Maybe an Aldohexose Oxidase

Oxidase substrate[a]	Hexose oxidase activity[b]				
	Mid-log	Memb. oxidase / Glucose oxid.	Stationary	Memb. oxidase / Glucose oxid.	Stationary / Mid-log
Glucose	104.2	1.00	361.1	1.00	3.47
Galactose	92.9	0.89	342.5	0.94	3.69
Mannose	81.2	0.78	280.7	0.78	3.46

[a]Bacteria were grown on glucose (0.1 M, Method B) and membranes prepared from mid-log and stationary phase cells as described in the Materials and Methods.

[b]The oxidase assay mixture contained (per ml): 30 µmoles potassium phosphate buffer, pH 6.7; 2 µmoles $MgSO_4$; membrane fraction; and 50 µmoles of the respective oxidase substrates. Activity expressed as nmoles O_2 consumed per min per mg membrane protein.

Figure 17

Dithionite reduced minus oxidized difference spectrum from stationary phase P. fluorescens malate grown (0.1 M) membrane material. Bacteria were grown using Method B, membranes prepared, and difference spectrum performed as described in Materials and Methods. Pooled glucose oxidase and gluconate oxidase membrane material was used to obtain reduced minus oxidized difference spectrum of the particulate fraction. The lower trace represents an oxidized minus oxidized trace. The light path was 10 mm and membrane protein concentration was 1.82 mg per ml. The discontinuity at approximately 600 nm was due to filter interference of the spectrophotometer.

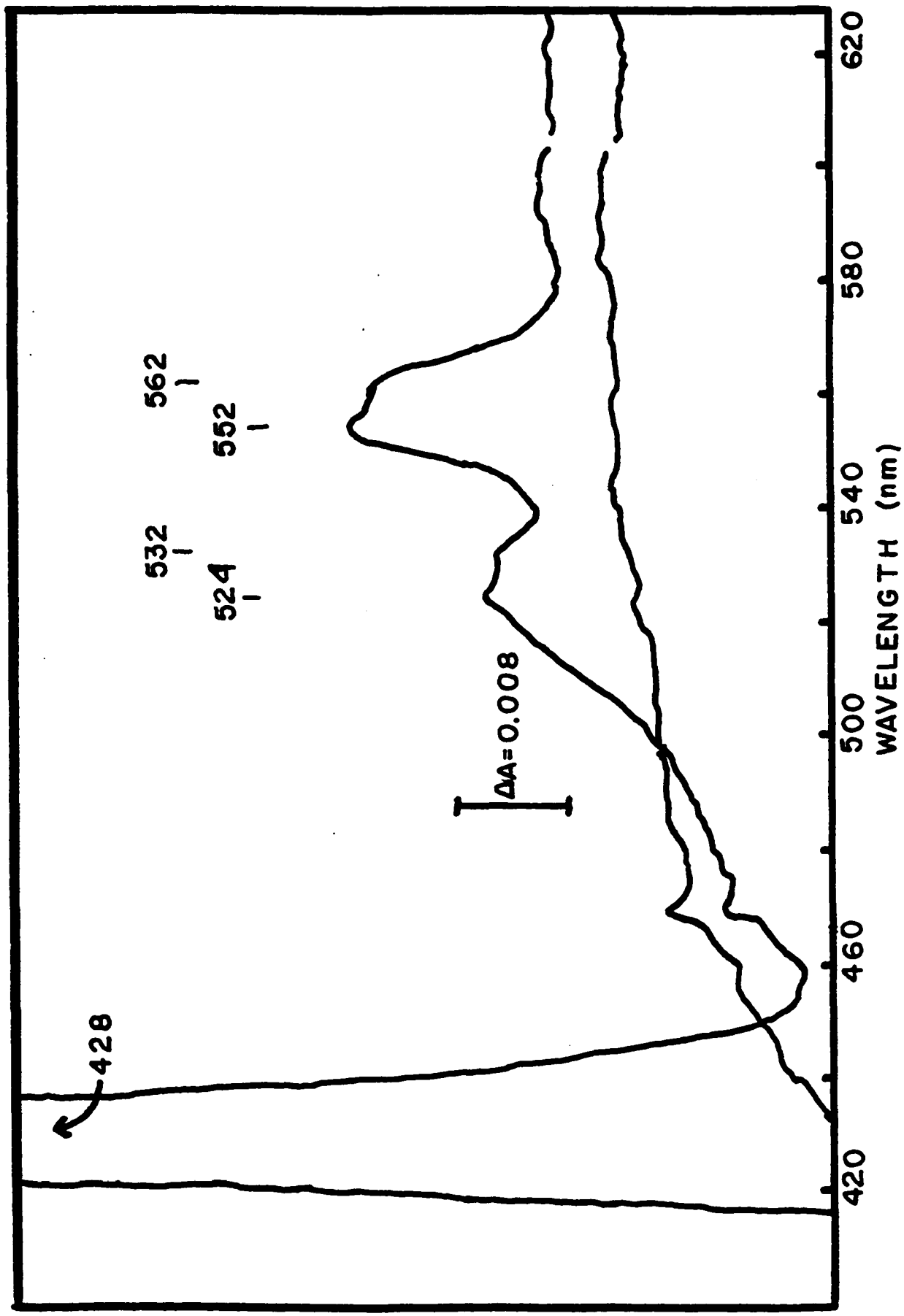


Figure 18

Dithionite reduced minus oxidized difference spectrum from stationary phase P. fluorescens glucose-grown (0.1 M) membrane material. Bacteria were grown using Method B, membranes prepared, and difference spectra performed as described in the Materials and Methods. Pooled glucose oxidase and gluconate oxidase membrane material was used to obtain the reduced minus oxidized difference spectrum of the particulate fractions. The relatively straight line represents an oxidized minus oxidized trace. The light path was 10 mm and membrane protein concentration was 2.83 mg per ml. The discontinuity at approximately 600 nm was due to filter interference of the spectrophotometer.

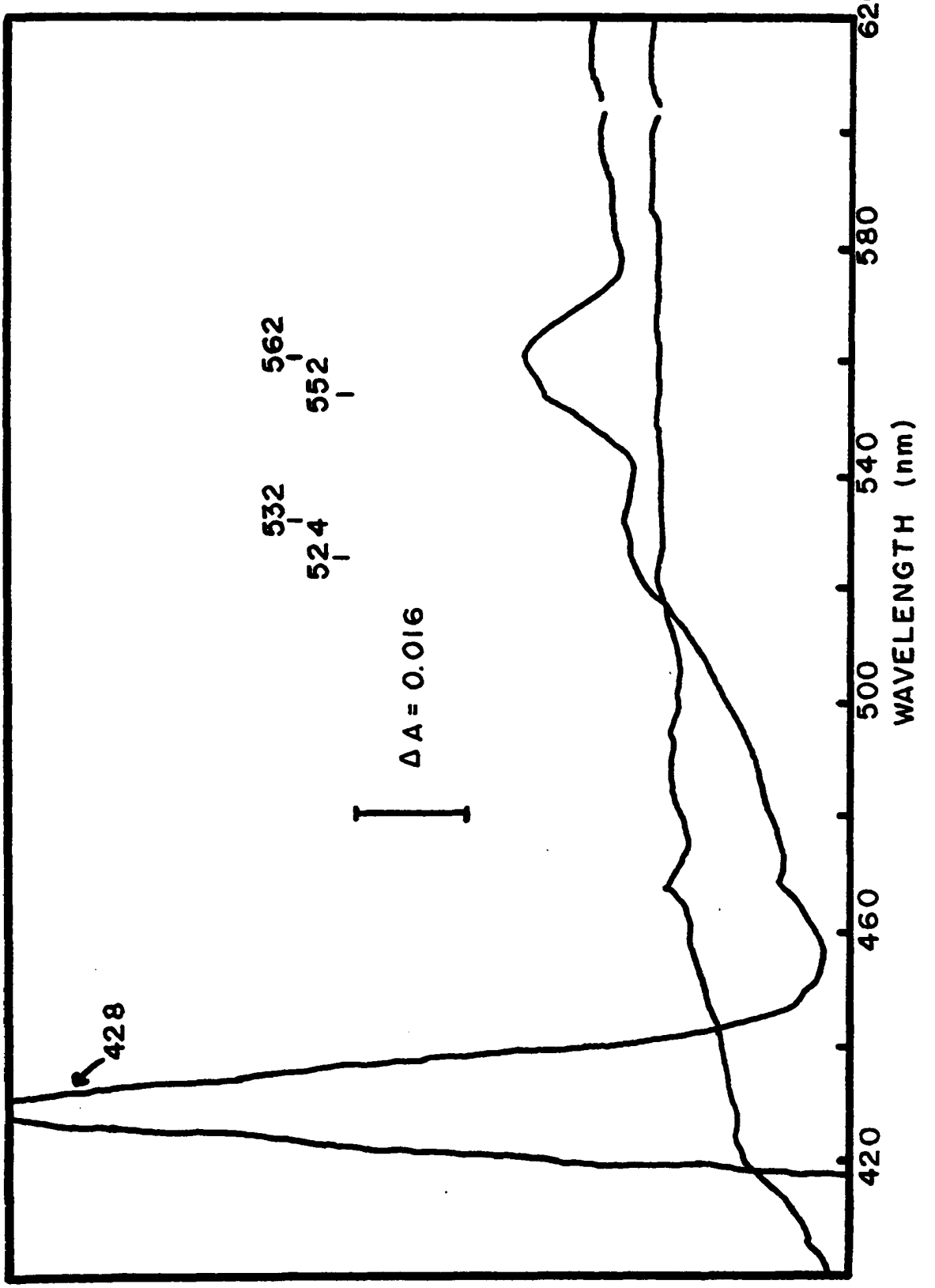
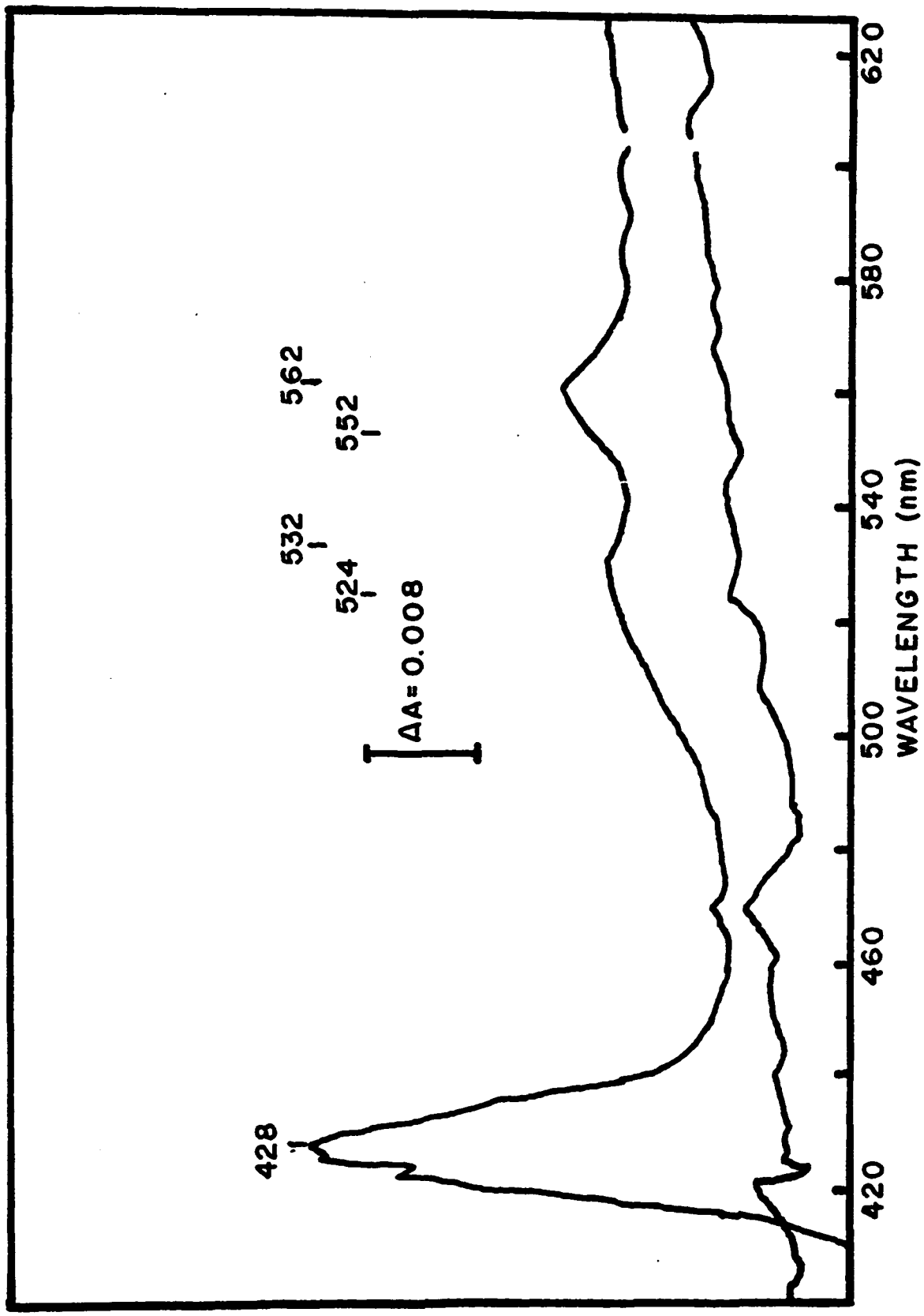


Figure 19

Dithionite reduced minus oxidized difference spectrum from stationary phase P. fluorescens Casamino Acids (0.25%) grown membrane material. Bacteria were grown using Method B, membranes prepared, and difference spectra performed as described in the Materials and Methods. Pooled glucose oxidase and gluconate oxidase membrane material was used to obtain the reduced minus oxidized difference spectrum of the particulate fraction. The lower line represents an oxidized minus oxidized trace. The light path was 10 mm and membrane protein concentration was 0.58 mg per ml. The discontinuity at approximately 600 nm was due to filter interference of the spectrophotometer.



The values reported for cytochrome composition as a function of culture age (Table 7) were obtained utilizing the wavelength pairs and millimolar extinction coefficients described in the Materials and Methods. Difference spectra of particles from L-malate and glucose grown P. fluorescens (Figures 17 and 18) definitely show the existence of a c-type cytochrome. Difference spectra of Casamino Acids grown P. fluorescens membrane particles, however, do not exhibit the characteristic peak or shoulder for cytochrome c at 551 nm - 553 nm. A value for cytochrome c from these membrane particles was reported, however, from wavelength pairs and millimolar extinction coefficients.

A CO-binding cytochrome, observed in dithionite reduced plus CO minus reduced difference spectra from L-malate, glucose and Casamino Acids grown P. fluorescens membrane particles, exhibited a trough in the 430 nm to 435 nm region and a peak in the 417 nm - 419 nm region. These results are shown in Figure 20.

From the results showing the dependence of oxidase activity and cytochrome content on both different growth substrates and phases of growth (Tables 6 and 7), it was deemed necessary to determine the extent of cytochrome reduction using physiological reductants. Cytochromes in the membrane particles from stationary phase gluconate-grown P. fluorescens were reduced by the physiological reductants glucose, gluconate, and L-malate. The glucose, gluconate and L-malate reduced minus oxidized difference spectra are indicated in Figures 21, 22, and 23, respectively. The three physiological reductants were also capable of reducing a CO-binding cytochrome o (Figure 24).

Figure 20

Dithionite reduced plus CO minus reduced difference spectra for P. fluorescens Casamino Acids (0.25%) grown (A), L-malate (0.1 M) grown (B), and glucose (0.1 M) grown (C) stationary phase cell membrane material. Bacteria were grown using Method B, membranes prepared, and difference spectra performed as described in the Materials and Methods. Pooled glucose oxidase and gluconate oxidase membrane material was used to obtain the reduced plus CO minus reduced difference spectra. The lines without the peak and trough 419 nm and 433 nm, respectively, represent the reduced minus reduced traces. The light path was 10 mm and the membrane protein concentrations of A, B, and C were as indicated in the legends to Figures 17, 18 and 19 respectively.

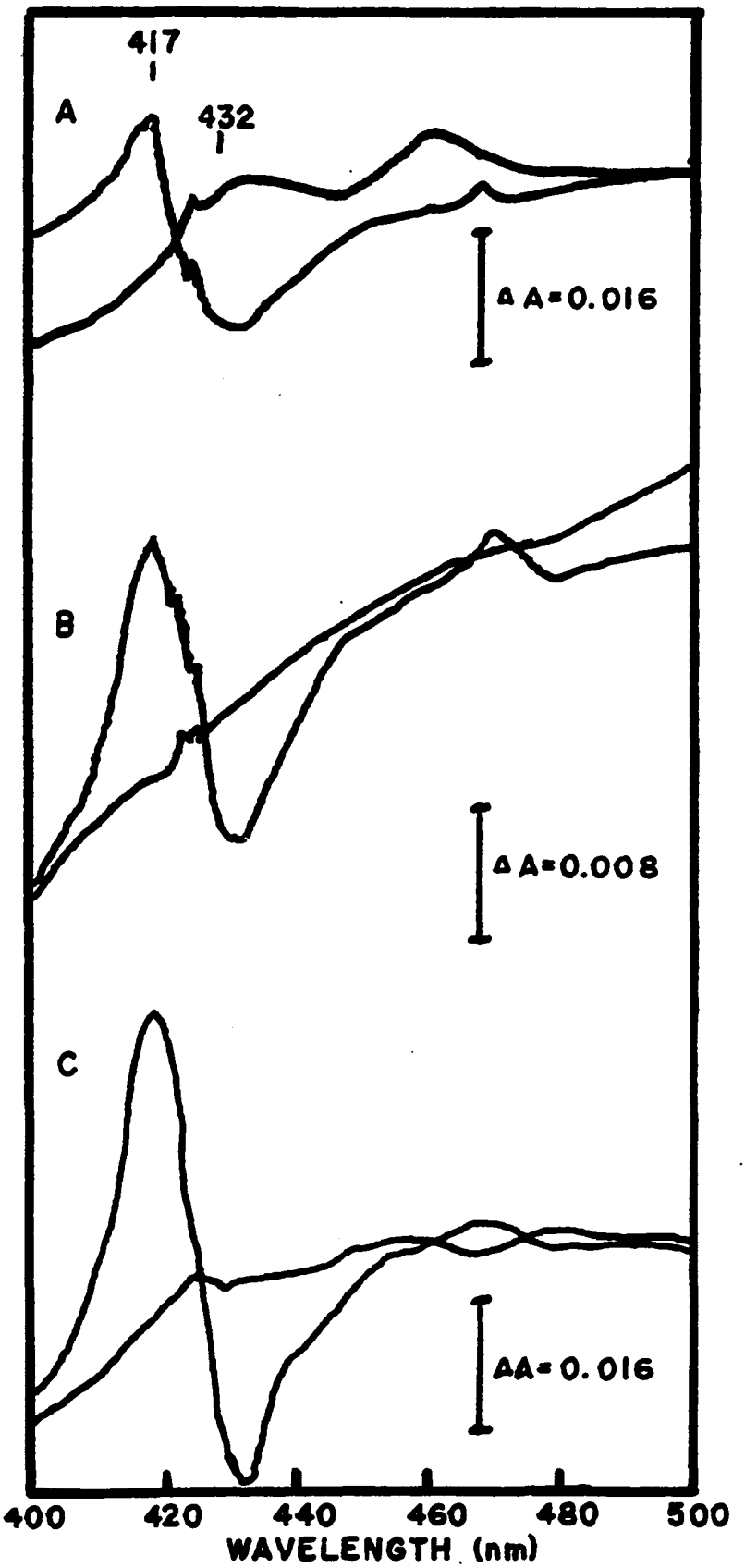


Figure 21

Glucose reduced minus oxidized difference spectrum from stationary phase P. fluorescens gluconate-grown (0.1 M) membrane material. Bacteria were grown using Method B, membranes prepared, and difference spectrum recorded as described in the Materials and Methods. Particles were reduced with 0.1 M glucose for 13 min. The light path was 10 mm and protein concentrations was 2.03 mg per ml. The discontinuity at approximately 600 nm was due to filter interference of the spectrophotometer.

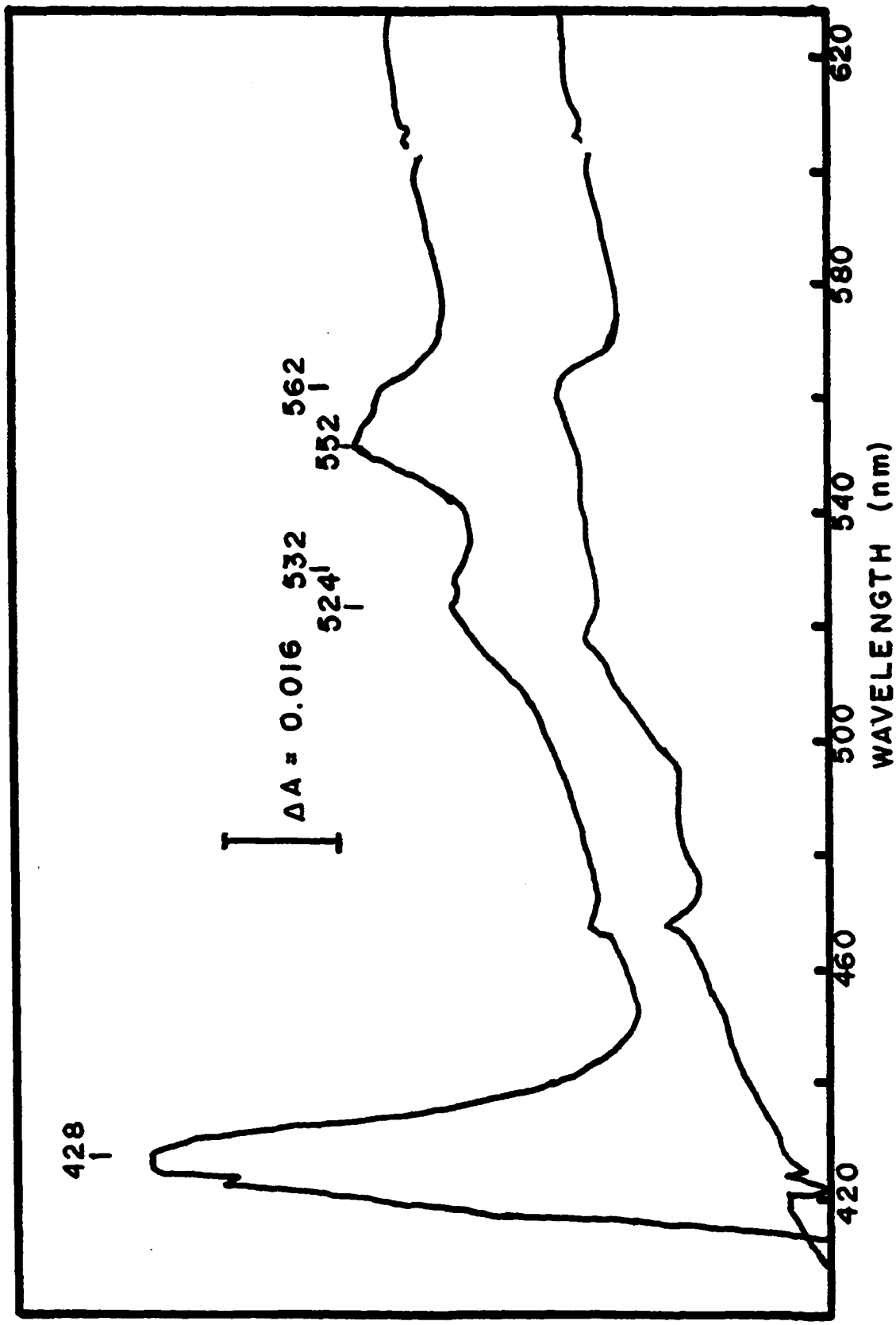


Figure 22

Gluconate reduced minus oxidized difference spectrum from stationary phase P. fluorescens gluconate-grown (0.1 M) membrane material. Bacteria were grown using Method B, membranes prepared, and difference spectrum recorded as described in the Materials and Methods. Particles were reduced with 0.1 M potassium gluconate for 13 min. The light path was 10 mm and protein concentration was 1.72 mg per ml. The discontinuity at approximately 600 nm was due to filter interference of the spectrophotometer.

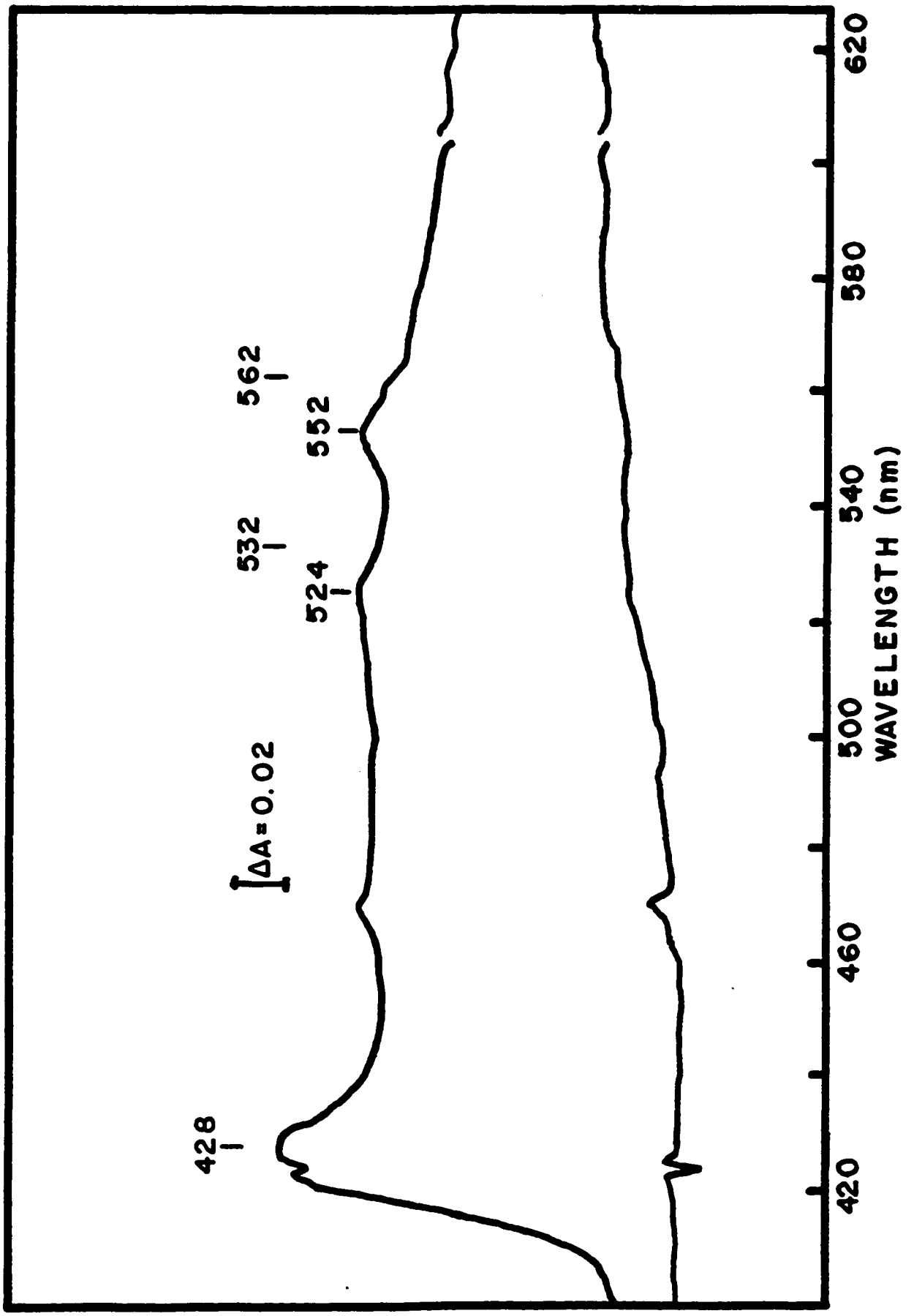


Figure 23

Malate reduced minus oxidized difference spectrum from stationary phase P. fluorescens, gluconate-grown (0.1 M) membrane material. Bacteria were grown using Method B, membranes prepared, and difference spectrum recorded as described in the Materials and Methods. Particles were reduced with 0.06 M L-malate for 13 min. The light path was 10 mm and protein concentration was 1.90 mg per ml. The discontinuity at approximately 600 nm was due to filter interference of the spectrophotometer.

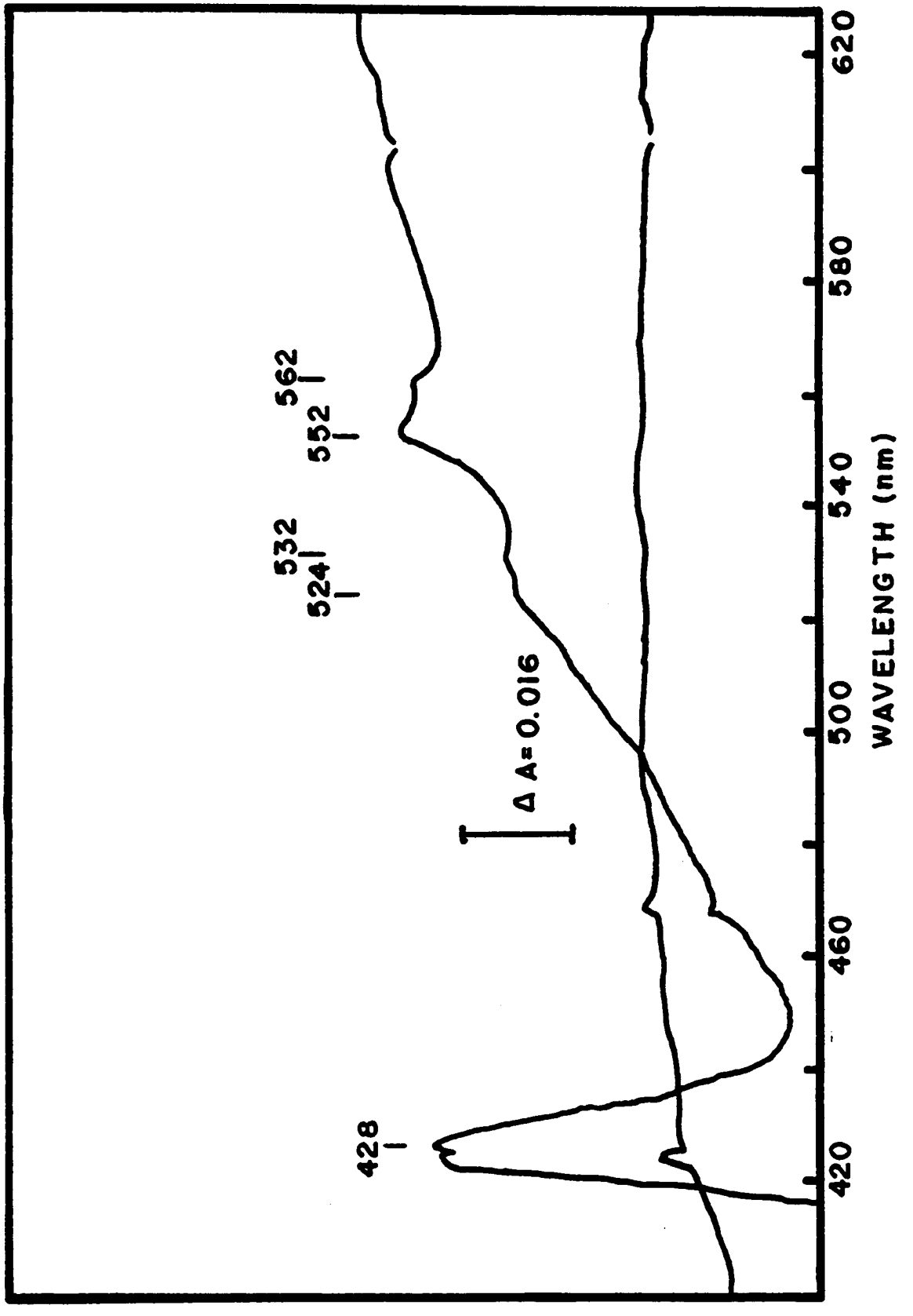


Figure 24

Substrate reduced plus CO minus reduced difference spectra from stationary phase P. flourescens gluconate-grown (0.1 M) membrane material. Bacteria were grown using Method B, membranes prepared, and difference spectra recorded as described in the Materials and Methods. Particles were reduced 13 min with 0.1 M glucose (A), 0.1 M gluconate (B), and 0.06 M L-malate (C) before spectra were recorded. The light path was 10 mm and protein concentration was: (A) 2.03 mg per ml, (B) 1.72 mg per ml, and (C) 1.90 mg per ml.

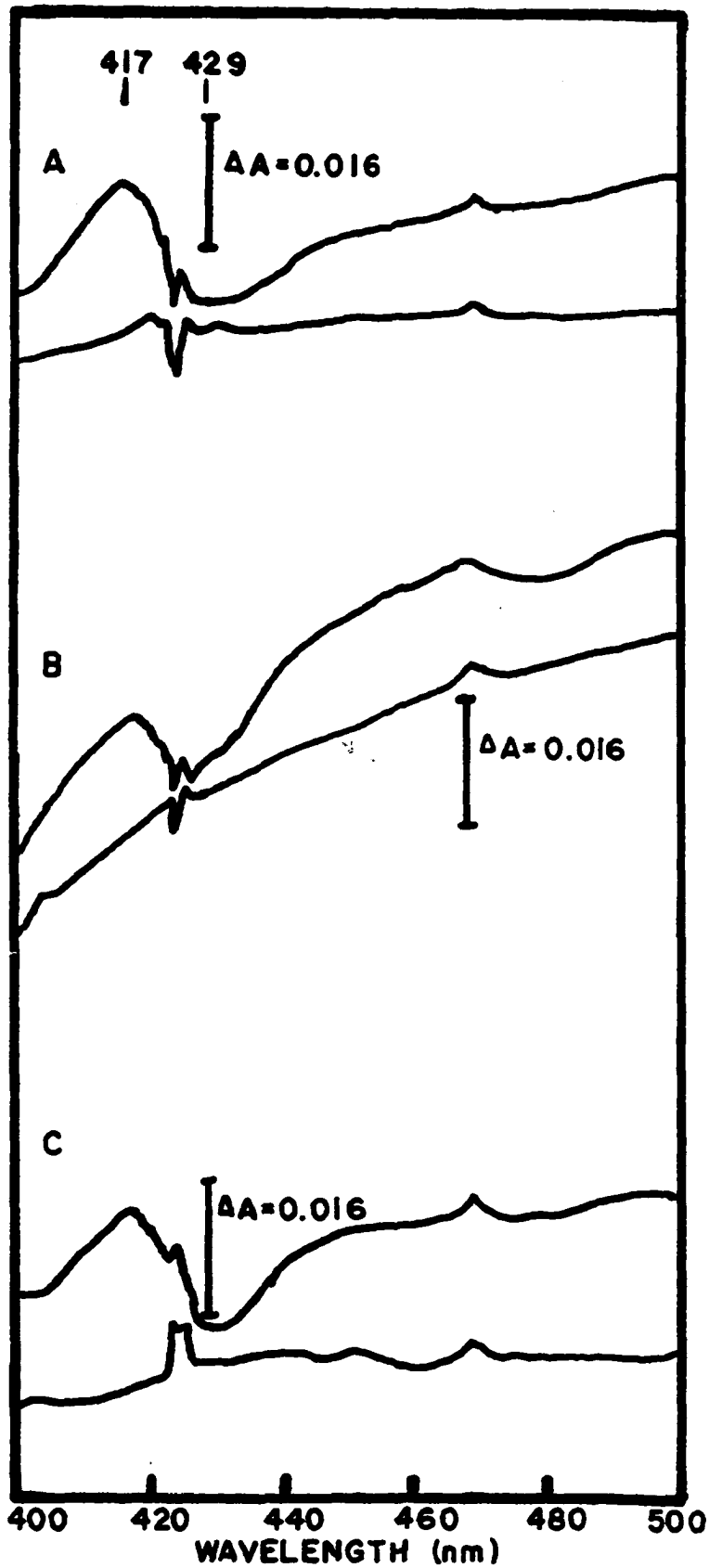


Table 9 shows the extent of cytochrome reduction using the physiological reductants compared to dithionite reduced cytochrome reduction. These results show the physiological reductants reduce the cytochromes almost as efficiently as dithionite.

Cytochrome and Glucose Oxidase Inhibition Studies

The effects of respiratory chain inhibitors on glucose oxidase activity are indicated in Table 10. The oxidase assays were performed using the optimum assay conditions already described. Cyanide and azide, typical inhibitors of cytochrome oxidases, demonstrated the expected inhibitory affect on glucose oxidation, although azide was not as effective an inhibitor at the concentrations used. HQNO, which inhibits the oxidation of cytochrome b in some bacteria (A. F. Brodie and J. Adelson, 1965 and W. Heinen, 1971) and inhibits transfer of electrons in mitochondrial preparations between cytochrome b and c, (E. C. Slater, 1967), caused approximately 86% inhibition of glucose oxidase activity. Atabrine, an inhibitor of flavoprotein enzymes (M. A. Faust and R. N. Doetsch, 1969), inhibited glucose oxidase activity between 39% and 65% over a ten fold concentration range. Acriflavin, a structural analog of flavins, also inhibited glucose oxidase although not as effectively as atabrine. The data for inhibition of glucose oxidase activity by 8-hydroxyquinoline, rotenone, and 2,4-DNP are included in Table 10, although the results must be interpreted with caution since the ethanol control alone caused approximately 72% inhibition of the glucose oxidase activity.

Table 9

Extent of cytochrome reduction by physiological reductants compared to dithionite reduced cytochromes. P. fluorescens was grown (0.1 M carbon source as indicated, Method B) and membranes prepared from stationary phase cells as described in the Materials and Methods. The difference spectra and oxidase assays were performed on membranes suspended in the appropriate assay buffers (see legends to Figures 2, 4, and 6). The cytochrome spectra were recorded in cuvettes with a 10 mm light path at room temperature (ca. 23°C) using a few crystals of dithionite for total cytochrome reduction and 0.1 M glucose, 0.1 M gluconate, and 0.06 M L-malate concentrations for reduction due to the physiological reductants.

Growth substrate and reductant	Cytochrome content		Oxidase activity
	comp.	Ratio: $\frac{\text{Subst. red}}{\text{Dithion. red}}$	nmoles O_2 consumed per min per mg prot.
Glucose	b	0.835	230.6
	c	0.949	
	o	1.000	
Gluconate	b	0.728	40.3
	c	0.847	
	o	0.930	
Malate	b	0.581	90.2
	c	0.814	
	o	0.693	

Table 10

Effect of Respiratory Chain Inhibitors on
P. fluorescens Glucose Oxidase Activity[a]

[a]Stationary phase P. fluorescens parent strain was grown on glucose (0.1 M) using Method B, and membranes prepared as described in the Materials and Methods. The reaction mixture contained (per ml): 30 µmoles potassium phosphate buffer, pH 6.7; 2 µmoles $MgSO_4$; 50 µmoles glucose; inhibitor (concentrations indicated in table); and 4.35 mg membrane protein.

[b]The inhibitors were added in solution after the buffer, magnesium, and membrane material. The reaction was initiated after a 3 min incubation period in the presence of inhibitor by the addition of glucose.

[c]Control oxidase activity was 361.1 nmoles O_2 consumed per min per mg membrane protein.

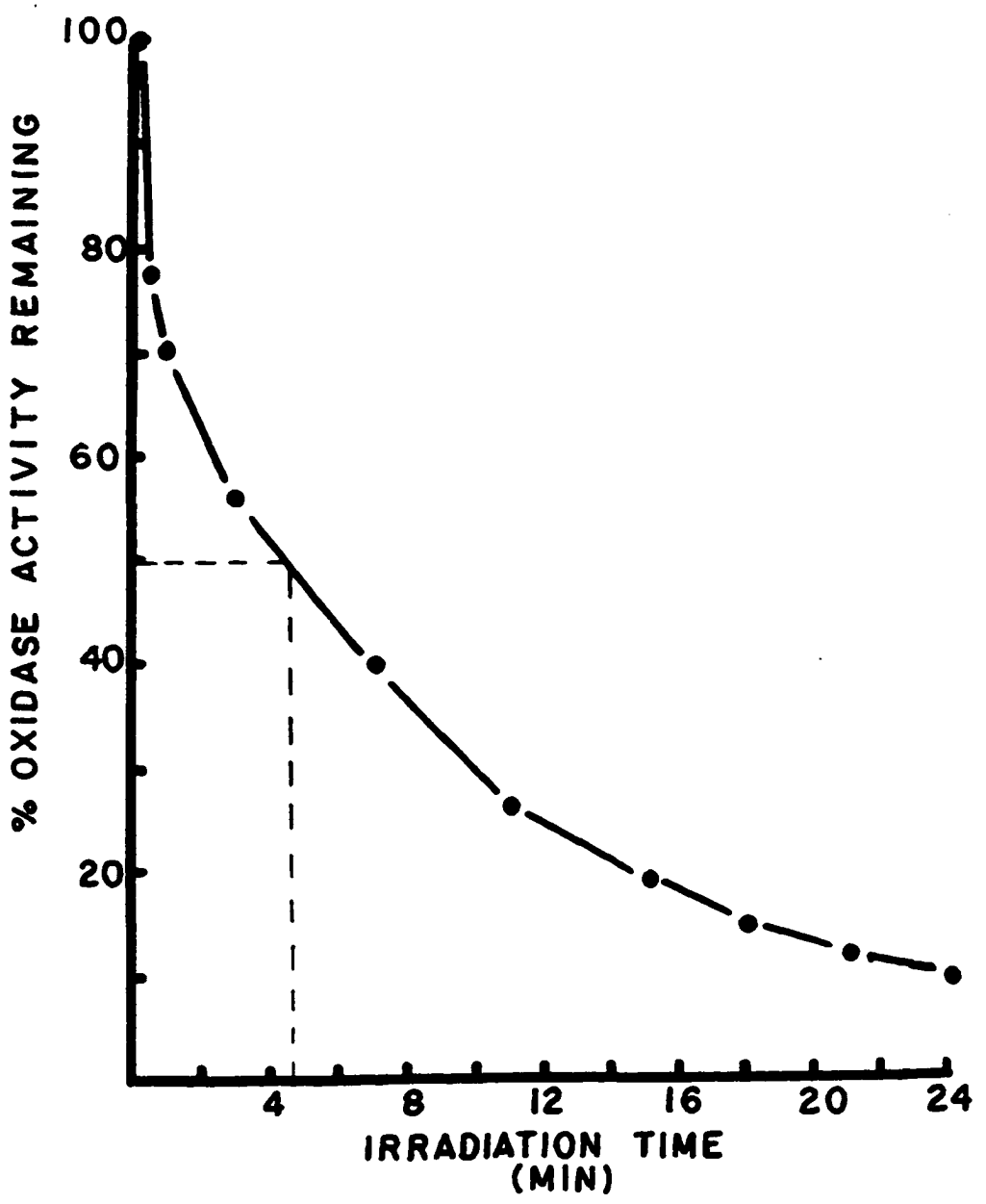
[d]HQNO was dissolved in 0.001 N KOH; the amount of alkali added had no affect on control oxidase activity.

Inhibitor[b]	Concentration	Percent of glucose[c] oxidase inhibited
Cyanide	10^{-2} M	99.3
	10^{-3} M	91.6
Azide	10^{-2} M	72.7
	10^{-3} M	24.2
Acriflavin	100 µg/ml	38.5
	33 µg/ml	7.7
Atabrine	10^{-3} M	65.3
	10^{-4} M	38.7
8-hydroxy-quinoline	EtOH	72.4
	EtOH + 10^{-2} M	94.1
	EtOH + 10^{-3} M	77.6
2-n-heptyl-4-[d] hydroxyquinoline -N-oxide	5×10^{-4} M	86.6
	1×10^{-4} M	85.6
	5×10^{-5} M	86.9
Rotenone	EtOH	72.1
	EtOH + 5×10^{-4} M	76.5
	EtOH + 5×10^{-5} M	72.8
2-4 DNP	EtOH	73.5
	EtOH + 10^{-3} M	73.5
	EtOH + 5×10^{-4} M	72.1
	EtOH + 10^{-4} M	72.8

Black-light irradiation kinetics of glucose oxidase activity are given in Figure 25. Loss of activity due to black-light irradiation (∿ 360 nm) can be attributed to the destruction of quinones in the ETS. Quinones play an important role in the electron transport chain since they serve as a co-factor shuttling electrons between flavin adenine dinucleotide (FAD) and cytochrome b. The data shows 50% inhibition (LD_{50}) after 4.5 min irradiation. Quinone reconstitution experiments were not included in this study.

Figure 25

Inhibition of stationary phase glucose-grown (0.1 M) P. fluorescens glucose oxidase activity by black-light irradiation. Bacteria were grown (Method B), membranes prepared, and irradiation performed as described in the Materials and Methods. Initial activity of glucose oxidase was 361.1 nmoles O_2 consumed per min per mg membrane protein. The reaction vessel contained (per ml): 30 µmoles potassium phosphate buffer, pH 6.7; 2 µmoles $MgSO_4$; 50 µmoles glucose and 4.35 mg membrane protein.



DISCUSSION

A number of bacteria have been reported to oxidize glucose to gluconate via a particulate glucose dehydrogenase (oxidase). These bacteria include Acetobacter suboxydans (T. E. King, and V. H. Cheldelin, 1957), Pseudomonas pseudomallei (formerly Malleomyces pseudomallei [J. H. Dawling and H. B. Levine, 1956]), Pseudomonas quercito-pyrogallica (R. Bentley and L. Slechta, 1960), Aerobacter aerogenes (A. Dalby and A. C. Blackwood, 1955), P. fluorescens A3.12 (W. A. Wood and R. F. Schwerdt, 1953), and Bacterium anitratum (J. G. Hauge, 1960). None of these particulate glucose oxidase enzymes required added coenzymes such as NAD or NADP to carry out glucose oxidation. This is similar to the glucose oxidase enzyme found in fungi which contains a tightly bound flavin moiety (R. Bentley, 1969). The reported pH optimum for the glucose oxidase enzymes varied from pH 5.5 for P. pseudomallei (J. H. Dawling, and H. B. Levine, 1956) to pH 7.0 for P. quercito-pyrogallica (R. Bentley and L. Slechta, 1960) using oxygen as the terminal electron acceptor. Figure 3 and Table 1 show the pH optimum for P. fluorescens ATCC 13525 to be in the above range with an optimum between 6.7 and 6.75. Dawling and Levine (1956), reported an apparent glucose oxidase K_m of 35.0×10^{-4} M in P. pseudomallei. This value contrasts the apparent K_m reported here of 5.9×10^{-4} Molar (Figure 2 and Table 1). This discrepancy could be accounted for by the procedures used to prepare and assay the membrane fractions. They disrupted their

cells with a Waring blender and had no magnesium salts in their assay buffer (phosphate buffer, pH 5.5). The data in Table 8 suggests the glucose oxidase enzyme in P. fluorescens might be described better as an aldohexose oxidase. This proposal, based on the constant ratio of aldohexose oxidase activity to glucose oxidase activity from mid-log and stationary phase cell membrane material, cannot be substantiated until the dehydrogenase is purified. Dahms and Anderson (1972) reported the purification and characterization of an inducible soluble, pyridine nucleotide linked, aldohexose dehydrogenase from a Pseudomonas species, designated pseudomonas MSU-1. This purified soluble dehydrogenase enzyme was able to oxidize a number of D- aldohexoses including D-fucose, D-glucose, D-galactose, D-mannose and 2-deoxy-D-glucose.

DeLey and Stouthamer (1959) reported three gluconate oxidizing systems in A. suboxydans. Only one system, however, was particle linked and it oxidized gluconate solely to 2-ketogluconate. This particulate gluconate oxidase enzyme appeared to be cytochrome linked and confirmed the reports of Wood and Schwerdt (1953). Glucose oxidase was also reported to be linked to the cytochrome system in P. fluorescens A3.12 (W. A. Wood and R. F. Schwerdt, 1953), B. anitratum (J. G. Hauge, 1960), and P. quercito-pyrogallica (R. Bentley and L. Slechta, 1960). The results presented in Table 9 are in agreement with these earlier studies.

Figure 12 shows the results of polarographically determined glucose and gluconate oxidase stoichiometries. These results confirm the manometrically determined stoichiometries of DeLey and Stouthamer

(1959), Wood and Schwerdt (1953), Dawling and Levine (1956), and Stokes and Campbell (1951). An important advantage obtained by determining the gluconate oxidase stoichiometry polarographically was that it could then be used as a rapid, quantitative assay for determining gluconate formation from glucose as a function of growth (see Figure 16).

The development of reproducible and optimum assay conditions for D(-) and L(+) lactate oxidase enzymes, based on saturation kinetics and pH optima, was included in this study following a report by Rizza and Hu (1973) where they proposed a novel pathway for glyceraldehyde-3-phosphate (G-3-P) metabolism in Pseudomonas saccharophila G6. As discussed previously, glucose and gluconate are catabolized via the Entner-Doudoroff pathway yielding G-3-P and pyruvate. The catabolism of G-3-P to pyruvate was assumed to follow the final reactions of the Embden-Myerhof pathway (J. DeLey, 1960; and R. A. Cooper, 1974). Rizza and Hu's proposed pathway for G-3-P metabolism is described below:

$$\text{G-3-P} \xrightarrow{-P_i} \text{Glyceraldehyde} \xrightarrow{-H_2O} \text{Methylglyoxal} \xrightarrow{+H_2O}$$

$$\longrightarrow \text{D,L-lactate} \xrightarrow{O_2} \text{Pyruvate}$$

This reaction sequence requires, by necessity, constitutive D(-) and L(+) lactate oxidase enzymes. These enzymes would be expected to be present at high levels when the bacteria were grown in glucose or gluconate as the sole carbon and energy source if these compounds are metabolized via a methylglyoxal pathway. The results in Table 6

do not support this conclusion, however, since the D(-) and L(+) lactate oxidases appear to be induced only by growth on racemic lactate as the sole carbon and energy source.

The L-malate oxidase enzyme was included in this study to serve as a reference for establishing induction of other oxidase activities since it has been demonstrated to be constitutive in Azotobacter vinelandii (E. M. Barnes, Jr., 1972) and a fluorescent Pseudomonas (D. J. Hopper, 1970); L-malate oxidase has also been shown to be localized on the cell wall membrane of Pseudomonas ovalis Chester (M. J. O. Francis et al., 1963). The results in Table 6 establish the constitutive nature of L-malate oxidase in P. fluorescens. The oxidation of L-malate was accompanied by spectral changes that indicated the involvement of flavoproteins and cytochromes b, c, and a CO-binding cytochrome (Table 9 and Figure 23). These results were similar to those reported by Stanier et al. (1953) for P. fluorescens A3.12 although the involvement of a CO-binding cytochrome was not reported. Involvement of b and c-type cytochrome during malate oxidation in P. ovalis Chester has also been reported (M. J. O. Francis et al., 1963).

Magnesium cations have been shown to be required for glucose oxidation in membrane preparations from Aerobacter aerogenes (A. Dalby and A. C. Blackwood, 1955) and a P. fluorescens strain (J. G. Hauge, 1961). Hauge postulated (1961) that divalent cations had a role in preserving the structural integrity of the oxidizing particle rather than participating directly in the dehydrogenation reaction. In more recent studies, Asbell and Eagon (1966) described

the formation of osmotically fragile rods of P. aeruginosa following incubation with ethylenediaminetetraacetate and tris(hydroxmethyl) aminomethane. These fragile rods could be restored to an osmotically stable form by the addition of multivalent cations. Some multivalent cations, however, resulted in a loss of ability to oxidize substrates. Eagon and Asbell (1969), continuing these studies, concluded that the inhibition of the oxidation of substrates by multivalent cations appeared to result from the inhibition of uptake of these substrates. Walker and Durham (1973), working with a P. fluorescens strain, showed that magnesium stimulated the respiratory rate. They concluded that Mg^{+2} acted at the cell envelope to hold transport and respiratory proteins in the proper tertiary and quaternary conformation. The results in Figure 13 have demonstrated the stabilizing affect of exogenously added Mg^{+2} on glucose and L-malate oxidase activities. The L-malate oxidase enzyme retained 65% more activity by the addition of 2 mM $MgSO_4$. Glucose oxidase retained 45% more activity by the addition of exogenous Mg^{+2}. A slightly different affect of Mg^{+2}-enzyme-membrane interactions was reported by Eisenberg et al. (1970). They were studying the membrane bound NADH oxidase and NADH-DCPIP oxidoreductase activities in B. megaterium KM protoplast preparations. It was demonstrated that when ghost membranes prepared in the presence of magnesium were washed in the absence of magnesium, increased levels of oxidase and DCPIP oxidoreductase activity was observed. It was suggested that the breakdown of membrane structure in the absence of Mg^{+2} lead to the unmasking of the enzyme activities.

Divalent cations are known to be involved in the structural integrity of bacterial membrane systems (M. Asbell and R. G. Eagon, 1966). Razin (1970), in an extensive review of membrane reconstitution, noted the importance of divalent cations in these types of studies. It was, therefore, imperative to determine if the exogenously added Mg^{+2} was resulting in membrane aggregation or vesicularization in our system. The kinetic results in Figure 13 indicated Mg^{+2} stabilized the membrane oxidases and did not result in vesicularization. The rationale of a kinetic approach to answer this question was similar to that reported by Hampton and Freese (1974).

The results for the induction studies in Table 4 suggested glucose oxidase inducibility when the specific activities from glucose grown cells were compared to the specific activities for glucose oxidase from malate, succinate, and asparagine grown cells. The specific activities from gluconate, glycerol, and Casamino Acids grown cells, however, indicate a more complex mechanism for glucose oxidase regulation than just simple induction. To ensure these growth substrates were not contaminated with sufficient glucose to cause induction of the glucose oxidase enzyme, they were assayed for glucose contamination using both the Nelson (1944) reducing sugar assay and the Glucostat assay kit (Worthington Biochemicals Corp.). All substrates used for sole carbon and energy sources in the induction studies were found to be free of contaminating glucose.

The 6-phosphogluconate dehydrase activity was also induced by glucose, gluconate, and glycerol (Table 4). These results confirm

the work of Lessie and Neidhardt (1967), Quay et al. (1972), and Hylemon and Phibbs (1972). It is interesting to note, however, that the specific activities for glucose oxidase, gluconate oxidase, and 6-phosphogluconate dehydrase indicate induction by growth on glucose gluconate, and glycerol as sole carbon and energy sources, whereas only the glucose oxidase enzyme has high levels of activity from cells grown on Casamino Acids.

Further characterization of the regulatory mechanisms for the biosynthesis of glucose and gluconate oxidase enzymes was based on measuring the specific activities of these enzymes as a function of growth. These results are shown in Table 6. It is apparent from the stationary to mid-log specific activity ratios that the glucose oxidase activity is very dependent on the age of the culture. The range of this ratio varied from 2.78 for gluconate grown cells to 21.7 for malate grown cells. Although the specific activity for glucose oxidase from malate grown cells is low the 20-fold increase in stationary phase malate grown cells is significant. The specific activities from stationary phase cells for gluconate oxidase from P. fluorescens grown on various carbon and energy sources confirms the inducibility of this enzyme (see Table 5). The ratio of the gluconate and malate oxidase activities obtained from cells grown on the various carbon sources were consistently less than unity except when the respective oxidases were assayed from bacteria grown on the oxidase substrate.

Data in Table 6 helps to explain the contradictory reports in the literature on the regulation of glucose oxidase. Entner and

Stanier (1951) reported glucose oxidase was constitutive in P. putida A3.12; other (Ng and Dawes, 1972; Von Tigerstrom and Campbell, 1966) reported glucose oxidase was inducible in P. aeruginosa. These data support and extend the above conflicting reports since the biosynthesis of glucose oxidase was dependent on both carbon and energy source and culture age.

There are several possible explanations which could account for the regulation of the glucose oxidase enzyme. If glycogen is assumed to be an endogenously stored reserve energy source its breakdown to glucose in old Casamino Acids grown cells could account for the increased glucose oxidase activity observed. This hypothesis, however, is not compelling since glycogen has not been reported to be a reserve energy source in these bacteria and if glucose is produced from glycogen degradation then increased levels of gluconate oxidase and 6-phosphogluconate dehydrase activities would be expected (see Table 6, glucose grown cell membrane enzyme activities) and this "expected induction" was not observed. A second possible explanation could be based on a starvation phenomenon. As the cells become "old" an unidentified inducer molecule derepresses the gene involved in transcribing the glucose oxidase enzyme and also serves to repress the biosynthesis of the gluconate and malate oxidase enzymes. The bacteria, therefore, would conserve necessary amino acids and energy by reducing gluconate and malate oxidase enzyme levels and yet have the capacity to utilize hexose if it is available. Having the first enzyme available for glucose catabolism could allow for the sequential induction (L. N. Ornston, 1966) of the remaining

glucose catabolic enzymes. The data in Figure 16, however, shed doubt on this hypothesis since stationary phase glucose grown P. fluorescens have approximately 50 mM glucose and 40 mM gluconate still available in the medium. The reason for the bacteria entering stationary phase with these significantly high remaining concentrations of potential carbon sources was not due to exhaustion of an energy source.

Preliminary studies indicate glucose uptake also depends on culture age since Casamino Acids grown P. fluorescens actively transport glucose (Eisenberg, unpublished data). In this case, glucose uptake did not begin until the cultures reached late exponential or early stationary phase. The possible involvement of cyclic adenosine-3',5'-monophosphate or cyclic guanidine-3',5'-monophosphate in the regulation of glucose oxidase and glucose uptake was not included in these studies. It has been reported previously that glucose uptake in P. fluorescens is glucose inducible.

Recently, McCowen and Phibbs (1974) reported a 7-fold increase in alanine dehydrogenase activity from alanine grown stationary phase Bacillus licheniformis. They suggested that this increased alanine dehydrogenase enzyme was involved in initiating sporulation in these bacteria. Increased levels of α-amylase activity from late stationary phase B. licheniformis has also been reported. (N. Saito and Y. Yamamoto, 1975). Increased enzyme levels in stationary phase cells may not be restricted to the spore-forming bacteria but may be a more general phenomenon which should be studied in other non-spore-forming bacteria.

Although no definite hypothesis for the regulation of glucose catabolic enzymes can be presented at this time, the evidence presented in this study suggests a complex regulation of glucose oxidase biosynthesis dependent on both growth substrates and culture age.

The cytochrome system in P. fluorescens strains have been studied. Smith (1954) reported the presence of cytochromes b, c, and $a+a_3$ in the respiratory chain. Stanier et al. (1953) reported the presence of cytochromes b and c. Lenhoff and co-workers (1953, 1956 a and b, 1963) reported the presence of a cytochrome peroxidase activity in P. fluorescens. Lenhoff et al. (1956b) concluded that the oxygen tension of the medium during the growth of P. fluorescens determined the alternate routes of terminal electron transfer which the organism forms; these studies suggested an iron-cytochrome peroxidase pathway when the organism is grown at low oxygen tension and a molybdoflavoprotein pathway as the terminal oxidase when the organism is grown at a high oxygen tension. It is very important to realize that although the above cytochrome systems were observed in P. fluorescens none of the investigators worked with P. fluorescens ATCC 13525 (proposed neotype strain, Stanier et al., 1966). Also, Lenhoff's strains were capable of anaerobic respiration using nitrate as the terminal electron acceptor. The strain used in this study, however, is an obligate aerobe, and can only use oxygen as the terminal electron acceptor (Stanier et al., 1966).

The results in Table 7 and Figures 17, 18 and 20 show the presence of cytochromes b, c and a CO-binding cytochrome oxidase.

The spectral traces in Figure 19, however, do not exhibit the characteristic peak or shoulder at 552 nm, the characteristic α-region for cytochrome c. Although a value for the amount of cytochrome c present in these Casamino Acids grown cell membranes was obtained, no supportive conclusions on the presence or absence of cytochrome c are possible until liquid nitrogen difference spectra are obtained or ascorbate-tetramethylphenylenediamine oxidase are performed.

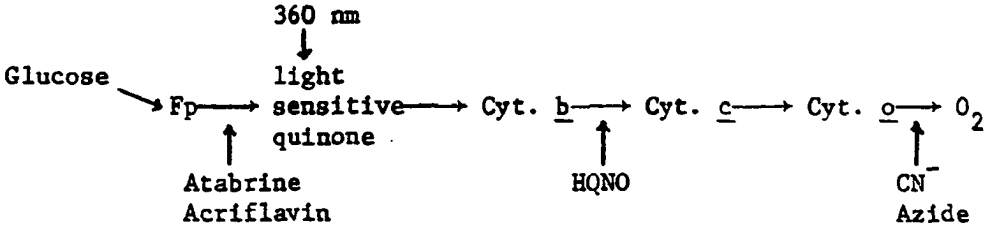
The presence of a CO-binding cytochrome, cytochrome o, has not been previously reported in P. fluorescens. Since P. fluorescnes does not contain cytochromes a (Table 7, Figures 17-24), the cytochrome o appears to function as the terminal oxidase in this bacterium. A number of bacteria including E. coli, Klebsiella pneumoniae (NCTC 5055), Acinetobacter lwoffi 4B, P. ovalis Chester (C. W. Jones et al., 1975) and A. suboxydans (R. M. Daniel, 1970) have been reported to contain cytochrome o as the only terminal oxidase in their cytochrome systems.

It has been reported that cytochrome oxidases (Pseudomonas cytochrome c: oxygen oxidoreductase, EC 1.9.3.2) functions both as a cytochrome oxidase and a nitrite reductase in P. aeruginosa (T. Yamanaka and K. Okunuki, 1963a, b, and c); when purified it was characterized as cytochrome cd (T. Kunonen and N. Ellfolk, 1972). This enzyme, however, has only been obtained from anaerobically grown P. aeruginosa using nitrate as terminal electron acceptor. The enzyme has never been isolated from cells grown aerobically in the absence of nitrate and therefore nitrite reductase activity is

evidently the physiological function of this enzyme (T. Kunonen and L. Ellfolk, 1972; and T. Yamanaka and K. Okunuki, 1963a, b, and c). Since P. fluorescens (ATCC 13525) does not grow anaerobically, this cytochrome c oxidase activity is probably not present.

The effect of growth conditions on the cytochrome composition in aerobic bacteria has been extensively studied (C. J. Knowles and E. R. Redfearn, 1969; R. F. Rosenberger and M. Kogut, 1958; D. C. White, 1962; D. C. White and L. Smith, 1964; and D. C. White and P. R. Sinclair, 1972). The cytochrome system in P. fluorescens (Table 7) also changes qualitatively and quantitatively as growth conditions vary. White (1967) concluded, from studies of the electron transport system in Haemophilus parainfuenzae, that the formation of all components of the electron transport system is controlled by the identity and concentration of the terminal electron acceptors present in the growth medium.

The flow of electrons through the cytochrome chain to oxygen, as judged by ETS inhibitors on glucose oxidase activity (Table 10), is similar to other bacteria. Reducing equivalents enter the system at the flavoprotein level, pass through a light-sensitive quinone and finally through cytochromes b, c, and o to oxygen. These reactions are summarized below:



Abbreviations: Cyt., cytochromes and Fp, flavoprotein.

It is readily apparent from this study on glucose and gluconate oxidases in P. fluorescens (ATCC 13525) that the need for well defined growth conditions when studying the regulation of these enzymes is important. Similar studies on the effect of cell ageing in the other fluorescent pseudomonads, P. aeruginosa and P. putida, are warranted since glucose oxidase activity may be induced in these species as a function of the cell growth cycle.

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